Eiichi Ishii *Editor*

Hematological Disorders in Children

Pathogenesis and Treatment

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Eiichi Ishii
Department of Pediatrics
Ehime University Graduate School of Medicine
Toon, Ehime
Japan

Preface

In recent years, the pathogenesis of hematological disorders in children has been clarified, which has led to remarkable progress in the treatment outcome for these disorders. In particular, molecular targeted therapy has been shown to have an effect on childhood leukemia refractory to conventional therapy. Further development of these strategies will enable us to use more effective and less toxic therapies for hematological disorders in the future. Here we describe the pathogenesis and treatment (present and future) of several representative hematological disorders in children, especially focusing on the genetic and molecular aspects.

Most hematological disorders in children arise from intrauterine endogenous or exogenous exposures, genetic susceptibility, or several factors after birth. A good example is the case of infant leukemia. Greaves et al. [1] clarified the etiology of infant leukemia; analysis of *MLL* gene rearrangements in identical leukemic twins suggested that the *MLL* gene undergoes prenatal rearrangement and leukemic cells are present in the blood of newborns. In utero exposure to some drugs and foods that possess functional similarity to topoisomerase-II inhibitors and certain environmental factors may affect *MLL* gene rearrangements, which contribute to leukemogenesis [2]. This conclusion can be expanded to B-cell precursor acute lymphoblastic leukemia (ALL) with *TEL-AML1* [3] or hyperdiploidy [4], acute myeloid leukemia (AML) with *AML1-ETO* [5]*, and* acute megakaryocytic leukemia with *GATA*1 mutations in Down syndrome [6]. Clarification of leukemogenesis after birth is needed to establish more appropriate treatment modalities in childhood leukemia. We have recently shown that the *MLL-miRNA let7b*-oncogene HMGA2 pathway plays an important role in the proliferation of leukemic cells and could be a possible molecular target for the therapy of infant leukemia [7].

Congenital bone marrow failure syndrome in children is an inherited disease, including Fanconi anemia (FA), dyskeratosis congenita, Shwachman–Diamond syndrome, Diamond–Blackfan syndrome, severe congenital neutropenia, and congenital amegakaryocytic thrombocytopenia**,** which are characterized by cytopenias in different hematologic lineages and several congenital abnormalities [8]. Early diagnosis of bone marrow failure syndrome is important for optimizing clinical management, anticipating possible complications that may develop later in life, and

providing appropriate genetic counseling for the family [9]. Many of these syndromes require multidisciplinary and multispecialty medical care for appropriate surveillance and management. As a representative disease, FA is caused by germline mutations in DNA repair genes with autosomal recessive inheritance. A major problem of FA is its high risk of cancer, which increases with age. In particular, the risk of myelodysplastic syndrome or AML is relatively high, indicating that most patients with FA need hematopoietic stem cell transplantation (HSCT) before the onset of malignancy [10]. However, the rate of treatment-related side effects and graft-versus-host disease, as well as the risk of head and neck cancers, is high in FA patients after HSCT, demonstrating the need for a more appropriate conditioning regimen [8].

A high risk of malignancy can also be seen in other types of congenital bone marrow failure syndrome, and the molecular pathology will be similar and sometimes overlap between different subtypes. Genetic advances have already led to improved diagnosis, particularly in cases wherein the presentation is atypical. These advances may also lead to new treatments. In the meantime, it is important to obtain accurate information on the incidence and natural history of each disorder in order to provide a more rational basis for the optimal provision of clinical services [11].

Acquired aplastic anemia (AA) is an uncommon, life-threatening disorder in childhood. Because of major advances in diagnosis and therapeutic approaches, nowadays AA is a disease that results in long-term survival in more than 90% of cases [12]. In recent years, an immune-mediated pathogenesis for AA has been suggested because immunosuppressive therapy is usually effective and bone marrow lymphocytes from patients can suppress normal marrow cells *in vitro* [13]. Numerous studies have established that HSCT, especially bone marrow transplantation, from HLA-matched donors is highly successful, with five-year survival rates of >90% [14]. Unfortunately, horse antithymocyte globulin (ATG) is no longer available and an alternative agent, rabbit ATG, is associated with a lower response rate for AA in children [15].

Hemophagocytic lymphohistiocytosis (HLH) is characterized by fever and hepatosplenomegaly associated with pancytopenia, hypertriglyceridemia, hypofibrinogenemia, and infiltration of histiocytes with hemophagocytic activity. HLH can be classified into two distinct forms, primary and secondary HLH; primary HLH includes familial hemophagocytic lymphohistiocytosis and several immunodeficiencies, while secondary HLH is usually associated with infections [especially Epstein–Barr virus (EBV) infection], immune disorders, or malignancy (especially non-Hodgkin lymphoma). In primary HLH, uncontrolled T lymphocyte activation by impairing defects of genes, such as *perforin* (*PRF1*) and *MUNC13-4*, results in large quantities of inflammatory cytokines that promote macrophage infiltration and formation of the cytokine network [16]. The pathogenesis of secondary HLH, especially EBV-HLH, is not fully understood. In EBV-HLH, inflammatory cytokines produced by EBV-infected T cells or natural killer cells are responsible for macrophage activation and subsequent development of HLH [17].

The treatment of HLH has been established in recent years. Immunochemotherapy followed by HSCT can be used for primary HLH, while the clinical course of

EBV-HLH varies among patients and treatment that is more appropriate should be organized for secondary HLH. Identification of all instigating mechanisms may prompt the development of novel approaches, including gene therapy, for this disorder in the future.

Langerhans cell histiocytosis (LCH) is a rare clonal disorder characterized by the proliferation of clonal CD1a-positive LCH cells in the skin, bone, lymph nodes, and other organs. LCH cells are immature dendritic cells, and the JAG-mediated Notch signaling pathway may play an important role in maintaining LCH cells in an immature state [18]. More than half of the LCH patients have the oncogene *BRAF* mutation, suggesting that LCH is a neoplastic disorder [19]. The outcome of LCH varies depending on the extent of organ involvement, and treatment should be planned according to the clinical subtype. In single-system disease, local corticosteroid therapy can be used for patients with single bones affected by lesions with no CNS risk [20], while chemotherapy is available for those with multiple affected bones or with CNS-risk lesions. In multisystem disease, on the other hand, chemotherapy including vincristine, cytarabine, and corticosteroid has been used with great success, with a mortality rate of only 10% [21].

Despite their successful treatment, LCH patients often develop long-term sequelae, including diabetes insipidus, orthopedic problems, hearing loss, neurological problems, growth-hormone deficiency, pulmonary fibrosis, and biliary cirrhosis [22]. The incidence of these sequelae increases with follow-up time. Further novel therapeutic measures are required to reduce these permanent sequelae.

Hematological disorders in children are usually rare, but clarifying their pathogenesis has progressed and appropriate treatment has been established in the recent years. More effective and less toxic therapies for these hematological disorders need to be developed in the near future.

Ehime, Japan   Eiichi Ishii

References

1. Greaves MF. Infant leukemia biology, aetiology and treatment. Leukemia. 1996; 10: 372–7.
2. Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. Blood. 2003; 102: 2321–33.
3. Wiemels JL, Cazzaniga G, Daniotti M, et al. Prenatal origin of acute lymphoblastic leukaemia in children. Lancet. 1999; 354: 1499–503.
4. Panzer-Grümayer ER, Fasching K, Panzer S, et al. Nondisjunction of chromosomes leading to hyperdiploid childhood B-cell precursor acute lymphoblastic leukemia is an early event during leukemogenesis. Blood. 2002; 100: 347–9.
5. Wiemels JL, Xiao Z, Buffler PA, et al. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. Blood. 2002; 99: 3801–5.
6. Ahmed M, Sternberg A, Hall G, et al. Natural history of GATA1 mutations in Down syndrome. Blood. 2004; 103: 2480–9.

7. Wu Z, Eguchi-Ishimae M, Yagi C, et al. HMGA2 as a potential molecular target in KMT2A-AFF1-positive infant acute lymphoblastic leukaemia. Br J Haematol. 2015; 171: 818–29.

8. Rivers A, Slayton WB. Congenital cytopenias and bone marrow failure syndromes. Semin Perinatol. 2009; 33: 20–8.

9. Khincha PP, Savage SA. Neonatal manifestations of inherited bone marrow failure syndromes. Semin Fetal Neonatal Med. 2016; 21: 57–65.

10. Alter BP, Giri N, Savage SA, et al. Malignancies and survival patterns in the National Cancer Institute inherited bone marrow failure syndromes cohort study. Br J Haematol. 2010; 150: 179–88.

11. Dokal I, Vulliamy T. Inherited bone marrow failure syndromes. Haematologica. 2010; 95: 1236–40.

12. Hartung HD, Olson TS, Bessler M. Acquired aplastic anemia in children. Pediatr Clin North Am. 2013; 60: 1311–36.

13. Kagan WA, Ascensao JA, Pahwa RN, et al. Aplastic anemia: presence in human bone marrow of cells that suppress myelopoiesis. Proc Natl Acad Sci U S A. 1976; 73: 2890–4.

14. Samarasinghe S, Steward C, Hiwarkar P, et al. Excellent outcome of matched unrelated donor transplantation in paediatric aplastic anemia following failure with immunosuppressive therapy: a United Kingdom multicenter retrospective experience. Br J Haematol. 2012; 157: 339–46.

15. Yoshimi A, Niemeyer CM, Fuhrer MM, et al. Comparison of the efficacy of rabbit and horse antithymocyte globulin for the treatment of severe aplastic anemia in children. Blood. 2013; 121: 860–1.

16. Ishii E, Ueda I, Shirakawa R, et al. Genetic subtypes of familial hemophagocytic lymphohistiocytosis: correlations with clinical features and cytotoxic T lymphocyte/natural killer cell functions. Blood. 2005; 105: 3442–8.

17. Kogawa K, Sato H, Asano T, et al. Prognostic factors of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children: Report of the Japan Histiocytosis Study Group. Pediatr Blood Cancer. 2014; 61: 1257–62.

18. Morimoto A, Oh Y, Shioda Y, et al. Recent advances in Langerhans cell histiocytosis. Pediatr Int. 2014; 56: 451–61.

19. Badalian-Very G, Vergilio JA, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. Blood. 2010; 116: 1919–23.

20. Morimoto A, Ishida Y, Suzuki N, et al. Nationwide survey of single-system single site Langerhans cell histiocytosis in Japan. Pediatr Blood Cancer. 2010; 54: 98–102.

21. Morimoto A, Shioda Y, Imamura T, et al. Intensified and prolonged therapy comprising cytarabine, vincristine and prednisolone improves outcome in patients with multisystem Langerhans cell histiocytosis: Results of the Japan Langerhans Cell Histiocytosis Study Group-02 Protocol Study. Int J Hematol. 2016;104:99–109.

22. Haupt R, Nanduri V, Calevo MG et al. Permanent consequences in Langerhans cell histiocytosis patients: a pilot study from the Histiocyte Society-Late Effects Study Group. Pediatr Blood Cancer. 2004; 42: 438–44.

Contents

Part I
Hematopoiesis

Chapter 1
Hematopoietic Stem Cells: The Basis of Normal and Malignant Hematopoiesis

Mariko Eguchi, Minenori Eguchi-Ishimae, and Eiichi Ishii

Abstract Hematopoietic stem cells (HSCs), which are responsible for producing all blood cell types, first appear in the early stage of embryonic development and transit through several different tissues, including the yolk sac, aorta-gonad-mesonephros (AGM) region, placenta, and fetal liver, before colonizing in the bone marrow where they reside throughout the individual's life. HSCs, characterized by the ability to self-renew and generate all types of blood cells, are supported by their specific environment called niches and depend on many developmental signaling pathways, molecules, and cytokines for their generation, maintenance, and expansion. Any disruption in this well-balanced system may cause aberrant HSC production, leading to malignant hematopoiesis. Leukemic stem cells (LSCs), originally identified using xenograft models of acute myeloid leukemia (AML), are a distinct cell population that can initiate leukemia in immunodeficient mice. LSCs are thought to emerge from HSCs or hematopoietic progenitors after obtaining multiple genetic changes that provide aberrant growth advantage and self-renewal ability. The emergence of LSCs is a multi-step event, including genetic diversification and clonal selection, resulting in genetic heterogeneity among leukemic cells. LSCs generally exist in the immature $CD34^+CD38^-$ leukemic population in most cases of AML and share some features with normal HSCs. However, recent studies have shown that in acute lymphoblastic leukemia (ALL), LSCs exist in B-lineage-committed progenitors expressing CD19. In contrast to that in AML, in which LSCs generate leukemic cells in a hierarchical order with LSCs at the top, leukemia propagation in ALL is better explained by a stochastic model.

M. Eguchi (✉) • M. Eguchi-Ishimae • E. Ishii
Department of Pediatrics, Ehime University Graduate School of Medicine,
Shitsukawa, Toon 791-0295, Ehime, Japan
e-mail: maeguchi@m.ehime-u.ac.jp

1.1 Introduction

Maintenance of the hematopoietic system relies on hematopoietic stem cells (HSCs), which have the ability to produce entire blood cells in a hierarchical manner and are simultaneously capable of self-renewing. HSCs are a very rare population among bone marrow cells and usually reside in the stem cell niche, a specific microenvironment that regulates stem cell fate. Well-regulated hematopoietic regulatory networks maintain the differentiation system of HSCs and homeostasis of the blood cell production. Any change that disturbs this balance in the maintenance and function of normal HSCs can cause aberrant HSC production, resulting in malignant hematopoiesis.

Malignant hematopoiesis that is observed in leukemia is also sustained by the presence of stem cells called leukemic stem cells (LSCs). Although, like that of normal HSCs, self-renewing ability is one of the hallmarks of LSCs; the latter possesses aberrant growth advantage over normal HSCs, rapidly dominating the bone marrow space.

LSCs were originally identified in acute myeloid leukemia (AML), using xenograft models, as a distinct cell population that can initiate leukemia in immunodeficient mice [1]. LSCs arise from pre-LSCs after obtaining genetic changes that confer self-renewal ability and some growth advantages. However, these genetic changes are not sufficient to fully transform pre-LSCs to cause overt leukemia. Since the existence of LSCs has been proven, identification of additional genetic changes that transform HSC into pre-LSCs and pre-LSCs into LSCs, and the identities of LSCs, has been intensively studied because LSCs and pre-LSCs are the most suitable targets of anticancer therapies [2–5].

In this chapter, recent knowledge on HSCs and LSCs and the role of genetic changes in transforming normal HSCs into malignant LSCs will be described, mainly focusing on childhood leukemia.

1.2 Hematopoietic Stem Cells in Normal Hematopoiesis

1.2.1 Shifting Site of Hematopoiesis

Although blood cells can be regenerated throughout the individual's life, the site for hematopoiesis, namely, residence of HSCs, shifts during embryonic development (Fig. 1.1). In mammals, hematopoiesis is recognized initially in the yolk sac at the very beginning of the fetal development. This initial hematopoiesis is termed as "primitive hematopoiesis," giving rise to circulating red blood cells that provide oxygen to tissues, necessary for embryonic growth. This primitive hematopoiesis does not produce HSCs that have the ability to reconstitute hematopoiesis in the later stage [6] and is rapidly replaced by "definitive" hematopoiesis, which is responsible for the continuous production of all the mature blood cells throughout the adult life [7, 8]. Around embryonic day (E) 11 in the mouse fetus, this definitive hematopoiesis commences in the embryo proper in the aorta-gonad-mesonephros (AGM) region, an area surrounding the dorsal aorta (Fig. 1.1). Clusters of hematopoietic cells appear in
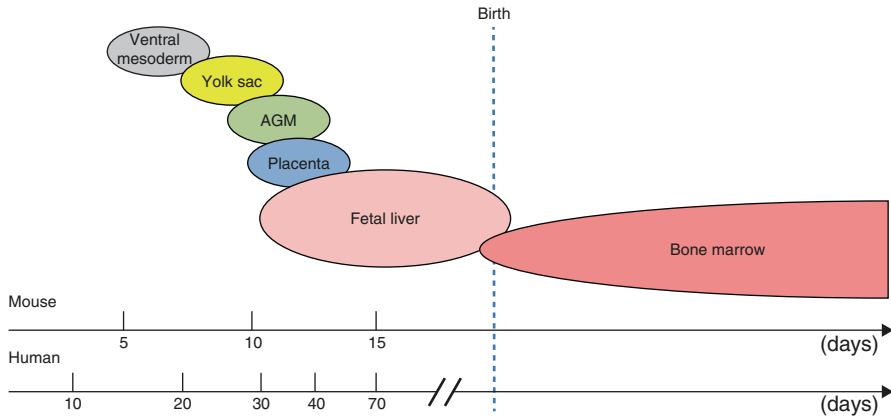
Fig. 1.1 Site of hematopoiesis during fetal development. Hematopoietic stem cells (HSCs) are thought to derive from the ventral mesoderm. The initial blood production termed as "primitive hematopoiesis" occurs in the yolk sac, producing red blood cells to supply oxygen for rapidly growing tissue. This primitive hematopoiesis is rapidly replaced by adult-type hematopoiesis termed as "definitive hematopoiesis," which produces HSCs that have the ability to give rise to all hematopoietic lineages. Definitive hematopoiesis, occurring in aorta-gonad-mesonephros (AGM) region, is also found in the placenta, where it moves from the fetal liver to the bone marrow to generate hematopoietic cells throughout life

the ventral wall following the formation of the aorta tube. High frequencies of HSCs were subsequently detected in the other sites such as umbilical arteries, indicating that the major arteries of the embryo are important sites for the emergence of definitive HSCs [9]. In addition, placenta was also shown to harbor adult-repopulating HSCs, beginning at E11 and expanding until E12.5–E13.5 of mouse embryo, containing more HSCs than the AGM [10–12]. Subsequently, accompanying the migration of HSCs, the site of definitive hematopoiesis moves onto the fetal liver, which serves as the major hematopoietic organ for the rest of the fetal development. During the fetal period, the HSC population expands in the fetal liver [13], and, finally, the site of hematopoiesis shifts to the bone marrow shortly before birth, where blood cells are produced during the entire life of the individual. Although HSCs in the fetal liver are proliferative, giving rise to both myeloid and B-lymphoid lineage cells, HSCs in the bone marrow seem to lose their proliferation tendency, to be a relatively quiescent HSC population [14–17].

1.2.2   Identification of HSCs

HSCs demonstrate long-lasting self-renewal ability and differentiate into all types of hematopoietic cells. Self-renewal is the ability of a cell to produce itself by cell division, whether symmetrical or asymmetrical, and this self-renewal ability is the essential characteristic of all stem cells, including HSCs.

HSCs are an extremely rare population, existing only one in a million bone marrow cells [18]. Owing to stem cell's self-renewal and reconstruction ability, *in vivo*

transplantation into conditioned hosts has been applied for the characterization of these stem cells. HSCs have shown self-renewal and differentiation capacity even in a single-cell level [19, 20]. In mice, HSC activity is evaluated and quantified by transplanting cells into myeloablative (usually by irradiation), syngeneic recipients. Availability of severe combined immunodeficient (SCID) mice made it possible to apply this transplantation strategy to human materials such as bone marrow and cord blood. Development of severe immunodeficient nonobese diabetic/SCID (NOD/SCID) mice and its subsequent generation of NOD/SCID/IL-2Rg null (NOG or NSG) mice allowed engraftment of a very small number of cells and greatly enhanced the isolation of HSCs [21–24]. Cells that generate all lineages but are capable of engrafting only hematopoietic lineages transiently are defined as short-term HSCs (ST-HSCs) or multipotent progenitors (MPPs), whereas HSCs that repopulate hematopoietic lineages beyond 12 weeks are defined as long-term HSCs (LT-HSCs) [20].

Lineage and differentiation stage-specific expression of surface proteins is notable in hematopoietic cells; therefore, HSCs and progenitor cells are defined by the cell surface phenotype, as described in Fig. 1.2 [20]. Prospective isolation of highly purified HSCs is made possible using monoclonal antibodies for these cell surface proteins and by precise fluorescence-activated cell sorting (FACS) [25]. Mouse HSCs were initially isolated as lineage marker-negative (Lin$^-$), c-kit$^+$, and Sca-1$^+$ (LSK) population [26, 27]. By extensively studied xenograft models, it was identified that a proportion in CD34$^-$ LSK cells, which show CD150$^+$CD48$^-$SLAM$^+$ phenotype, possesses LT-HSCs activity in mouse [25, 28]. By contrast, CD34$^+$ cells, which are found in less than 5% of hematopoietic cells, are enriched for HSCs and progenitors in humans. While phenotype of human HSCs was described as Lin$^-$CD34$^+$CD38$^-$CD90(Thy1)$^+$CD45RA$^-$ [21, 29–31], Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$, which has lost CD90 expression, represents its descendant multipotent progenitors [32]. In addition, a recent study showed an essential role of CD49f in separation of HSCs [23]. Human HSCs are currently thought to be phenotypically Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$CD49f$^+$. When HSCs give rise to MPPs, first, CD49f expression is lost, and then they differentiate into immature progenitors and mature hematopoietic cells with specific cell surface phenotype under the influence of various transcriptional controls (Fig. 1.2) [20].

Within the CD34$^+$CD38$^-$ population in human, CD90$^-$CD45RA$^-$ cells are multipotent progenitors (MPP) with transient multilineage engraftment capacity [32]. CD90$^-$CD45RA$^+$ cells show differentiation to all lymphoid lineages as well as some of the myeloid lineage without self-renewal ability, functionally similar to murine lymphoid-primed multipotent progenitors (LMPP) [33] corresponding to multi-lymphoid progenitor (MLP) in human.
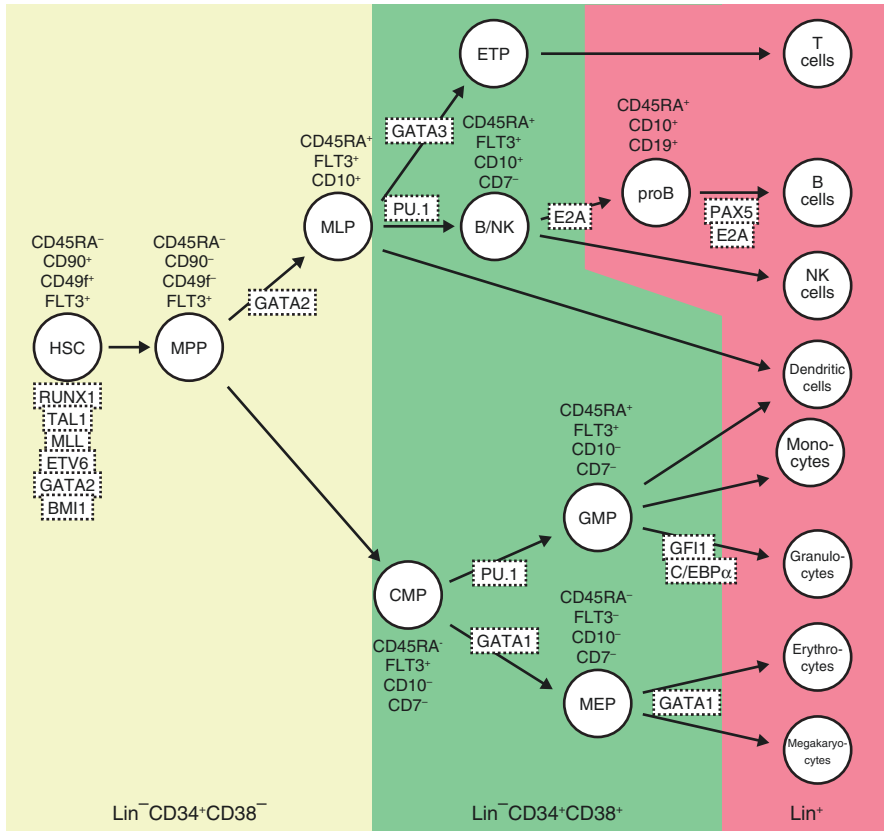
Fig. 1.2 Hematopoietic hierarchies and their regulators in human. Using specific antibodies and applying precise fluorescence-activated cell sorting (FACS) technique, HSCs, progenitor cells, and differentiated hematopoietic cells can be defined by cell surface phenotypes as shown. Transcription factors and signaling pathway known to be involved in hematopoietic cell differentiation are depicted. Hematopoietic development is blocked in the absence of many of these factors, as determined by gene knockout studies. *MPPs* multipotent progenitors, *MLP* multi-lymphoid progenitors, *ETPs* earliest thymic progenitors, *CMPs* common myeloid progenitors, *GMPs* granulocyte-macrophage progenitors, *MEP* megakaryocyte/erythroid progenitors, *Lin* cocktail of cell surface markers for all terminally differentiated hematopoietic cells. Modified from [20]

1.2.3 Transcriptional Control of HSCs: Hematopoietic Hierarchies and Lineage Determination

Blood cells are produced in developmental hierarchy, with multipotent HSCs at the apex and terminally differentiated cells at the bottom [20]. Key regulators controlling hematopoietic cell maintenance and differentiation differ in various stages of hematopoietic cell development, and many of these regulators are known to be transcription factors that play a central role in determining cell fate intracellularly. To date, more than 50

transcription factors have shown to affect HSCs survival and differentiation, forming transcriptional hierarchies active during hematopoietic differentiation (Fig. 1.2). Through the analysis of conventional gene knockout mice, some transcription factors have proven to play a crucial role in each stage of hematopoietic cell development. These crucial transcription factors have been often identified as targets of leukemia-specific gene abnormalities such as chromosome translocations, resulting in fusion gene formation.

HSCs and progenitor cells also respond to some external signals by activating a series of signaling cascades inside the cells, which may also activate other signaling pathways to form complex networks, finally activating lineage determinant transcription factor(s) [34, 35]. External signals, which work as determinants for stem cell fate decision, include interactions with surrounding cells through cell adhesion molecules and secreted molecules such as cytokines. Thus, microenvironments in the stem cell niche are critical for regulating the fate of HSCs [36, 37].

Growth factor receptor FLK1, as well as its ligand VEGF, and transcription factor TAL1 (SCL), with its protein partner LMO2, are essential factors for HSC formation and function in the early hematopoietic stage of development such as in the embryonic yolk sac. TAL1 and its protein partner LMO2 are necessary for the development of both primitive and definitive hematopoiesis because they specify blood lineage from the hemangioblast [38]. In AGM and fetal liver stage, AML1 (RUNX1), GATA2, GATA3, LMO2, TEL (ETV6) transcription factors, histone methyltransferase MLL (KMT2A), and KIT with its ligand SCF (KITLG) are required for hematopoietic cell survival, proliferation, and differentiation [35, 39–42]. ETS transcription factor TEL initiates adult hemangioblast program in the dorsal lateral plate mesoderm by switching on the FLK1 ligand VEGFA, where ETS factor FLI1 together with GATA2 ensures the expression of FLK1 [43].

The pluripotent HSCs generate mature hematopoietic cells via multipotent progenitors and committed precursors. Key regulators that control hematopoietic cell maintenance and differentiation differ in various stages of hematopoietic cell development (Fig. 1.2). During fate decision of hematopoietic cells, key lineage-restricted factors promote their own lineage differentiation and simultaneously act against factors that drive cells into other linages [35]. GATA1 promotes erythroid, megakaryocytic, and eosinophil differentiation, whereas PU.1 (SPI1) promotes myeloid differentiation in common myeloid progenitors (CMP). This GATA1 and PU.1 antagonism promotes cell differentiation to their specific lineage. This kind of lineage determination is also known between EKLF and FLI1 for erythroid and megakaryocytic lineages in megakaryocyte/erythroid progenitors (MEP), GFI1 and PU.1 for neutrophil versus monocyte differentiation in granulocyte/macrophage progenitors (GMP), and C/EBP and FOG1 for eosinophil and multipotent cell fate [35].

For B-cell development, PAX5 is required for B-cell commitment [44], and other B-cell transcription factors such as E2A and EBF1 specify gene activation toward the B-lineage. In T-cell development, NOTCH1 commits cells to T-cell lineage, whereas other transcription factors such as GATA3 support T-cell differentiation under NOTCH signaling. Further, GATA3 and T-bet (TBX21) antagonize in T-cell differentiation to promote Th1 and Th2 cells [45]. It is now known that these lineages can be reprogrammed upon expression of critical transcription factors such as GATA1, C/EBP, and GATA3 [35].

Notably, in HSCs and progenitor cells, alternative cell fate potentials are preserved by keeping simultaneous low expression of key genes for several lineages [46]. Differentiation of HSCs to certain lineage takes place by selecting and stabilizing the expression of a subset of genes, which are essential for that direction from a wide range of expression of lineage-oriented genes and, at the same time, downregulating expression of other irrelevant genes under the effects of selected dominantly expressed gene(s). Thus, to maintain a hierarchy of hematopoietic differentiation from HSCs, precise regulation of transcription factors expression is critical.

This multiple co-expression of lineage-oriented genes, such as transcription factors in HSCs and progenitor cells, is essential for maintaining multipotentiality and flexibility in cell fate decisions. Loss of expression of one lineage determinant transcription factor and upregulation of another lineage-specific gene in already lineage-committed cells can induce lineage conversion in normal hematopoiesis [46, 47] and also in leukemogenesis [48], indicating lineage plasticity.

1.2.4   Microenvironment of HSCs: The Niche

HSCs undergo a wide range of cell fate, such as self-renewal, quiescence, and differentiation into all mature blood cells. Fate of HSCs is tightly regulated by external stimuli provided by the HSC microenvironment, known as niche, and by intercellular regulatory programs. The adult bone marrow niche is best studied among various stages of fetal and adult hematopoietic development.

The major components of the niche that support HSC fate in the bone marrow are the osteoblastic and vascular niches, which lie in close proximity and are thought to influence HSCs in a cooperative manner (Fig. 1.3) [49].

HSCs exist adjacent to the osteoblasts, which align bone surface of the bone marrow, and are under the regulation of bone morphogenetic protein (BMP)—the osteoblastic niche. Various studies using mouse models showed that osteoblastic niche maintains HSCs thorough various signaling molecules, including Notch1, Tie-2, N-cadherin, and Mpl [49]. HSCs are also found to migrate to blood vessels within the bone marrow—the vascular niche. LT-HSCs in mice are defined through SLAM markers (CD150$^+$, CD41$^-$, CD48$^-$, cKit$^+$, Sca-1$^+$, lineage-) and lie adjacent to the bone marrow sinusoidal endothelial cells that express Notch ligands, Jagged-1 and Jagged-2, and KIT ligand SCF. The bone marrow also contains other stromal cells that support HSCs and hematopoietic progenitors by producing cytokines such as SCF. Cells surrounding the vasculature, CXC chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells, and Nestin$^+$ mesenchymal stem cells have been identified as the independent niche components (the perivascular niche). CAR cells exert their niche functions through SCF and CXCL12 secretion into the microenvironment [49–51], regulating the proliferation of HSCs. Nestin$^+$ cells possess the ability of multilineage differentiation into various mesenchymal cell lineages, including osteoblasts. Nestin$^+$ cells highly express chemokine/cytokines such as CXCL12, SCF, angiopoietin-1, and osteopontin that are involved in the regulation of HSCs with
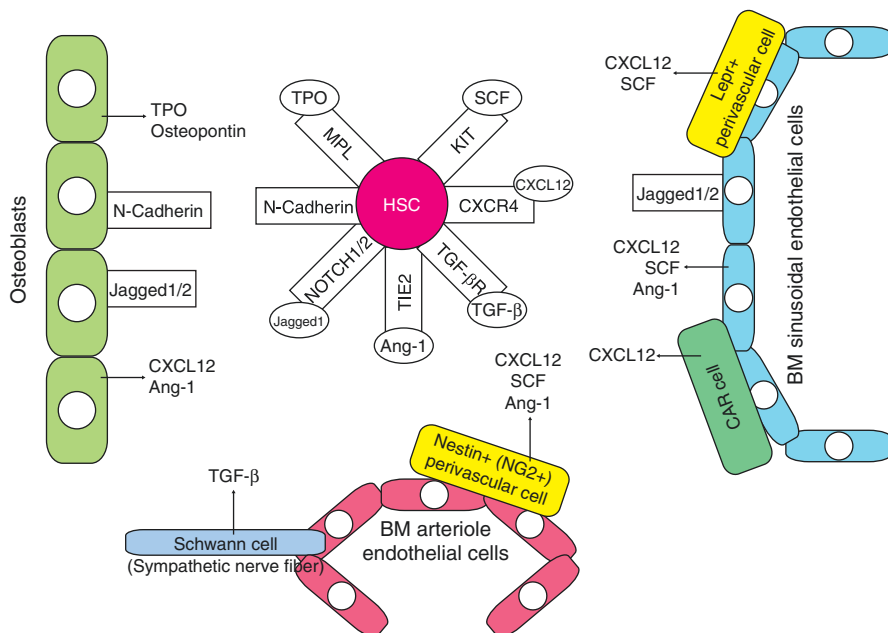
Fig. 1.3 Stem cell niche in adult bone marrow. The major components of the niche that support HSCs cell fate in the bone marrow are the osteoblastic and vascular niches that lie in close proximity and are thought to influence HSCs in a conjoined manner. HSCs exist adjacent to the osteoblasts, which align bone surface of the bone marrow, and are under the regulation of bone morphogenetic protein (BMP) (the osteoblastic niche). HSCs are also found to have migrated to blood vessels within the bone marrow—the vascular niche. Bone marrow also contains other stromal cells that support HSCs and hematopoietic progenitors by producing cytokines such as KIT ligand SCF. *CAR cell* CXCL12-abundant reticular cell, *Ang-1* angiopoietin-1, *OPN* osteopontin

other niche components. Bone marrow macrophages, non-myelinating Schwann cells [52], and extracellular matrix protein such as tenascin-C (TNC) [53] have been recently reported to possess niche function in the bone marrow.

1.3 Stem Cells in Abnormal Hematopoiesis

1.3.1 Disruption of Transcription Network Leads to Hematopoietic Malignancy

The transcription factors that are critical for hematopoiesis are mainly various classes of DNA-binding proteins, and perturbation of the function of these proteins is known to cause hematopoietic defect and malignancies. Many of these transcription factors involved in hematopoiesis are known to be disrupted by chromosome translocations, deletions, or mutations, resulting in defective normal cell

differentiation and/or acquisition of cell proliferation, which consequently leads to leukemia or other hematopoietic malignancies. Leukemia-associated chromosome translocation often produces either chimeric fusion proteins with abnormal function or deregulated expression of certain genes that drives cell transformation. Some of the common examples of chimeric fusion proteins are MLL fusion proteins such as MLL-AF9, TEL (ETV6)-associated fusion proteins such as TEL-AML1 (ETV6-RUNX1), and AML1 (RUNX1)-associated fusion proteins such as AML1-ETO (RUNX1-RUNX1T1), which are thought to affect normal HSC development leading to leukemia. Examples of chromosome translocation contributing to leukemogenesis by causing aberrant expression of transcription factors are TAL1 (SCL) and LMO2 in T-cell acute leukemia, caused by translocation with TCR genes. These aberrant transcription factors generated by the chromosome translocation cause improper activation or repression of their target genes and inhibit function of other critical factors, exerting multiple downstream effects that lead to aberrant hematopoiesis. Somatic mutation in transcription factors itself such as PU.1 and C/EBPα (CEBPA) is also known to cause myeloid leukemia [54–56]; mutations in PAX5 [57–59] and other B-cell factors lead to B-cell leukemia, and NOTCH1 mutation leads to T-cell leukemia [60].

1.3.2 Cancer Stem Cells and Xenograft Transplantation Models

It is widely accepted nowadays that most tumors, including leukemia, are sustained by a subpopulation of the self-renewing stem cells, called cancer stem cells (CSCs). The presence of these CSCs maintain the clonal architecture of cancer itself, and in clinical terms, failure to eradicate CSCs by therapy definitely causes post-therapy relapse, i.e., reconstruction of clonal architecture of cancer by CSCs.

By definition, CSC is a cell within a tumor that possesses the ability to self-renew and simultaneously produce heterogeneous lineages of cancer cells that comprise the tumor [61]. Therefore, CSCs can only be defined experimentally by demonstrating their ability to recapitulate the generation of a continuously growing tumor. This ability is best assayed using xenotransplantation models and measuring tumor formation in serially transplanted recipients [2, 19, 61, 62]. To prove the self-renewal ability of CSCs, isolated CSCs from tumor formed in the first recipient animal should generate tumor in the second recipient and continuously generate original tumor in the succeeding recipients (serial transplantation). Tumors generated in the recipient animal should maintain the heterogeneity observed in the original tumor, containing descendant cells without self-renewal ability. In this experimental system, CSCs are termed as "tumor-initiating cell (T-IC)" or leukemia-initiating cell (L-IC) in case of leukemia. AML represents the very first example of human cancers from which CSCs have been identified as SCID leukemia-initiating cell (SL-IC) [1].

Therefore, description of subpopulation of cells that sustain whole cancer cells as CSCs or LSCs is basically conceptual, in contrast to xenotransplant assay-based term T-IC or L-IC. Although these terms are considered to be largely overlapping, consistency between these two terms from different standpoints is currently a key issue [2, 62].

1.3.3 Preleukemic Stem Cells as the Reservoir of LSCs

Development of leukemia, like that of other cancers, is a multi-step process based on the consecutive acquisition of several genetic changes such as driver mutations. Generally, a single driver mutation is not enough to cause leukemia, and at least another cooperating mutation is necessary for the initiation to the formation of LSCs. There are animal models showing that some leukemia-specific fusion such as TEL-AML1 or AML-ETO is not sufficient to drive leukemia by themselves, and additional genetic hit(s) are necessary for generation of leukemia [63–67]. Therefore, on the pathway of leukemogenesis, preleukemic stem cells (pre-LSCs) and their descendant preleukemic cells, which do not harbor enough number of driver mutations, are present before proceeding to LSCs. In accord with this, expression of some leukemia-specific fusion genes is detectable in nonleukemic blood samples. The *TEL-AML1* and *AML1-ETO* fusion genes present in the cord blood are reported to be 10^{-3} to 10^{-4} in frequency, suggesting the presence of cells with significant ability of clonal expansion and indicative of preleukemic nature [68–70]. In addition, there are reports showing that not only *TEL-AML1* and *AML1-ETO* but also *BCR-ABL1* and *BCL2-IGH* fusion genes exist in a detectable amount in the blood cells of normal adults [71–75], indicating that the formation of preleukemic cells by fusion genes is a fairly common phenomenon. By contrast, frequency of *TEL-AML1* and the amount of *TEL-AML1*-positive cells in a healthy individual are reported to decrease with age, indicating that preleukemic *TEL-AML1*-positive clones do not persist in adulthood [76].

As for *MLL-AF4*, functional significance in normal individual is doubtful. Detection of *MLL-AF4* fusion gene by RT-PCR at high frequency (~25%) in the fetal liver and fetal bone marrow has been reported [77], but these findings were not subsequently confirmed by others. Although *MLL-AF4*-positive leukemia is found in high frequency in infant leukemia and is proven to be formed in utero, it is not detected in normal individual, which suggests that single *MLL-AF4* fusion may give rise to leukemic stem cell by itself, and preleukemic stage does not exist or exists only for a very short period. By contrast, there are controversial results from murine models where *MLL* fusion gene requires protracted latency before leukemic transformation [78, 79]. Additional studies are needed to draw any conclusion regarding the functional role of *MLL-AF4* in preleukemic and leukemic stem cells.

In *AML1-ETO*-positive AML, it is known that the fusion gene can be detected during remission and can also exist in the normal hematopoietic stem cell compartment [80–82]. As this *AML1-ETO*-positive hematopoietic stem cell possesses the

ability to differentiate not only to myeloid lineage but also to B-cell lineage, the existence of preleukemic stem cell is suggested. *NPM1* and *TET2* mutations, detected in high frequency in AML, are reported to exist during remission and also in the normal hematopoietic stem cell compartment, similar to that in *AML1-ETO* [83–85]. These cells do not possess second genetic changes such as *FTL3* internal tandem duplication (FLT3-ITD), indicating preleukemic potential of these cells.

The same phenomenon was observed in AML with *DNMT3A* and *IDH1/IDH2* mutations [85]. These leukemogenic driver mutations were persistently present in the patients' blood in remission. Since co-occurring mutations such as those in *NPM1* or *FLT3* were not present, these cells should be considered preleukemic, without the ability to fully transform to cause overt leukemia. In addition, *DNMT3A* mutation was also detectable in non-myeloid lineage cells, indicating multipotent HSC origin. HSCs with *DNMT3A* mutations persistently and clonally expand more effectively than normal HSCs, indicating retention of advanced self-renewal ability. These cells can be called preleukemic stem cells (pre-LSCs).

Persistence and limited expansion of *TEL-AML1*-positive cells for several years before reaching the diagnosis of ALL, and persistence of cells with *AML1-ETO*, *DNMT3A*, or *IDH1/IDH2* mutations in remission supported the presence of functional pre-LSCs. These pre-LSCs may be resistant to chemotherapy and work as a reservoir to generate another leukemic clone at relapse [86].

1.3.4   *In Utero Generation of Pre-LSCs/LSCs in Childhood Leukemia*

It is well recognized nowadays that leukemic cells in some of the childhood leukemia arise in utero [87, 88], and it was first identified by the studies of monozygotic twins with concordant leukemia, carrying identical genetic marker for chimeric fusion genes formed in utero [87, 89]. This was followed by retrospective studies using neonatal blood spots, demonstrating that common fusion genes such as *MLL-AF4* in infant ALL, *TEL-AML1* in childhood ALL, and *AML1-ETO* in childhood AML arise predominantly in utero and are present before and at the time of birth in circulating blood. In utero, origin of leukemic stem cell is proven not only in infant or childhood B-ALL with *TEL* or *MLL* fusion genes but also in ALL with high hyperdiploidy [90], T-ALL with *NOTCH1* mutation [91], and AML with *AML1-ETO* [92] usually occurring later in the childhood.

This indicates that the fusion gene-positive cells represent expanded preleukemic clones, which will remain pathologically and clinically silent in the absence of additional postnatal second genetic hits, according to the classical "two-hit" model of Knudson for noninheritable cancer in childhood (Fig. 1.4) [69, 93]. The presence of these nonleukemic, preleukemic clones is also supported by the observation of *TEL-AML1* and *AML1-ETO* fusion genes, which were shown to exist in the cord blood at a frequency of 1%, almost 100-fold higher than the actual risk of clinically diagnosed leukemia with the same fusion gene.
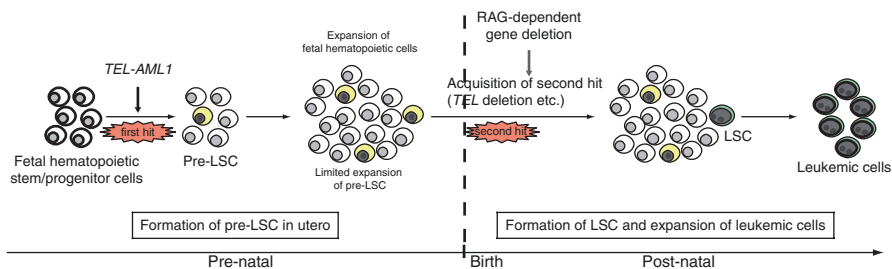
Fig. 1.4 In utero origin of ALL and multi-step leukemogenesis. A hypothesis for preleukemic and leukemic stem cell formation in *TEL-AML1*-positive ALL. A preleukemic stem cell (pre-LSC) is generated by the acquisition of *TEL-AML1* translocation, but the cell is not completely transformed. Overt leukemia develops when one or more additional genetic changes (i.e., second hit, third hit, and so on) occur in the pre-LSCs after a relatively long latency period. The commonly observed secondary genetic changes are thought to be induced by recombination activating gene (RAG)-dependent gene deletions, which may explain why LSCs exist only in B-cell precursors and not in immature hematopoietic cells in *TEL-AML1*-positive ALL. Note that the same hypothesis may be applied to ALL with *MLL-AF4*, high hyperdiploidy, *NOTCH1* mutation, and in some type of AML such as *AML1-ETO* (which also originate in utero). The cells in *yellow* indicate pre-LSCs that are formed as a result of the first genetic change. The cells in *green* indicate LSCs that acquire proliferative capacity by additional genetic change(s)

1.3.5 Genetic Profiles of Leukemic Stem Cells and Their Progeny

In the past decade, advances in next-generation sequencing technique have resulted in an enormous amount of newly identified genetic abnormalities to better understand the pathogenesis and etiology of malignant transformation of HSCs. In colon and breast cancer, 50–80 genetic changes were detected in the coding region of associated genes [94], whereas in AML and ALL, these genetic changes were much less compared to that in most other adult cancers: only around ten mutations in average [95–99]. Excluding common genetic changes such as *FLT3*, *NPM1*, and *NRAS* mutation (called driver mutation), the rest is not recurrent and thought to contribute in determining characteristic of the cells rather than in cell transformation itself (i.e., "passenger" mutation). Preleukemic cells, acquiring various random genetic and epigenetic changes, transform into leukemic stem cells, following the initiating events that are crucial for cell transformation.

There are several questions raised with respect to cell transformation by genetic changes. First, what is the cell of origin where leukemia arises? Next, how many and what kind of genetic changes are required for leukemic transformation? Finally, what clinical implications can we learn from the biology of leukemic stem cells? The origin and evolution of genetic mutation have been studied thoroughly in acute promyelocytic leukemia (APL, FAB M3 subtype) or AML without maturation (FAB M1 subtype) with normal karyotype by performing whole genome sequencing [98]. They found that the number of recurrent mutations required to generate malignant founding clone is three in M1 subtype and two in M3 subtype (one of which is *PML-RARA*). Other additional mutations obtained in these founder clones are

cooperating mutations, which contribute to disease progression and/or relapse. This result is in accord with the previous suggestion that introduction of class I mutation that cause cell proliferation and class II mutation that results in block in differentiation is sufficient for cell transformation [100, 101].

In ALL, SNP array and whole exome or whole genome analysis revealed that *TEL-AML1*-positive leukemic cells do not often carry mutations in tyrosine kinase. By contrast, deletions in genes associated with B-cell development, such as *TEL*, *CDKN2A*, *BTG1*, *PAX5*, and *RAG2*, were frequent, suggesting that gene deletion mainly contributes to cell transformation in *TEL-AML1*-positive ALL. The number of additional genetic changes required in cell transformation is six in average in *TEL-AML1*-positive ALL and one in *MLL* fusion-positive ALL. These results suggest that although some variation exists among fusion genes, the average number of mutations required for transforming preleukemic cell to leukemic stem cells is one or two in average.

1.3.6 Clonal Evolution of Preleukemic Cells to Leukemic Stem Cells

Pre-LSC is generated from hematopoietic stem/progenitor cell by acquisition of one driver mutation which could provide or enhance its self-renewal/survival capacity. Transition from pre-LSC to LSC, which gives rise to overt leukemia, usually needs acquisition of another driver mutation that can complete the leukemogenic transformation. Acquisition of mutation(s) usually occurs randomly in any gene(s) and is essential to have effective mutation in proper gene that can act as a leukemogenic driver for the generation of LSC. Because of this random, continuous acquisition of gene mutation(s), there is always a possibility that descendant of single pre-LSC clone gets different driver mutation producing different subclones of LSC in a single patient. Same phenomenon can also happen in each LSC clone resulting in further generation of different subclones of LSCs with different characteristics such as chemoresistance, phenotype, and so on. This clonal evolution, due to genomic diversity, is universally observed in cancer including leukemia [102] and among several LSC clones. LSC with outstanding growth is selected and becomes a dominant clone within leukemic cell population. The presence of these genetically distinct LSC clones often causes a clonal change at relapse or during chemotherapy.

1.4 Characteristics of Leukemic Stem Cells

1.4.1 Leukemic Stem Cells in AML

AML is an aggressive disease predominantly seen in adult, which represents poor prognosis of 30–40% 5-year overall survival. In order to study the cell of origin of leukemia, surface antigen has been used as markers to separate and enrich the LSC subset from the leukemic population by using technological advanced
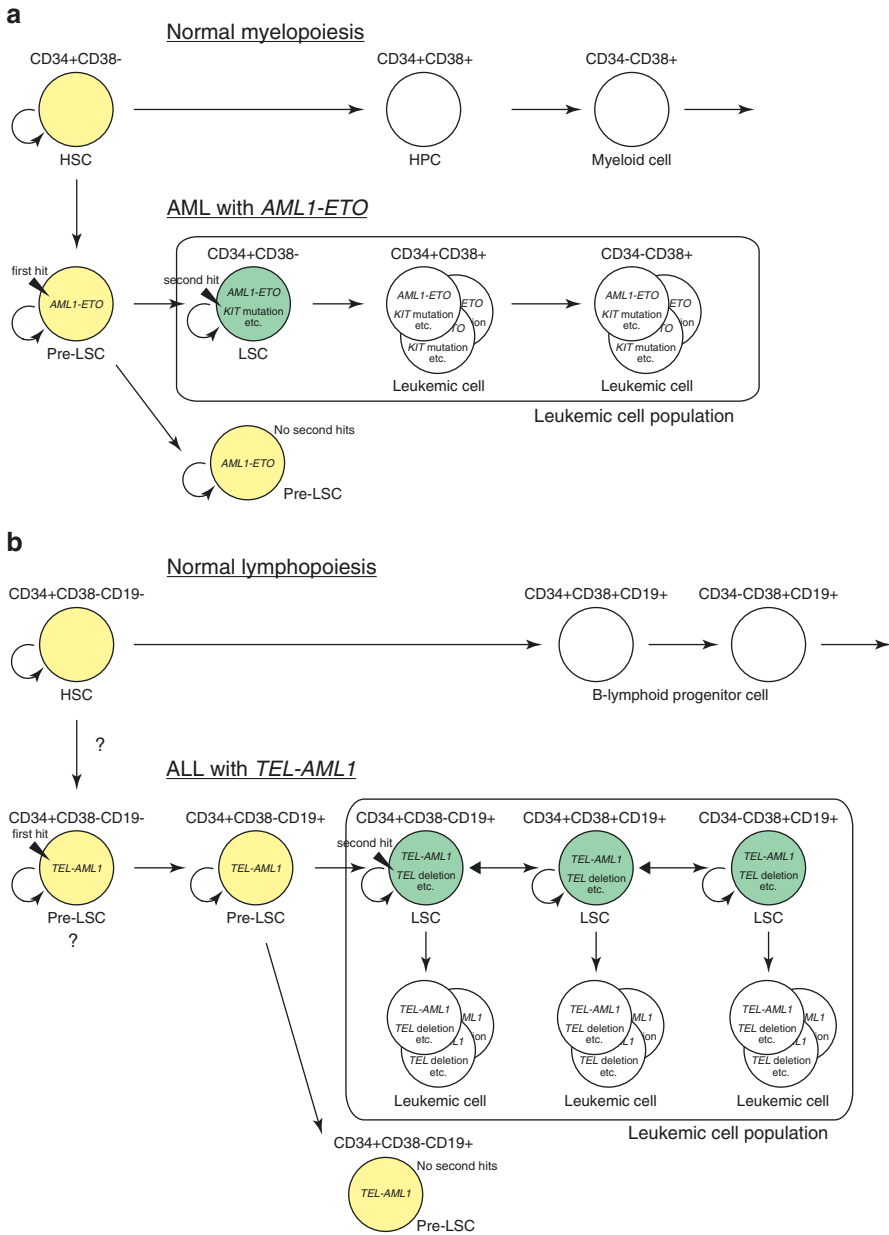
fluorescence-activated cell sorting (FACS). Regardless of the heterogenetic characteristics of the leukemic blasts, CD34$^+$CD38$^-$ phenotype similar to normal HSCs was originally defined as LSC [1] in all subtypes of AML (except APL). This phenotype was identified as cells with self-renewal and proliferative capacities that cause leukemia (L-ICs) in NOD/SCID mice, technically termed as SCID leukemia-initiating cell (SL-IC). Like the normal hematopoietic system, leukemic clone is organized as a hierarchy in AML with LSCs among the CD34$^+$CD38$^-$ cells on the apex (Fig. 1.5). The number of LSCs in AML varies among patients but is estimated to be approximately 0.2–200 in a million AML cells [1].

LSCs of CD34$^+$CD38$^-$ phenotype in AML can be further divided into CD45RA$^+$CD90$^-$ and CD45RA$^-$CD90$^-$ populations. These cells can be derived from normal HSCs population that possesses Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$ phenotype. CD45RA$^+$CD90$^-$ in LSCs resembles LMPP (lymphoid-primed multipotent progenitor), whereas CD45RA$^-$CD90$^-$ resembles GMP (granulocyte-macrophage progenitor) in normal hematopoietic development. In leukemic hierarchy, LMPP-like cells (equivalent of MLP in normal hematopoietic development) stay primitive to GMP-like cells within LSC fraction [33].

While L-ICs in SCID xenotransplant assay were enriched in the Lin$^-$CD34$^+$CD38$^-$ fraction compared with CD38$^+$ and the other fractions analyzed as originally reported, L-ICs in Lin$^-$CD34$^+$CD38$^-$ fraction represented only one-third, at the maximum, of all L-ICs present in the whole specimen in most of the AML cases. These results indicate that human LSCs in AML are enriched in Lin$^-$CD34$^+$CD38$^-$ fraction, sharing the phenotype with normal primitive hematopoietic cells but are not restricted to this fraction with immature phenotype suggesting a plasticity of the cancer stem cell phenotype [103].

Fig. 1.5 Leukemic stem cell in AML and ALL. (**a**) Hierarchy of leukemic stem cells (LSCs) in AML. AML with *AML1-ETO* fusion is shown as an example. Pre-LSCs are formed as a result of the first genetic change (i.e., generation of *AML1-ETO*). There is no overt leukemia unless pre-LSCs acquire secondary genetic changes to form LSCs. LSCs exist in immature CD34$^+$CD38$^-$ population that can give rise to its progeny and accounts for majority of leukemic cells. The cells in *yellow* indicate dormant cells that have self-renewal capacity but lack robust proliferation capacity, such as normal hematopoietic stem cells (HSCs) and pre-LSCs. The cells in *green* indicate LSCs that have acquired proliferative capacity and can thus induce leukemia. The cells in *white within the box* are the majority of leukemic cells that does not possess self-renewing capacity to induce leukemia on its own. *HPC* hematopoietic progenitor cell. (**b**) Stochastic formation of ALL stem cells. ALL with *TEL-AML1* fusion is shown as an example. In childhood ALL, pre-LSCs (shown in *yellow*) are formed by the acquisition of the first genetic change, i.e., the formation of *TEL-AML1* fusion gene, which may occur at the hematopoietic stem cell level. Pre-LSCs possibly differentiate into aberrant CD34$^+$CD38$^-$CD19$^+$ cells that are not present during normal hematopoiesis. Pre-LSCs then acquire a second genetic change during various stages of differentiation to form LSCs (shown in *green*) that have the ability to dedifferentiate and self-renew. LSCs are reported to account for 1–24% of total leukemic cells in ALL, and each LSC not only self-renew but also produce progeny that does not possess self-renewing ability (shown in *white*), resulting in the development of clinically overt leukemia. These LSCs may differentiate or dedifferentiate, making the mixed population of various phenotypes. The accurate stage of cell development where first and second hits are acquired is currently unknown

a

Normal myelopoiesis

CD34+CD38-   CD34+CD38+   CD34-CD38+

HSC   HPC   Myeloid cell

first hit

AML1-ETO

Pre-LSC

AML with *AML1-ETO*

CD34+CD38-   CD34+CD38+   CD34-CD38+

second hit

AML1-ETO
KIT mutation
etc.

LSC

AML1-ETO
KIT mutation
etc.
ETO
on
KIT mutation
etc.

Leukemic cell

AML1-ETO
KIT mutation
etc.
ETO
on
KIT mutation
etc.

Leukemic cell

Leukemic cell population

No second hits

AML1-ETO

Pre-LSC

b

Normal lymphopoiesis

CD34+CD38-CD19-   CD34+CD38+CD19+   CD34-CD38+CD19+

HSC   B-lymphoid progenitor cell

?

ALL with *TEL-AML1*

CD34+CD38-CD19-   CD34+CD38-CD19+   CD34+CD38-CD19+   CD34+CD38+CD19+   CD34-CD38+CD19+
first hit

TEL-AML1   TEL-AML1   second hit
                                         TEL-AML1
                                         TEL deletion
                                         etc.

TEL-AML1
TEL deletion
etc.

TEL-AML1
TEL deletion
etc.

Pre-LSC   Pre-LSC   LSC   LSC   LSC
?

TEL-AML1
TEL deletion
etc.
ML1
on
TEL deletion
etc.

Leukemic cell

TEL-AML1
TEL deletion
etc.
ML1
on
TEL deletion
etc.

Leukemic cell

TEL-AML1
TEL deletion
etc.
ML1
on
TEL deletion
etc.

Leukemic cell

Leukemic cell population

CD34+CD38-CD19+

No second hits

TEL-AML1

Pre-LSC

In AML, while LSCs may arise from normal HSCs by acquiring leukemogenic driver mutation(s), some of the LSCs may arise from the differentiated, myeloid-committed progenitors by recovering self-renewal capacity, as a result of obtained driver mutation(s). Some driver mutations do not have transforming potential unless they occur in hematopoietic stem cells. Expression of *BCR-ABL1* fusion gene must occur in HSCs to cause CML in murine models, providing evidence that LSCs reside at the HSC level [104]. As HSCs originally have self-renewing capacity, *AML1-ETO*, which blocks cell differentiation, can transform HSCs into LSCs with other driver mutations such as *RAS* and *FLT3* and provide growth advantage. On the other hand, *MLL-ENL* [105], *MOZ-TIF2* [104], *MLL-AF9* [106], and *PML-RARA* [107, 108] can provide self-renewing capacity to the cells, initiating leukemia even when it occurred in more lineage-restricted progenitors.

In case of *AML1-ETO*-positive AML, isolated CD34$^+$CD90$^-$CD38$^-$ cells with *AML1-ETO* fusion give rise to multilineage progenitor with normal differentiation capacity as shown in vitro hematopoietic progenitor assay, whereas more mature CD34$^+$CD90$^-$CD38$^+$ with *AML1-ETO* form blast colonies [82]. These results indicate that *AML1-ETO* is formed in primitive stem cell, but subsequent events occur in committed progenitor level, giving rise to LSCs.

Not much is known about LSCs of childhood AML. From LSCs with disease-type-specific fusion genes, such as *AML1-ETO*, one can speculate that the rules obtained from adult AML can be applied also in childhood AML. On the other hand, mutation in epigenetic regulator, such as *DNMT3A*, *IDH1*, and *IDH2*, are rare in childhood AML [109]. It has been reported that gene mutation in HSCs accumulate with age and that most of the mutations present in AML genomes were already present in the hematopoietic cell that was "transformed" by the initiating mutation in adult AML [98]. As young HSCs do not carry as much number of mutations as the old HSCs, some mechanism may vary in transformation of the young HSCs.

1.4.2   Leukemic Stem Cells in Acute Lymphoid Leukemia (ALL)

ALL is the most common hematologic malignancy in childhood. Generally leukemic cells respond well to combination chemotherapy resulting in over 80% of 5-year event-free survival in children.

Leukemic stem cells in ALL was first described in *BCR-ABL1*-positive ALL, and similar to AML, L-IC activity was identified in immature Lin$^-$CD34$^+$CD38$^-$ population by NOD/SCID xenotransplantation assay [110]. However, several studies showed different views about L-IC activity in ALL.

TEL-AML1 is the most frequent fusion gene in childhood ALL formed as a result of chromosome translocation t(12;21)(p13;q22). So far, several xenotransplantation studies of *TEL-AML1*-positive ALL were reported with varying results. Early report indicated that, in at least some of the ALL patients with *TEL-AML1*, CD34$^+$CD19$^-$ cells, but not CD19$^+$ cells, showed L-IC activity [111]. However, later report

contradicted those early results and showed that CD19+ cells are the main source of L-IC activity [112–114]. Some reports even denied the presence of L-IC activity in CD34+CD19− cells [113]. FISH (fluorescence *in situ* hybridization) analysis of sorted leukemic cells partly supported the absence of L-IC activity in immature CD34+CD19− cells in *TEL-AML1*-positive ALL and also showed an absence of *TEL-AML1* fusion on CD34+CD19− cell population [115].

As aforementioned, *TEL-AML1* is frequently (almost always) generated in utero, and *TEL-AML1*-harboring hematopoietic cells exist as preleukemic (stem) cells for several years until overt leukemia develops after acquisition of additional genetic mutations sufficient for transformation. In the detailed study of twin leukemia case, *TEL-AML1*-positive preleukemic cells existed in CD34+CD38−CD19+CD10− population and were also present in nonleukemic twin as well as the leukemic twin [114]. These findings are also comparable with LSCs in CD19+ cell population in *TEL-AML1*-positive ALL, suggesting CD19+ B-lineage-committed cells as the target for the leukemogenic transformation by *TEL-AML1* fusion.

On the other hand, some of the experimental mouse model of *TEL-AML1*-positive leukemia showed that immature hematopoietic stem cell is the target of leukemogenic transformation by *TEL-AML1* [116]. The fact that *TEL-AML1* needs to be introduced into hematopoietic stem cells, not into lymphocyte-primed progenitors, to cause overt leukemia in conditional knock-in mouse *in vivo* simply indicates that the immature stem cells, rather than CD19-positive B-primed progenitor, may be the origin of LSCs in *TEL-AML1*-positive ALL.

In *TEL-AML1*-positive ALL, most of the additional genetic changes that are necessary for full leukemic transformation are thought to be derived by aberrant activity of V(D)J-recombinase, RAG1 and RAG2 enzymes, that are active only after commitment to lymphoid lineage [117]. As a differentiating factor, block and self-renewing capacity conferred by *TEL-AML1* fusion are only valid in B-lymphoid lineage progenitor cells [116], and final transformation may take place at the B-lymphocyte-primed progenitor stage, even if *TEL-AML1* is generated at the more immature stem cell stage.

Rearrangement of *MLL*, resulting in formation of *MLL* fusion gene, is frequently observed in ALL as well as AML and is especially frequent in leukemia during infant period. Also, there are some discrepancies between reports concerning population with LSC activity: in early reports, immature CD34+CD19− cells were reported to have L-IC activity [113], but in later reports [118], CD19+ cells were identified as cells with repopulating potential in most of them [113, 118, 119]. Further detailed analysis is required to understand the nature of LSCs in *MLL*-rearranged ALL.

De novo ALL with minor *BCR-ABL1* fusion, which is observed in only ALL, shows that LSCs exist in CD19+ B-lineage progenitor, just like *TEL-AML1*-positive ALL, whereas CD34+CD19− immature population seems to have no leukemic activity [112]. On the contrary, ALL with major *BCR-ABL1* fusion, which has its breakpoint within major *BCR* region and causes chronic myeloid leukemia (CML) and ALL, has its LSC activity in immature CD34+CD19− population, CD19+ leukemic population, and even in CD33+CD19− myeloid population [112]. However, CD19−

non-lymphoid cells, isolated from ALL with major *BCR-ABL1*, never recapitulate the original ALL in xenotransplantation assay [112], indicating that some additional driver mutation generated in CD19+ cells is necessary to cause ALL in *BCR-ABL1*-positive ALL, like *TEL-AML1*- or *MLL* fusion-positive ALL.

In *TEL-AML1*-positive ALL, L-IC is mainly present in the more mature CD19+ population rather than in lineage marker-negative (Lin-) immature population. On the other hand, in aggressive ALL with *MLL-AF4* or *BCR-ABL1*, L-IC activity is observed in CD34+CD38−CD19− immature population as well as in CD19+ population. Although these contradicting results may be simply due to the difference in methods used for transplantation (i.e., intravenous or intrafemoral), or due to the difference in the immunodeficient mice used for the assay, or even due to the different leukemogenic pathway provided by the fusion genes itself, these results may indicate the heterogeneity of LSCs in ALL. At any rate, retention of LSC activity in CD19+ cells, the descendant of hematopoietic stem cells without any robust self-renewal capacity in normal circumstances, is the major characteristic of ALL in most cases.

One more significant difference of LSCs between AML and ALL is LSCs frequency among leukemic cell population. In xenotransplantation assay, estimation of cells with LSC activity measured by limiting dilution assay showed around 1% of frequency of LSC in most of the primary ALL cases and cell lines that were analyzed. However, one primary case showed as high as 24% of LSC frequency [120]. Considering that the frequency of LSCs in AML was reported to be less than 0.1% in most of the reports [1, 121, 122], LSCs in ALL exist at much higher frequency.

The hierarchy model, in which the rare population of LSCs reside at the top of hierarchy sustaining leukemic cells, well explain the LSC model in AML but does not necessarily fit to the characteristics of LSCs observed in ALL. Considering the fact that CD19+ cell fraction at various stages of differentiation have the LSC potential in ALL and the fact that relatively higher frequency of LSCs exist, the stochastic model rather than the hierarchy model better explains the characteristics of LSCs in ALL (Fig. 1.5).

1.4.3   Clinical Implications and Future Directions

Understanding the role and characteristics of LSCs and their normal counterpart HSCs will not only expand our knowledge but can also lead to development of novel therapies or strategies to fight leukemia. As the prognosis improves along with development of new and stronger chemotherapies and radiotherapies against hematopoietic malignancies, accompanying complications resulting from these intensive therapies in surviving children are also becoming more severe and causing decline in quality of life (QOL). Revealing the mechanism underlying stem cell transformation and its sequential event toward leukemogenesis is essential to develop novel targeted therapies and improve the long-term QOL of these patients.
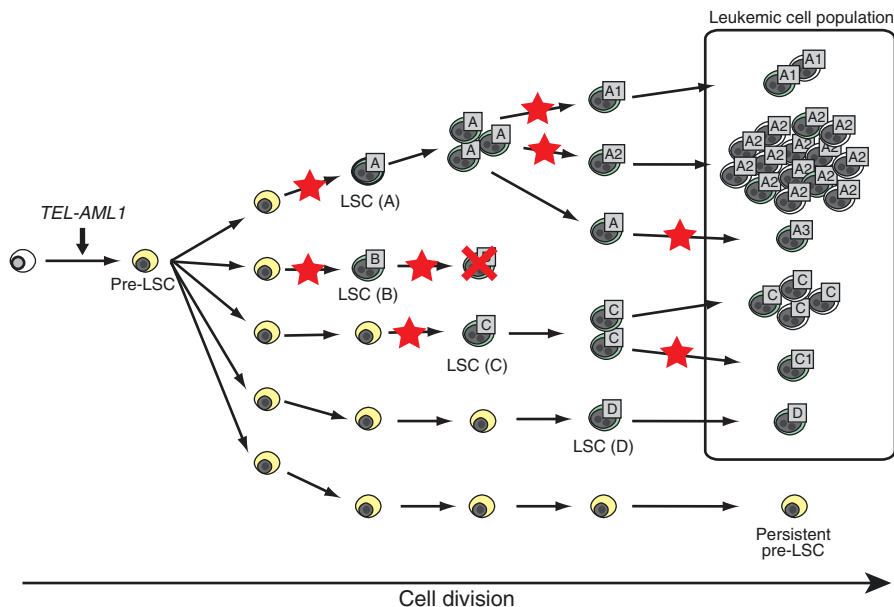
Fig. 1.6 Clonal evolution of ALL. Clonal evolution of ALL based on the successive acquisition of genetic changes. The hematopoietic stem cell, which has acquired the first genetic change, leads to leukemogenesis such as *TEL-AML1* and stays as dormant preleukemic stem cell (pre-LSC) until the second genetic event occurs (*red star*). Such pre-LSCs may show limited expansion. Each pre-LSC may acquire different additional genetic change(s) eventually forming several leukemic stem cell (LSC) clones (A–D) and its progeny with addition of another genetic change(s) (A1, A2, A3, and C1) at various stages of differentiation. In *TEL-AML1*-positive ALL, most of the additional genetic changes are deletions that have resulted in inactivation of tumor suppressors or B-lymphopoiesis-related transcriptional factors. These deletions are often due to aberrant activity of V(D)J recombinase RAG proteins [102, 117]. Since RAG proteins are continuously active in pre-LSCs and in LSCs that are already committed to the B-lineage, additional genetic changes may generate continuously and randomly in any cell. This results in a mixture of genetically heterogeneous LSCs and their progenies in a single patient with ALL. Common additional genetic changes in *TEL-AML1*-positive ALL include loss of normal *TEL* allele or *CDKN2A* and acquisition of additional *AML1* or *TEL-AML1* fusion gene [102, 117]. Most leukemic cells can be eliminated by anticancer chemotherapy, but resistant clones may result from the minor LSC clones that survived the intensive chemotherapy or pre-LSC that eventually acquire other additional genetic change(s)

Leukemia develops as a result of multiple genetic and epigenetic hits, some of which drive malignant cell transformation, growth, and survival and some contribute to the characteristics changes in the malignant cells, sometimes conferring resistance to treatment (Fig. 1.6). In a way, it resembles Darwinian evolution, i.e., subclones that have suitable characteristics for surviving in the existing selective pressure can expand [123]. LSCs exist in the apex of these malignant clones. In contrast to AML, in which LSCs supply malignant clone in a hierarchal manner, LSCs in childhood ALL are formed in a stochastic manner, producing various LSCs that can proliferate and self-renew. In this case, molecular targeting therapy must target the common molecule that promotes the main driver genetic changes, which

the LSCs share in common. To save patients from therapy-resistant disease, it is important to identify such molecules and clarify the signal transduction pathways that support LSC survival.

References

1. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730–7.
2. Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV, Chomienne C, Ishikawa F, Schuringa JJ, Stassi G, et al. Cancer stem cell definitions and terminology: the devil is in the details. Nat Rev Cancer. 2012;12(11):767–75.
3. Corces-Zimmerman MR, Majeti R. Pre-leukemic evolution of hematopoietic stem cells: the importance of early mutations in leukemogenesis. Leukemia. 2014;28(12):2276–82. doi:10.1038/leu.2014.211.
4. Stuckey DW, Shah K. Stem cell-based therapies for cancer treatment: separating hope from hype. Nat Rev Cancer. 2014;14(10):683–91. doi:10.1038/nrc3798.
5. Wang T, Shigdar S, Gantier MP, Hou Y, Wang L, Li Y, Shamaileh HA, Yin W, Zhou SF, Zhao X, Duan W. Cancer stem cell targeted therapy: progress amid controversies. Oncotarget. 2015;6(42):44191–206. doi:10.18632/oncotarget.6176.
6. Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. Immunity. 2001;15(3):477–85.
7. Lemischka IR. Clonal, in vivo behavior of the totipotent hematopoietic stem cell. Semin Immunol. 1991;3(6):349–55.
8. Spangrude GJ, Smith L, Uchida N, Ikuta K, Heimfeld S, Friedman J, Weissman IL. Mouse hematopoietic stem cells. Blood. 1991;78(6):1395–402.
9. de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 2000;19(11):2465–74. doi:10.1093/emboj/19.11.2465.
10. Gekas C, Dieterlen-Lievre F, Orkin SH, Mikkola HK. The placenta is a niche for hematopoietic stem cells. Dev Cell. 2005;8(3):365–75. doi:10.1016/j.devcel.2004.12.016.
11. Mikkola HK, Gekas C, Orkin SH, Dieterlen-Lievre F. Placenta as a site for hematopoietic stem cell development. Exp Hematol. 2005;33(9):1048–54. doi:10.1016/j.exphem.2005.06.011.
12. Ottersbach K, Dzierzak E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Dev Cell. 2005;8(3):377–87. doi:10.1016/j.devcel.2005.02.001.
13. Kumaravelu P, Hook L, Morrison AM, Ure J, Zhao S, Zuyev S, Ansell J, Medvinsky A. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. Development. 2002;129(21):4891–9.
14. Wilpshaar J, Bhatia M, Kanhai HH, Breese R, Heilman DK, Johnson CS, Falkenburg JH, Srour EF. Engraftment potential of human fetal hematopoietic cells in NOD/SCID mice is not restricted to mitotically quiescent cells. Blood. 2002;100(1):120–7.
15. Wilpshaar J, Falkenburg JH, Tong X, Noort WA, Breese R, Heilman D, Kanhai H, Orschell-Traycoff CM, Srour EF. Similar repopulating capacity of mitotically active and resting umbilical cord blood CD34(+) cells in NOD/SCID mice. Blood. 2000;96(6):2100–7.
16. Wilpshaar J, Noort WA, Kanhai HH, Willemze R, Falkenburg JH. Engraftment potential into NOD/SCID mice of CD34+ cells derived from human fetal liver as compared to fetal bone marrow. Haematologica. 2001;86(10):1021–8.
17. Gothot A, Pyatt R, McMahel J, Rice S, Srour EF. Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0/G1 phase of the cell cycle. Blood. 1997;90(11):4384–93.

18. Wang JCY, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood. 1997;89(11):3919–24.

19. Goyama S, Wunderlich M, Mulloy JC. Xenograft models for normal and malignant stem cells. Blood. 2015;125(17):2630–40. doi:10.1182/blood-2014-11-570218.

20. Doulatov S, Notta F, Laurenti E, Dick John E. Hematopoiesis: a human perspective. Cell Stem Cell. 2012;10(2):120–36. doi:10.1016/j.stem.2012.01.006.

21. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A. 1997;94(10):5320–5.

22. Cashman JD, Lapidot T, Wang JC, Doedens M, Shultz LD, Lansdorp P, Dick JE, Eaves CJ. Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. Blood. 1997;89(12):4307–16.

23. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science. 2011;333(6039):218–21. doi:10.1126/science.1201219.

24. Hogan CJ, Shpall EJ, Keller G. Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. Proc Natl Acad Sci U S A. 2002;99(1):413–8. doi:10.1073/pnas.012336799.

25. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121(7):1109–21. doi:10.1016/j.cell.2005.05.026.

26. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241(4861):58–62.

27. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A. 1992;89(4):1502–6.

28. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 1996;273(5272):242–5.

29. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med. 1998;4(9):1038–45. doi:10.1038/2023.

30. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci U S A. 1997;94(18):9836–41.

31. Lansdorp PM, Sutherland HJ, Eaves CJ. Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. J Exp Med. 1990;172(1):363–6.

32. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007;1(6):635–45. doi:10.1016/j.stem.2007.10.001.

33. Goardon N, Marchi E, Atzberger A, Quek L, Schuh A, Soneji S, Woll P, Mead A, Alford KA, Rout R, Chaudhury S, Gilkes A, Knapper S, Beldjord K, Begum S, Rose S, Geddes N, Griffiths M, Standen G, Sternberg A, Cavenagh J, Hunter H, Bowen D, Killick S, Robinson L, Price A, Macintyre E, Virgo P, Burnett A, Craddock C, Enver T, Jacobsen SE, Porcher C, Vyas P. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell. 2011;19(1):138–52. doi:10.1016/j.ccr.2010.12.012.

34. Pina C, Enver T. Differential contributions of haematopoietic stem cells to foetal and adult haematopoiesis: insights from functional analysis of transcriptional regulators. Oncogene. 2007;26(47):6750–65. doi:10.1038/sj.onc.1210759.

35. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132(4):631–44. doi:10.1016/j.cell.2008.01.025.

56. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009;113(13):3088–91. doi:10.1182/blood-2008-09-179895.

57. Familiades J, Bousquet M, Lafage-Pochitaloff M, Bene MC, Beldjord K, De Vos J, Dastugue N, Coyaud E, Struski S, Quelen C, Prade-Houdellier N, Dobbelstein S, Cayuela JM, Soulier J, Grardel N, Preudhomme C, Cave H, Blanchet O, Lheritier V, Delannoy A, Chalandon Y, Ifrah N, Pigneux A, Brousset P, Macintyre EA, Huguet F, Dombret H, Broccardo C, Delabesse E. PAX5 mutations occur frequently in adult B-cell progenitor acute lymphoblastic leukemia and PAX5 haploinsufficiency is associated with BCR-ABL1 and TCF3-PBX1 fusion genes: a GRAALL study. Leukemia. 2009;23(11):1989–98. doi:10.1038/leu.2009.135.

58. Shah S, Schrader KA, Waanders E, Timms AE, Vijai J, Miething C, Wechsler J, Yang J, Hayes J, Klein RJ, Zhang J, Wei L, Wu G, Rusch M, Nagahawatte P, Ma J, Chen SC, Song G, Cheng J, Meyers P, Bhojwani D, Jhanwar S, Maslak P, Fleisher M, Littman J, Offit L, Rau-Murthy R, Fleischut MH, Corines M, Murali R, Gao X, Manschreck C, Kitzing T, Murty VV, Raimondi SC, Kuiper RP, Simons A, Schiffman JD, Onel K, Plon SE, Wheeler DA, Ritter D, Ziegler DS, Tucker K, Sutton R, Chenevix-Trench G, Li J, Huntsman DG, Hansford S, Senz J, Walsh T, Lee M, Hahn CN, Roberts KG, King MC, Lo SM, Levine RL, Viale A, Socci ND, Nathanson KL, Scott HS, Daly M, Lipkin SM, Lowe SW, Downing JR, Altshuler D, Sandlund JT, Horwitz MS, Mulligan CG, Offit K. A recurrent germline PAX5 mutation confers susceptibility to pre-B cell acute lymphoblastic leukemia. Nat Genet. 2013;45(10):1226–31. doi:10.1038/ng.2754.

59. Hyde RK, Liu PP. Germline PAX5 mutations and B cell leukemia. Nat Genet. 2013;45(10):1104–5. doi:10.1038/ng.2778.

60. Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269–71. doi:10.1126/science.1102160.

61. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells – perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res. 2006;66(19):9339–44. doi:10.1158/0008-5472.CAN-06-3126.

62. Kreso A, Dick JE. Evolution of the cancer stem cell model. Cell Stem Cell. 2014;14(3):275–91. doi:10.1016/j.stem.2014.02.006.

63. Yuan Y, Zhou L, Miyamoto T, Iwasaki H, Harakawa N, Hetherington CJ, Burel SA, Lagasse E, Weissman IL, Akashi K, Zhang DE. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. Proc Natl Acad Sci U S A. 2001;98(18):10398–403. doi:10.1073/pnas.171321298.

64. Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell. 2002;1(1):63–74.

65. Grisolano JL, O'Neal J, Cain J, Tomasson MH. An activated receptor tyrosine kinase, TEL/PDGFbetaR, cooperates with AML1/ETO to induce acute myeloid leukemia in mice. Proc Natl Acad Sci U S A. 2003;100(16):9506–11. doi:10.1073/pnas.1531730100.

66. van der Weyden L, Giotopoulos G, Rust AG, Matheson LS, van Delft FW, Kong J, Corcoran AE, Greaves MF, Mulligan CG, Huntly BJ, Adams DJ. Modeling the evolution of ETV6-RUNX1-induced B-cell precursor acute lymphoblastic leukemia in mice. Blood. 2011;118(4):1041–51. doi:10.1182/blood-2011-02-338848.

67. Li M, Jones L, Gaillard C, Binnewies M, Ochoa R, Garcia E, Lam V, Wei G, Yang W, Lobe C, Hermiston M, Passegue E, Kogan SC. Initially disadvantaged, TEL-AML1 cells expand and initiate leukemia in response to irradiation and cooperating mutations. Leukemia. 2013;27(7):1570–3. doi:10.1038/leu.2013.15.

68. Eguchi-Ishimae M, Eguchi M, Ishii E, Miyazaki S, Ueda K, Kamada N, Mizutani S. Breakage and fusion of the TEL (ETV6) gene in immature B lymphocytes induced by apoptogenic signals. Blood. 2001;97(3):737–43.

69. Mori H, Colman SM, Xiao Z, Ford AM, Healy LE, Donaldson C, Hows JM, Navarrete C, Greaves M. Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci U S A. 2002;99(12):8242–7. doi:10.1073/pnas.112218799.

70. Zuna J, Madzo J, Krejci O, Zemanova Z, Kalinova M, Muzikova K, Zapotocky M, Starkova J, Hrusak O, Horak J, Trka J. ETV6/RUNX1 (TEL/AML1) is a frequent prenatal first hit in childhood leukemia. Blood. 2011;117(1):368–9. doi:10.1182/blood-2010-09-309070.

71. Limpens J, Stad R, Vos C, de Vlaam C, de Jong D, van Ommen GJ, Schuuring E, Kluin PM. Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. Blood. 1995;85(9):2528–36.

72. Liu Y, Hernandez AM, Shibata D, Cortopassi GA. BCL2 translocation frequency rises with age in humans. Proc Natl Acad Sci U S A. 1994;91(19):8910–4.

73. Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. Blood. 1995;86(8):3118–22.

74. Maes B, Vanhentenrijk V, Wlodarska I, Cools J, Peeters B, Marynen P, De Wolf-Peeters C. The NPM-ALK and the ATIC-ALK fusion genes can be detected in non-neoplastic cells. Am J Pathol. 2001;158(6):2185–93.

75. Quina AS, Gameiro P, Sa da Costa M, Telhada M, Parreira L. PML-RARA fusion transcripts in irradiated and normal hematopoietic cells. Genes Chromosomes Cancer. 2000;29(3):266–75.

76. Olsen M, Madsen HO, Hjalgrim H, Gregers J, Rostgaard K, Schmiegelow K. Preleukemic TEL-AML1-positive clones at cell level of 10(−3) to 10(−4) do not persist into adulthood. J Pediatr Hematol Oncol. 2006;28(11):734–40. doi:10.1097/01.mph.0000243652.33561.0f.

77. Uckun FM, Herman-Hatten K, Crotty ML, Sensel MG, Sather HN, Tuel-Ahlgren L, Sarquis MB, Bostrom B, Nachman JB, Steinherz PG, Gaynon PS, Heerema N. Clinical significance of MLL-AF4 fusion transcript expression in the absence of a cytogenetically detectable t(4;11)(q21;q23) chromosomal translocation. Blood. 1998;92(3):810–21.

78. Chen W, Li Q, Hudson WA, Kumar A, Kirchhof N, Kersey JH. A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy. Blood. 2006;108(2):669–77. doi:10.1182/blood-2005-08-3498.

79. Metzler M, Forster A, Pannell R, Arends MJ, Daser A, Lobato MN, Rabbitts TH. A conditional model of MLL-AF4 B-cell tumourigenesis using invertor technology. Oncogene. 2006;25(22):3093–103. doi:10.1038/sj.onc.1209636.

80. Kusec R, Laczika K, Knobl P, Friedl J, Greinix H, Kahls P, Linkesch W, Schwarzinger I, Mitterbauer G, Purtscher B, et al. AML1/ETO fusion mRNA can be detected in remission blood samples of all patients with t(8;21) acute myeloid leukemia after chemotherapy or autologous bone marrow transplantation. Leukemia. 1994;8(5):735–9.

81. Miyamoto T, Nagafuji K, Akashi K, Harada M, Kyo T, Akashi T, Takenaka K, Mizuno S, Gondo H, Okamura T, Dohy H, Niho Y. Persistence of multipotent progenitors expressing AML1/ETO transcripts in long-term remission patients with t(8;21) acute myelogenous leukemia. Blood. 1996;87(11):4789–96.

82. Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. Proc Natl Acad Sci U S A. 2000;97(13):7521–6.

83. Jan M, Majeti R. Clonal evolution of acute leukemia genomes. Oncogene. 2013;32(2):135–40. doi:10.1038/onc.2012.48.

84. Ploen GG, Nederby L, Guldberg P, Hansen M, Ebbesen LH, Jensen UB, Hokland P, Aggerholm A. Persistence of DNMT3A mutations at long-term remission in adult patients with AML. Br J Haematol. 2014;167(4):478–86. doi:10.1111/bjh.13062.

85. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, Kennedy JA, Schimmer AD, Schuh AC, Yee KW, McLeod JL, Doedens M, Medeiros JJ, Marke R, Kim HJ, Lee K, McPherson JD, Hudson TJ, Consortium HP-LGP, Brown AM, Yousif F, Trinh QM, Stein LD,

Minden MD, Wang JC, Dick JE. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014;506(7488):328–33. doi:10.1038/nature13038.

86. Seton-Rogers S. Leukaemia: a pre-leukaemic reservoir. Nat Rev Cancer. 2014;14(4):212–2. doi:10.1038/nrc3706.

87. Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. Blood. 2003;102(7):2321–33. doi:10.1182/blood-2002-12-3817.

88. Greaves MF, Wiemels J. Origins of chromosome translocations in childhood leukaemia. Nat Rev Cancer. 2003;3(9):639–49. doi:10.1038/nrc1164.

89. Greaves M. Pre-natal origins of childhood leukemia. Rev Clin Exp Hematol. 2003;7(3):233–45.

90. Maia AT, van der Velden VH, Harrison CJ, Szczepanski T, Williams MD, Griffiths MJ, van Dongen JJ, Greaves MF. Prenatal origin of hyperdiploid acute lymphoblastic leukemia in identical twins. Leukemia. 2003;17(11):2202–6. doi:10.1038/sj.leu.2403101.

91. Eguchi-Ishimae M, Eguchi M, Kempski H, Greaves M. NOTCH1 mutation can be an early, prenatal genetic event in T-ALL. Blood. 2008;111(1):376–8. doi:10.1182/blood-2007-02-074690.

92. Wiemels JL, Xiao Z, Buffler PA, Maia AT, Ma X, Dicks BM, Smith MT, Zhang L, Feusner J, Wiencke J, Pritchard-Jones K, Kempski H, Greaves M. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. Blood. 2002;99(10):3801–5.

93. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer. 2001;1(2):157–62. doi:10.1038/35101031.

94. Fox EJ, Salk JJ, Loeb LA. Cancer genome sequencing—an interim analysis. Cancer Res. 2009;69(12):4948–50. doi:10.1158/0008-5472.CAN-09-1231. pii:1158/0008-5472.CAN-09-1231.

95. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363(25):2424–33. doi:10.1056/NEJMoa1005143.

96. The-Cancer-Genome-Atlas-Research-Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368(22):2059–74. doi:10.1056/NEJMoa1301689.

97. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, Koboldt DC, Fulton RS, Delehaunty KD, McGrath SD, Fulton LA, Locke DP, Magrini VJ, Abbott RM, Vickery TL, Reed JS, Robinson JS, Wylie T, Smith SM, Carmichael L, Eldred JM, Harris CC, Walker J, Peck JB, Du F, Dukes AF, Sanderson GE, Brummett AM, Clark E, McMichael JF, Meyer RJ, Schindler JK, Pohl CS, Wallis JW, Shi X, Lin L, Schmidt H, Tang Y, Haipek C, Wiechert ME, Ivy JV, Kalicki J, Elliott G, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson MA, Baty J, Heath S, Shannon WD, Nagarajan R, Link DC, Walter MJ, Graubert TA, DiPersio JF, Wilson RK, Ley TJ. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009;361(11):1058–66. doi:10.1056/NEJMoa0903840.

98. Welch John S, Ley Timothy J, Link Daniel C, Miller Christopher A, Larson David E, Koboldt Daniel C, Wartman Lukas D, Lamprecht Tamara L, Liu F, Xia J, Kandoth C, Fulton Robert S, McLellan Michael D, Dooling David J, Wallis John W, Chen K, Harris Christopher C, Schmidt Heather K, Kalicki-Veizer Joelle M, Lu C, Zhang Q, Lin L, O'Laughlin Michelle D, McMichael Joshua F, Delehaunty Kim D, Fulton Lucinda A, Magrini Vincent J, Mcgrath Sean D, Demeter Ryan T, Vickery Tammi L, Hundal J, Cook Lisa L, Swift Gary W, Reed Jerry P, Alldredge Patricia A, Wylie Todd N, Walker Jason R, Watson Mark A, Heath Sharon E, Shannon William D, Varghese N, Nagarajan R, Payton Jacqueline E, Baty Jack D, Kulkarni S, Klco Jeffery M, Tomasson Michael H, Westervelt P, Walter Matthew J, Graubert Timothy

A, DiPersio John F, Ding L, Mardis Elaine R, Wilson Richard K. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150(2):264–78. doi:10.1016/j.cell.2012.06.023.

99. White BS, DiPersio JF. Genomic tools in acute myeloid leukemia: from the bench to the bedside. Cancer. 2014;120(8):1134–44. doi:10.1002/cncr.28552.

100. Deguchi K, Gilliland DG. Cooperativity between mutations in tyrosine kinases and in hematopoietic transcription factors in AML. Leukemia. 2002;16(4):740–4. doi:10.1038/sj.leu.2402500.

101. Gilliland DG. Molecular genetics of human leukemias: new insights into therapy. Semin Hematol. 2002;39(4 Suppl 3):6–11. doi:10.1053/shem.2002.36921.

102. Anderson K, Lutz C, van Delft FW, Bateman CM, Guo Y, Colman SM, Kempski H, Moorman AV, Titley I, Swansbury J, Kearney L, Enver T, Greaves M. Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature. 2011;469(7330):356–61.

103. Sarry JE, Murphy K, Perry R, Sanchez PV, Secreto A, Keefer C, Swider CR, Strzelecki AC, Cavelier C, Recher C, Mansat-De Mas V, Delabesse E, Danet-Desnoyers G, Carroll M. Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD/SCID/IL2Rgammac-deficient mice. J Clin Invest. 2011;121(1):384–95. doi:10.1172/JCI41495.

104. Huntly BJ, Shigematsu H, Deguchi K, Lee BH, Mizuno S, Duclos N, Rowan R, Amaral S, Curley D, Williams IR, Akashi K, Gilliland DG. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell. 2004;6(6):587–96.

105. Cozzio A, Passegue E, Ayton PM, Karsunky H, Cleary ML, Weissman IL. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. Genes Dev. 2003;17(24):3029–35. doi:10.1101/gad.1143403.

106. Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, Levine JE, Wang J, Hahn WC, Gilliland DG, Golub TR, Armstrong SA. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature. 2006;442(7104):818–22. doi:10.1038/nature04980.

107. Wojiski S, Guibal FC, Kindler T, Lee BH, Jesneck JL, Fabian A, Tenen DG, Gilliland DG. PML-RARalpha initiates leukemia by conferring properties of self-renewal to committed promyelocytic progenitors. Leukemia. 2009;23(8):1462–71. doi:10.1038/leu.2009.63.

108. Guibal FC, Alberich-Jorda M, Hirai H, Ebralidze A, Levantini E, Di Ruscio A, Zhang P, Santana-Lemos BA, Neuberg D, Wagers AJ, Rego EM, Tenen DG. Identification of a myeloid committed progenitor as the cancer-initiating cell in acute promyelocytic leukemia. Blood. 2009;114(27):5415–25. doi:10.1182/blood-2008-10-182071.

109. Radtke I, Mulligan CG, Ishii M, Su X, Cheng J, Ma J, Ganti R, Cai Z, Goorha S, Pounds SB, Cao X, Obert C, Armstrong J, Zhang J, Song G, Ribeiro RC, Rubnitz JE, Raimondi SC, Shurtleff SA, Downing JR. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc Natl Acad Sci U S A. 2009;106(31):12944–9. doi:10.1073/pnas.0903142106.

110. Cobaleda C, Gutierrez-Cianca N, Perez-Losada J, Flores T, Garcia-Sanz R, Gonzalez M, Sanchez-Garcia I. A primitive hematopoietic cell is the target for the leukemic transformation in human Philadelphia-positive acute lymphoblastic leukemia. Blood. 2000;95(3):1007–13.

111. Cox CV, Evely RS, Oakhill A, Pamphilon DH, Goulden NJ, Blair A. Characterization of acute lymphoblastic leukemia progenitor cells. Blood. 2004;104(9):2919–25. doi:10.1182/blood-2004-03-0901.

112. Castor A, Nilsson L, Astrand-Grundstrom I, Buitenhuis M, Ramirez C, Anderson K, Strombeck B, Garwicz S, Bekassy AN, Schmiegelow K, Lausen B, Hokland P, Lehmann S, Juliusson G, Johansson B, Jacobsen SE. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat Med. 2005;11(6):630–7. doi:10.1038/nm1253.

113. le Viseur C, Hotfilder M, Bomken S, Wilson K, Rottgers S, Schrauder A, Rosemann A, Irving J, Stam RW, Shultz LD, Harbott J, Jurgens H, Schrappe M, Pieters R, Vormoor J. In childhood acute lymphoblastic leukemia, blasts at different stages of immunophenotypic maturation have stem cell properties. Cancer Cell. 2008;14(1):47–58. doi:10.1016/j.ccr.2008.05.015.

114. Hong D, Gupta R, Ancliff P, Atzberger A, Brown J, Soneji S, Green J, Colman S, Piacibello W, Buckle V, Tsuzuki S, Greaves M, Enver T. Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. Science. 2008;319(5861):336–9. doi:10.1126/science.1150648.

115. Hotfilder M, Rottgers S, Rosemann A, Jurgens H, Harbott J, Vormoor J. Immature CD34+CD19-progenitor/stem cells in TEL/AML1-positive acute lymphoblastic leukemia are genetically and functionally normal. Blood. 2002;100(2):640–6.

116. Schindler JW, Van Buren D, Foudi A, Krejci O, Qin J, Orkin SH, Hock H. TEL-AML1 corrupts hematopoietic stem cells to persist in the bone marrow and initiate leukemia. Cell Stem Cell. 2009;5(1):43–53.

117. Papaemmanuil E, Rapado I, Li Y, Potter NE, Wedge DC, Tubio J, Alexandrov LB, Van Loo P, Cooke SL, Marshall J, Martincorena I, Hinton J, Gundem G, van Delft FW, Nik-Zainal S, Jones DR, Ramakrishna M, Titley I, Stebbings L, Leroy C, Menzies A, Gamble J, Robinson B, Mudie L, Raine K, O'Meara S, Teague JW, Butler AP, Cazzaniga G, Biondi A, Zuna J, Kempski H, Muschen M, Ford AM, Stratton MR, Greaves M, Campbell PJ. RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia. Nat Genet. 2014;46(2):116–25. doi:10.1038/ng.2874.

118. Aoki Y, Watanabe T, Saito Y, Kuroki Y, Hijikata A, Takagi M, Tomizawa D, Eguchi M, Eguchi-Ishimae M, Kaneko A, Ono R, Sato K, Suzuki N, Fujiki S, Koh K, Ishii E, Shultz LD, Ohara O, Mizutani S, Ishikawa F. Identification of CD34+ and CD34- leukemia-initiating cells in MLL-rearranged human acute lymphoblastic leukemia. Blood. 2015;125(6):967–80. doi:10.1182/blood-2014-03-563304.

119. Kong Y, Yoshida S, Saito Y, Doi T, Nagatoshi Y, Fukata M, Saito N, Yang SM, Iwamoto C, Okamura J, Liu KY, Huang XJ, Lu DP, Shultz LD, Harada M, Ishikawa F. CD34+CD38+CD19+ as well as CD34+CD38-CD19+ cells are leukemia-initiating cells with self-renewal capacity in human B-precursor ALL. Leukemia. 2008;22(6):1207–13. doi:10.1038/leu.2008.83.

120. Morisot S, Wayne AS, Bohana-Kashtan O, Kaplan IM, Gocke CD, Hildreth R, Stetler-Stevenson M, Walker RL, Davis S, Meltzer PS, Wheelan SJ, Brown P, Jones RJ, Shultz LD, Civin CI. High frequencies of leukemia stem cells in poor-outcome childhood precursor-B acute lymphoblastic leukemias. Leukemia. 2010;24(11):1859–66. doi:10.1038/leu.2010.184.

121. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367(6464):645–8. doi:10.1038/367645a0.

122. Ishikawa F, Yoshida S, Saito Y, Hijikata A, Kitamura H, Tanaka S, Nakamura R, Tanaka T, Tomiyama H, Saito N, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat Biotechnol. 2007;25(11):1315–21.

123. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012;481(7381):306–13. doi:10.1038/nature10762.

Part II
White Blood Cell Disorders

Chapter 2
Acute Lymphoblastic Leukemia

Daisuke Tomizawa and Nobutaka Kiyokawa

Abstract Acute lymphoblastic leukemia (ALL) accounts for a quarter of malignant neoplasms in children and adolescents. With continuous effort on developing risk-stratified multi-agent chemotherapy through cooperative clinical trials worldwide, survival rate of childhood ALL increased from less than 10% in the 1960s to approximately 90% nowadays. Recent advance in genomic analyses is rapidly increasing our understanding of the pathobiology of ALL, which may lead to a development of novel molecular targeted therapy and finally to overcome the disease in future.

2.1 Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells and the most common type of malignant neoplasms in children and adolescents. Development of multi-agent chemotherapy and risk stratification based on leukemia biology and early treatment response have improved the overall cure rate of childhood ALL to 80% or higher, which is one of the most successful story in the history of human medicine (Table 2.1) [1–12]. In this chapter, advances in the pathobiology and clinical management of ALL in children will be described.

D. Tomizawa, M.D., Ph.D. (✉)
Children's Cancer Center, National Center for Child Health and Development, Tokyo, Japan
e-mail: tomizawa-d@ncchd.go.jp

N. Kiyokawa, Ph.D.
Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, Tokyo, Japan

Table 2.1 Results of the recently reported trials for pediatric ALL

Study group	Trial	Years	ALL subtypes	No. of patients	Age	EFS, % (year)	OS, % (year)	Reference
AIEOP/BFM	AIEOP-BFM ALL 2000	2000–2006	B-ALL	4016	1–18	80.4 (7)	91.8 (7)	Conter et al. [1]
			T-ALL	464		75.9 (7)	80.7 (7)	Schrappe et al. [2]
MRC	UKALL 2003	2003–2011	All	3126	1–24	87.2 (5)	91.5 (5)	Vora et al. [3]
			B-ALL	2731		–	–	
			T-ALL	388		–	–	
DCOG	DCOG Protocol ALL-9	1997–2004	All	859	1–18	81 (5)	86 (5)	Veerman et al. [4]
			B-ALL	701		82 (5)	–	
			T-ALL	90		72 (5)	–	
EORTC-CLG	EORTC CLG 58591	1998–2008	All	1947	1–18	82.7 (5)	89.7 (5)	Domenech et al. [5]
			B-ALL	1650		–	–	
			T-ALL	296		–	–	
NOPHO	ALL-2000	2002–2007	All	1023	1–15	79 (5)	89 (5)	Schmiegelow et al. [6]
			B-ALL	906		–	–	
			T-ALL	115		–	–	
COG	Various CCG, POG, and COG trials	2000–2005	All	7153	0–22	–	90.4 (5)	Hunger et al. [7]
			B-ALL	5982		–	91.1 (5)	
			T-ALL	459		–	81.6 (5)	
SJCRH	Total therapy XV	2000–2007	All	498	1–18	85.6 (5)	93.5 (5)	Pui et al. [8]
			B-ALL	422		86.9 (5)	94.6 (5)	
			T-ALL	76		78.4 (5)	87.6 (5)	

DFCI	DFCI 05-001	2005–2010	All	551	1–18	85 (5)	91 (5)	Place et al. [9]
			B-ALL	482		85 (5)	91 (5)	
			T-ALL	69		87 (5)	91 (5)	
Ma-Spore	Ma-Spore ALL 2003	2002–2011	All	556	0–18	80.6 (6)	88.4 (6)	Yeoh et al. [10]
			B-ALL	507		80.7 (6)	–	
			T-ALL	49		80.5 (6)	–	
TCCSG	TCCSG L99-15	1999–2003	All	754	1–18	78.2 (4)	87.6 (4)	Hasegawa et al. [11]
			B-ALL	664		80.5 (4)	–	Manabe et al. [12]
			T-ALL	90		66.0 (4)	–	

AIEOP Associazione Italiana Ematologia Oncologia Pediatrica, *BFM* Berlin-Frankfurt-Münster, *MRC* Medical Research Council, *DCOG* Dutch Children's Oncology Group, *EORTC-CLG* European Organization for Research and Treatment of Cancer—Children's Leukemia Group, *NOPHO* Nordic Society of Paediatric Hematlpogy and Oncology, *COG* Children's Oncology Group, *SJCRH* St. Jude Children's Research Hospital, *DFCI* Dana-Farber Cancer Institute, *Ma-Spore* Malasya-Singapore, *TCCSG* Tokyo Children's Cancer Study Group
EFS event-free survival, *OS* overall survival

2.2   Epidemiology

Registry of the Japanese Society of Pediatric Hematology (currently, the Japanese Society of Pediatric Hematology and Oncology) covers more than 90% of the population under age 20 with hematological malignancies in Japan. 2463 patients are diagnosed as ALL between 2006 and 2010, that is, approximately 500 new cases are diagnosed every year [13]. As annual number of childhood cancer in Japan is estimated as 2000–2500, ALL accounts for 20–25% of all childhood cancers and 72% of all childhood leukemias. Male/female ratio is 1.3:1, and the peak incidence of ALL occurs between 2 and 5 years old. Immunophenotypically, 86% is B-cell precursor, 11% is T cell, 2% is mature B cell, and 1% is unknown.

Several inherited syndromes are known to be associated with an increased risk of ALL. Among all, most well recognized is Down syndrome which is 10–20 times more likely to develop ALL compared to non-Down syndrome children [14]. In addition, several rare germline mutations, such as *PAX5* and *ETV6*, have been identified to be a genetic predisposition to B-ALL in familial leukemia kindreds [15, 16]. Although it has been long recognized that majority of ALL patients have no apparent clinical inherited factors, recent genome-wide association studies have identified several germline single-nucleotide polymorphisms (SNPs) in several genes (including *IKZF1*, *ARID5B*, *CEBPE*, *CDKN2A*, *GATA3*, etc.) that are associated with significantly higher risk of developing ALL [17–20].

Environmental factors, such as ionizing radiation, electromagnetic field, certain diets (e.g., bioflavonoids), seem to have little association in most of the ALL cases [21–23]. However, two infection-based hypotheses, Greaves' "delayed infection" hypothesis and Kinlen's "population-mixing" hypothesis that childhood ALL arise as a consequence of an abnormal immune response in susceptible individuals to common infections, are well supported by epidemiological data [24, 25].

2.3   Pathobiology

It is considered that ALL occurs as a consequence of malignant transformation of an abnormal single lymphoid progenitor cell via multi-step genetic alterations. It is not entirely clear how and when these genetic events take place. However, it is believed that causation of ALL is by chance, and normal allelic variation in inherited genes or inherited ALL predisposing syndromes in a small portion of cases, and exposures to various exogenous and endogenous factors all contribute to leukemogenesis [26].

Identification of genetic abnormalities plays a pivotal role in understanding the biology and treatment of ALL. Cytogenetic analysis by karyotyping which was established in 1960s and by fluorescence in situ hybridization (FISH) in 1980s is still necessary in the modern diagnostic evaluation in ALL. However, newer array-based technologies enabled us to analyze global gene expression, DNA copy numbers, SNPs, and methylation status. In addition, recent advance in genome-wide sequencing which include whole-genome sequencing, transcriptome sequencing
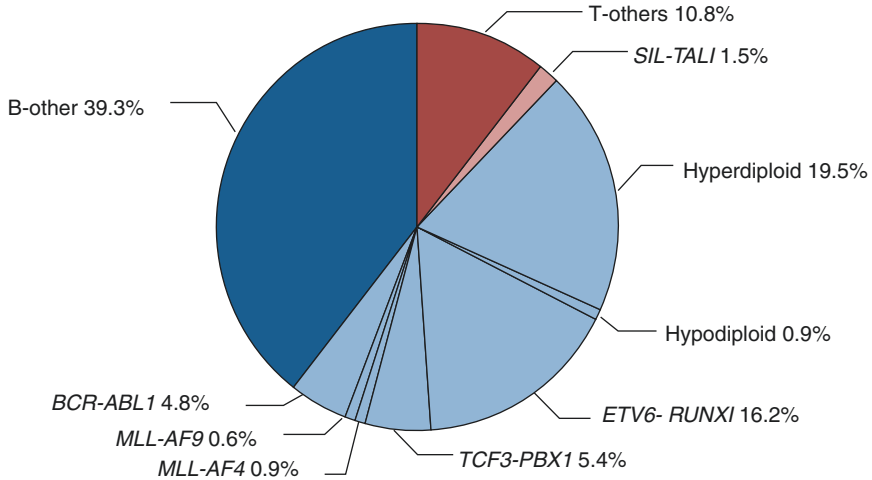
Fig. 2.1 Cytogenetic and molecular genetic abnormalities in pediatric ALL. (data provided from the TCCSG L04-16 and L07-16 studies)

(RNA-seq), and whole-exome sequencing have helped us to approach the true nature of ALL. Although these novel genomic techniques are still used as research discovery tools, it is expected that they would be increasingly utilized for clinical applications in the near future.

Recurrent genetic alterations in ALL include aneuploidy (changes in chromosome number) and structural abnormalities. The molecular and clinical features of specific alterations are discussed below (Fig. 2.1).

2.3.1   Aneuploidy

Ploidy (number of chromosomes of a cell) could be determined by direct counting of the chromosome numbers in a metaphase karyotype preparation by G-banding technique. Clinically, high hyperdiploidy is defined as chromosome number greater than 50 and hypodiploidy as less than 45 chromosomes. DNA index (DI) is an alternative method of measuring DNA content by flow cytometry; DI is 1.0 in normal diploid cells, while it is 1.16 or higher in high hyperdiploid cases.

2.3.1.1   Hyperdiploidy

High hyperdiploidy accounts for 20–30% of childhood ALL. There is a distinct pattern of chromosomes gained at each modal number of chromosomes: most commonly gained is chromosome 21 (ch21), followed by ch4, chX, ch10, ch6, ch14, ch18, and ch17 [27]. Previous clinical studies have demonstrated that certain combination of gained chromosome are associated with better prognosis: "double

trisomy" of ch4 and ch10 in the Pediatric Oncology Group (POG); "triple trisomy" of ch4, ch10, and ch17 in Children's Oncology Group (COG); trisomy of ch11 and ch17 in the Tokyo Children's Cancer Study Group (TCCSG) [27–29]. Although prognostic significance of specific gained chromosomal combination is inconsistent among different study groups, high hyperdiploidy itself is associated with CD10-positive B-cell precursor phenotype, low leukocyte (WBC) count, younger age at diagnosis, and excellent prognosis.

2.3.1.2 Hypodiploidy

In contrast, hypodiploidy accounts for only 1% of childhood ALL and is a strong predictor of poor prognosis [30, 31]. Recent genomic analysis has identified *TP53* mutations in 91% of low-hypodiploid ALL (32–39 chromosomes) [32]. Surprisingly, the mutation was also present in germline in 43% of the cases, which is a hallmark of Li-Fraumeni syndrome. Occasionally, there are cases with "masked hypodiploidy," which occurs as a result of doubling of a hypodiploid clone [33]. Caution is needed because they do not appear to be different from non-masked hypodiploidy in clinical and prognostic features but could be misdiagnosed as "hyperdiploidy" harboring opposite prognosis.

2.3.2 Structural Chromosomal Abnormalities

Structural chromosomal abnormalities, most commonly translocations, are frequently observed in childhood ALL. They are considered to be initiating events in leukemogenesis. Several occur in utero, nearly 100% of *MLL-AF4*, similar to hyperdiploid cases, and 75% of *ETV6-RUNX1*, but none of *TCF3-PBX1* [34]. This is supported by evidences of the high rate of concordance of leukemia in monozygotic twins and the prenatal origin of ALL demonstrated directly by the detection of unique fusion in neonatal blood spots on Guthrie cards who later developed ALL [35, 36]. Generally, two functional classes of translocations exist. One is juxtaposition of oncogenes with regulatory regions of actively transcribed genes causing its dysregulated target gene expression, such as *c-MYC* to immunoglobulin gene in Burkitt's lymphoma/leukemia. Another is fusion of the genes at the translocation breakpoints to encode a novel chimeric protein with oncogenic function, *ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1*, rearrangements of mixed-lineage leukemia (*MLL* or *KMT2A*) gene, etc.

2.3.2.1 ETV6-RUNX1 (TEL-AML1)

t(12;21)(p13;q22.1) accounts for 15–20% of childhood ALL. It is cryptic in most cases and could be detected by FISH or real-time quantitative polymerase chain reaction (PCR). The translocation results in fusion of two hematopoietic transcription factor genes, *ETV6* (formerly known as *TEL*) and *RUNX1* (*AML1*).

ETV6-RUNX1 appears to arise in utero, but the fusion solely itself is not sufficient to cause the leukemia and subsequent events are required to full progression [37]. Children with *ETV6-RUNX1* ALL is associated with excellent outcome similar to high hyperdiploid ALL [38].

2.3.2.2 TCF3-PBX1 (E2A-PBX1)

t(1;19)(q23;p13.3) is the second most common translocation and accounts for 5% of childhood ALL. The translocation results in the fusion of two transcription factor genes, *TCF3* (formerly known as *E2A*) and *PBX1*. *TCF3-PBX1* ALL appears to be pre-B cell phenotype with positive cytoplasmic μ. It was once associated with poor prognosis but has lost its prognostic significance in the context of modern ALL chemotherapy especially that contains high-dose methotrexate [39]. However, *TCF3-PBX1* is associated with higher risk of central nervous system (CNS) relapse [40].

There is a rare subtype of *TCF3* gene involved ALL with fusion partner *hepatic leukemia factor* (*HLF*) gene which arise from t(17;19)(q22;p13). *TCF3-HLF* only occurs in less than 1% of childhood ALL but has unique characteristics such as hypercalcemia, an increased risk of disseminated intravascular coagulation (DIC), and, moreover, very poor prognosis [41].

2.3.2.3 KMT2A (MLL) Gene Rearrangements

KMT2A (*MLL*) gene, located at chromosome band 11q23, encodes a histone methyltransferase that is involved in epigenetic regulation of blood cell development via expression of multiple *Hox* genes [42]. Rearrangements of *MLL* occur as a result of balanced chromosomal translocations that fuse the *MLL* gene to one of more than 70 known partner genes. The most common in ALL is *MLL-AF4* (*KMT2A-AFF1*) derived from t(4;11)(q21;q23), followed by *MLL-ENL* (*KMT2A-MLLT1*) from t(11;19)(q23;p13), and *MLL-AF9* (*KMT2A-MLLT3*) from t(9;11)(p22;q23) [43]. *MLL* rearrangement is particularly common in ALL in infants (age <1 year old), which accounts for nearly 80% of the cases and associated with CD10-negative pro-B cell phenotype, high leukocyte count at diagnosis, and very poor prognosis (Table 2.2) [44–48]. ALL with *MLL* rearrangements have very few additional somatic mutations, one of the lowest of any sequenced cancers [49].

2.3.2.4 BCR-ABL1

t(9;22)(q34;q11.2) results in formation of Philadelphia (Ph) chromosome, which is one of the first leukemic translocations described. Ph encodes BCR-ABL1, an activated tyrosine kinase. It is the most common translocation in adult ALL (25% of the cases) but less common in children (less than 5% of the cases). Ph-positive (Ph+) ALL was one of the most difficult to cure ALL with only 30–40% event-free survival (EFS) rate despite intensive chemotherapy and allogeneic hematopoietic stem cell

Table 2.2 Results of the recently reported trials for infants with ALL

Study group	Trial	Years	ALL subtypes	No. of patients	No. of patients received Allo-SCT in 1CR	EFS, % (year)	OS, % (year)	Reference
Interfant	Interfant-99	1999–2005	All	482	37	47.0 (4)	55.3 (4)	Pieters et al. [44]
			MLL-r	314		36.9 (4)	–	
			MLL-g	82		74.1 (4)	–	
CCG	CCG 1953	1996–2000	All	115	37	41.7 (5)	44.8 (5)	Hilden et al. [45]
			MLL-r	79		33.6 (5)	–	
			MLL-g	36		60.3 (5)	–	
COG	COG P9407 (cohort 3)	2001–2006	All	147	0	42.3 (5)	52.9 (5)	Dreyer et al. [46]
			MLL-r	100		35.5 (5)	–	
			MLL-g	35		69.7 (5)	–	
JILSG	MLL96 and MLL98	1995–2001	All	102	49	50.9 (5)	60.5 (5)	Tomizawa et al. [47]
			MLL-r	80		38.6 (5)	50.8 (5)	
			MLL-g	22		95.5 (5)	95.5 (5)	
JPLSG	MLL03	2004–2009	MLL-r	62	44	43.2 (4)	67.2 (4)	Koh et al. [48]

CCG Children's Cancer Group, *COG* Children's Oncology Group, *JILSG* Japan Infant Leukemia Study Group, *JPLSG* Japanese Pediatric Leukemia/Lymphoma Study Group
CR complete remission, *EFS* event-free survival, *HSCT* hematopoietic stem cell transplantation, *MLL-g* germline *MLL* gene, *MLL-r* rearranged *MLL* gene, *OS* overall survival

transplantation [50]. However, introduction of imatinib, a selective tyrosine kinase inhibitor (TKI), has revolutionized the therapy for Ph+ ALL (Table 2.3) [51–54].

2.3.2.5 Philadelphia (Ph)-like ALL

Ph-like or *BCR-ABL1*-like ALL is a newly recognized entity, harboring similar gene expression profile to Ph+ ALL but without *BCR-ABL1* fusion gene [55, 56]. Recent genomic analyses have revealed diverse range of genetic alterations that activate tyrosine kinase signaling in 90% of the cases. The most commonly identified alterations are rearrangements involving *ABL1*, *ABL2*, *CRLF2*, *CSF1R*, *EPOR*, *JAK2*, *NTRK3*, *PDGFRB*, *PTK2B*, *TSLP*, or *TYK2* and sequence mutations involving

Table 2.3 Results of the recently reported trials for children with Philadelphia chromosome positive ALL in the TKI era

Study group	Trial	Years	Subgroups	No. of patients	No. of patients received Allo-SCT in 1CR	DFS, % (year)	OS, % (year)	Reference
EsPhALL	EsPhALL	2004–2009	All	178	137	61.9 (4)	72.1 (4)	Biondi et al. [51]
			Good-risk with imatinib	46	37	72.9 (4)	–	
			Good-risk without imatinib	44	32	61.7 (4)	–	
			Poor-risk	70	59	53.5 (4)	63.5 (4)	
COG	AALL0031 (cohort 5)	2002–2006	Chemotherapy + imatinib	28	0	70 (5)	–	Schultz et al. [52]
			Sibling donor BMT	21	21	65 (5)	–	
			Unrelated donor BMT	13	13	59 (5)	–	
JPLSG	ALL-Ph04	2004–2008	All	42	26	54.1ª (4)	78.1 (4)	Manabe et al. [53]

COG Children's Oncology Group, *JPLSG* Japanese Pediatric Leukemia/Lymphoma Study Group *Allo-SCT* allogeneic hematopoietic stem cell transplantation, *1CR* first complete remission, *DFS* disease-free survival, *OS* overall survival, *BMT* bone marrow transplantation
ªEFS

FLT3, *IL7R*, or *SH2B3* [57]. Importantly, "ABL-class" kinases (ABL1, ABL2, CSF1R, and PDGFRβ) could be targeted with TKI such as imatinib and dasatinib and alterations that activate JAK-STAT signaling (*JAK1*, *JAK2*, *JAK3*, *CRLF2*, *EPOR*, *TSLP*, and *IL7R*) with JAK inhibitors [58].

2.3.2.6 iAMP21

ALL with intrachromosomal amplification of chromosome 21 (iAMP21) is characterized by amplification of a portion of ch21, which could be detected by FISH analysis using a probe for the *RUNX1* gene that reveals five or more copies of the gene (or three extra copies on a single abnormal ch21 in metaphase FISH) [59]. It occurs in 2% of childhood ALL and is associated with poor prognosis [60].

2.3.2.7 IKZF1, CRLF2, and JAK

Except for *MLL*-rearranged ALL in infants, many of the subtypes have multiple additional genetic alterations in general. These alterations commonly target genes encoding proteins involved in cell signaling, tumor-suppression functions, and lymphoid differentiation.

Most commonly targeted genes involved in B-lymphoid development are *PAX5* and *IKZF1* that are mutated in 31% and 15% of children with B-ALL, respectively, [61]. Notably, recurrent deletions and inactivating mutations in *IKZF1*, which encodes the hematopoietic transcription factor IKAROS, are associated with very poor prognosis in B-ALL [56]. Genetic alterations of *IKZF1* occur more frequently in high-risk cases, including Ph+ ALL and Ph-like ALL [62].

Cytokine receptor-like factor 2 (CRLF2) forms a heterodimeric cytokine receptor with interleukin-7 receptor α (IL7Rα) that mediates B-cell precursor proliferation and survival via activation of downstream JAK/STAT pathways. Rearrangements of *CRLF2* gene as *IGHa-CRLF2* or *P2RY8-CRF2* result in *CRLF2* overexpression and found in 5–8% of pediatric ALL, 10–15% of adult ALL, and more than 50% of Down syndrome ALL [63–65]. It has been reported as poor prognostic factor but still controversial.

JAK2 mutations are found in 18–35% of Down syndrome ALL cases and 11% of non-Down high-risk *BCR-ABL1*-negative ALL cases [66]. The mutation cause constitutive JAK-STAT activation. There is overlap among *IKZF1* and *CRLF2* alterations [67].

2.3.2.8   ETP-ALL

In contrast to genetic alterations discovered in B-ALL, many of the genetic alterations in T-ALL have not been found to have prognostic value yet. However, one subset of T-ALL with unique biology, early T-cell precursor (ETP) ALL that accounts for 10–15% of pediatric T-ALL, is recognized as a new entity [68]. ETP-ALL was originally identified by its unique gene expression pattern and immunophenotype with very early T-cell progenitor features: absence of T-cell markers CD1a and CD8; dim or absent CD5; combined with cytoplasmic, but not surface, CD3; and positive for one or more myeloid/stem cell markers (CD34, CD117, HLA-DR, CD13, CD33, CD11b, or CD65). By whole-genome sequencing, gene mutations similar to myeloid leukemias including RAS signal pathways were identified [69].

2.3.2.9   Genetic Alterations at Relapse

Genomic studies of matched diagnosis and relapsed ALL sample pairs have revealed that only 42% of the cases had evolved from diagnosis clone (8% was same and 34% was clonal evolution from diagnosis clone), but majority (52%) was rather evolved from ancestral clones, and the remaining 6% was genetically distinct secondary leukemia [70]. By further sequencing studies, mutations including *CREBBP*, *TP53*, and *NT5C2* were identified [71–74]. Focal deletion and sequence mutations in *CREBBP* were found in 19% of children with relapsed B-ALL. The mutation causes impaired histone acetylation and transcriptional regulation of *CREBBP* targets, thus impairing the normal *CREBBP*-mediated transcriptional response to glucocorticoids and possibly results in steroid therapy resistance of the relapsed clone.

NT5C2 encodes as 5′-nucleotidase enzyme that is involved in metabolisms of 6-mercaputopurine. Its mutation causes increased enzyme activity and resistance to nucleoside analog therapy. *TP53* alterations were identified in 12% of relapsed B-ALL and 6% of relapsed T-ALL cases in the Berlin-Frankfurt-Münster (BFM) study, which was enriched compared to the initial diagnosis samples and was associated with poor response to the therapy and inferior outcome.

2.4 Clinical Management

2.4.1 Clinical Presentation

Anemia, thrombocytopenia, and/or neutropenia are typically observed among ALL patients, which reflect failure of normal hematopoiesis; pallor, fatigue, bleeding (e.g., petechiae or purpura), and fever (usually as leukemia-related rather than infection) are often present. Hepatosplenomegaly, lymphadenopathy, and bone pain are frequently manifested. Although not common at the time of initial diagnosis, involvement of extramedullary sites such as CNS, testis, and skin might be present simultaneously. Duration of these symptoms may vary from days to months; however, one may face life-threatening "oncologic emergency" situation at initial presentation, superior vena cava syndrome/superior mediastinal syndrome in T-ALL patients, disseminated intravascular coagulation, tumor lysis syndrome (renal failure, elevated levels of serum uric acid, potassium, and/or phosphate), and CNS hemorrhage or thrombosis induced by hyperviscosity syndrome, both often associated with hyperleukocytosis (>100,000/μL in peripheral blood), etc.

2.4.2 Diagnostic Procedures

Diagnosis of ALL is established by morphological detection of more than 25% leukemic blasts in bone marrow by aspirated bone marrow smears. It is preferable to perform bone marrow biopsy in case of dry tap. In addition to morphological examination, immunophenotyping, karyotype, and genetic analyses are essential for ALL diagnosis.

Evaluation of extramedullary leukemia, especially involvement in CNS, is necessary. CNS status at diagnosis is defined as follows: CNS1, no detectable blast cells in cerebrospinal fluid (CSF); CNS2, fewer than five leukocytes per μL with detectable blasts; and CNS3, the presence of overt CNS leukemia [75]. Although CNS2 had an adverse prognostic impact on CNS relapse in previous pediatric ALL trials, it has lost its prognostic effect in contemporary trials that include more effective systemic and CNS-directed treatment. Traumatic lumbar puncture at diagnosis is an issue, because it could cause iatrogenic CNS leukemia by mixing patients' CSF with bloods with abundant circulating blasts, thus lead to increased risk of

CNS relapse [76, 77]. In order to reduce the risk of traumatic lumbar puncture at diagnosis, correction of thrombocytopenia and coagulopathy prior to the procedure, keeping patients steady under deep sedation, and immediate administration of intrathecal therapy (e.g., methotrexate) after the collection of CSF are strongly recommended [78].

2.4.3 Treatment

Principle of ALL treatment is based on "total cell kill" theory, an attempt to eradicate every leukemic blast inside the patient's body. This is mostly accomplished by multi-agent combination chemotherapy including CNS-directed therapy. Hematopoietic stem cell transplantation (SCT) is additionally combined in a small portion of cases with very high risk of relapse. Risk stratification, means of optimizing therapy for the patients by evaluating relapse risk with known prognostic factors, has also contributed to improve survival rate in children with ALL.

Treatment algorithm of pediatric ALL proposed in the guideline of the Japanese Society of Pediatric Hematology and Oncology is shown in Fig. 2.2. Mature
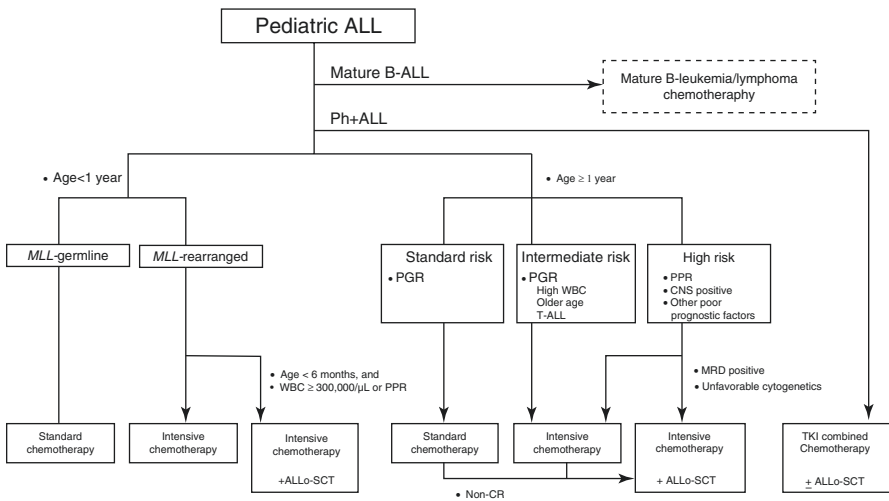
Fig. 2.2 Algorithm of treatment for children with ALL. *Ph+ ALL* Philadelphia chromosome-positive ALL, *PPR* prednisone poor responder, *PGR* prednisone good responder, *WBC* leukocyte count, *CNS* central nervous system, *MRD* minimal residual disease, *TKI* tyrosine kinase inhibitor, *Allo-SCT* allogeneic hematopoietic stem cell transplantation. [Adapted from Clinical Guideline for Pediatric Leukemia and Lymphoma (ver.3) Japanese Society of Pediatric Hematology and Oncology]

B-cell ALL or Burkitt ALL, usually presented with French-American-British (FAB) L3 blasts, surface antigen expression of kappa or lambda detected by flow cytometry, and presence of cytogenetic abnormalities of t(8;14)(q24;q32), t(2;8) (p11-p12;q24), or t(8;22)(q24;q11), should be treated separately with short intensive non-Hodgkin lymphoma-oriented chemotherapy [79–81]. Ph+ ALL with t(9;22)(q34;q11.2) or *BCR-ABL1* is currently treated with TKI-combined chemotherapy, thus should be treated independently [51, 52, 54]. Infants (age at diagnosis below 1 year old) with ALL are another special subgroup requiring specific care [44, 48, 82]. The rest of the children with ALL should be treated with appropriate risk-stratified therapies.

2.4.3.1   Prognostic Factors

Prognostic factors are the factors that are predictive of disease outcome and are usually used to tailor therapy ("risk-stratified therapy") with the intention to minimize toxic events for patients with better prognosis and to maximize the treatment effect for patients with poorer prognosis. The currently well-recognized prognostic factors could be categorized into three groups.

Clinical Features at Initial Diagnosis

WBC count, which reflects tumor burden, and age at initial diagnosis have been traditionally used as most reliable prognostic factors. The NCI-Rome criteria defined patients with WBC <50,000/μL and age 1–9 years old as "standard risk" and patients with either WBC ≥50,000/μL or age ≥10 years old as "high risk" [83]. Even in the context of modern therapies, WBC count and age continue to be significant prognostic factors especially in B-ALL; however, its importance became limited in T-ALL. Infants younger than 1 year old are a special group with worse prognosis; among infants, younger age at diagnosis (e.g., age <6 months old) is an independent poor prognostic factor [44].

Biologic and Genetic Features

As discussed in the pathobiology section, a number of recurrent genetic alterations are associated with the outcome of children with ALL. High hyperdiploidy and *ETV6-RUNX1* are associated with a favorable outcome. In contrast, hypodiploidy, *MLL* rearrangement, *BCR-ABL1*, and recently Ph-like ALL and ETP-ALL are associated with high-risk clinical features and a poor outcome.

Early Treatment Response

After 4–6 weeks of initial remission-induction chemotherapy, 97–98% of the children with ALL achieve morphological remission. In other words, only 2–3% of the cases experience induction failure, and their prognosis is poor with overall survival rate of only 32% in a large international retrospective cooperative study [84].

Early clearance of leukemic blasts within 1–2 weeks of initial induction is predictive of good prognosis. "Prednisone response" established by the BFM group, evaluating residual leukemic blasts in peripheral blood following 7 days of prednisone monotherapy with single intrathecal methotrexate injection, clearly segregates good responder ($\geq$1000 blasts/μL, 91% of the pediatric ALL cases) with 87% EFS rate and poor responder (<1000 blasts/μL, 9% of the cases) with 55% EFS rate in 6 years [85]. Because of the principles that children with ALL should be treated with combination chemotherapy and that response should be evaluated in bone marrow, Children's Cancer Group (CCG) defined rapid early responder (25% or fewer bone marrow blasts) and slow early responder (more than 25% bone marrow blasts) evaluated on days 8 or 15 of induction therapy that was also predictive of favorable and poor prognosis, respectively [86].

In recent years, measurement of minimal residual disease (MRD) using flow cytometry detecting aberrant combinations of leukemic cell-surface antigen or PCR amplification targeting leukemic clone-specific rearrangement of immunoglobulin heavy chain (IgH) or T-cell receptor (TCR) genes is superseding morphological response [87, 88]. These techniques are able to detect submicroscopic levels of residual leukemia, one leukemia cell per 10^3–10^4 normal cells in flow-MRD and one per 10^4–10^5 cells in PCR-MRD. With MRD-guided therapy, outcome of poor prognostic subgroups such as Ph-like ALL or ETP-ALL could be significantly improved [89, 90]. Thus, MRD is currently utilized as the most powerful tool to predict prognosis in childhood ALL therapy. In German and Italian cooperative study, Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP)-BFM ALL 2000, all the children with ALL were stratified to either of the three risk groups (low-, intermediate-, and high-risk) only by treatment response indicators: prednisone response, state of bone marrow remission after the end of induction, and IgH/TCR-targeted PCR-MRD results after end of induction and end of early consolidation [1]. This study showed different MRD kinetics relevant to the outcome between B-ALL and T-ALL patients: significantly higher proportion of T-ALL patients showed slower blast clearance than B-ALL patients, and risk of relapse was strongly associated with MRD level of end of early consolidation in T-ALL, while it was with MRD level of end of induction in B-ALL [2]. In approximately 10% of the cases, the PCR target cannot be identified, thus MRD is not measurable. In contrast, flow-MRD is applicable to larger proportion of patients with other advantages such as less expensiveness and faster availability. In fact, study groups in North America (e.g., COG, St. Jude Children's Research Hospital) are using flow-MRD in their ALL trials [91]. However, newer

technologies such as next-generation sequencing-based methods identifying MRD with specific molecular signatures might solve all the technical issues problematic in the current methods such as availability, sensitivity, and rapidity [92].

2.4.3.2 Chemotherapy for ALL

Chemotherapy is a mainstay of ALL treatment. History of ALL chemotherapy begins from the first description of temporary remission of leukemia by folic acid antagonist, aminopterin, reported by Sidney Farber in 1948 [93]. Since then, most of the chemotherapeutic agents that are still in current clinical use, such as 6-mercaptopurine, methotrexate, prednisone, dexamethasone, cyclophosphamide, vincristine, cytarabine, L-asparaginase, and daunorubicin, have been developed before the 1970s. By the use of these conventional drugs, along with recognition and introduction of multi-agent combination chemotherapy, CNS-directed therapy, post-induction intensification, and risk stratification, through step-wise efforts of clinical trials worldwide, have improved the outcome of children with ALL (Table 2.1).

ALL chemotherapy typically spans 2–3 years and consists of three phases; remission-induction therapy and post-remission therapy consisted of consolidation and maintenance therapy. CNS-directed therapy is included throughout the therapy except maintenance phase.

Remission-Induction Therapy

The aim of remission-induction therapy is to achieve morphological remission, defined as less than 5% blasts in bone marrow with regeneration of normal hematopoiesis and no evidence of residual extramedullary disease, which could be achieved in 97–98% of the cases. The therapy generally spans 4–6 weeks and consists of glucocorticoid (prednisone or dexamethasone), vincristine, asparaginase, intrathecal therapies (methotrexate with or without cytarabine and corticosteroid), and optional use of anthracyclines (the most common, daunorubicin).

Prednisone (40–60 mg/m^2 per day) is the most commonly used glucocorticoid; however, substitution with dexamethasone has been of strong interest, because dexamethasone has five- to sixfold higher cytotoxic potency than prednisone in *in vitro* assays, longer plasma half-life, and better cerebrospinal fluid penetration. In the CCG study for NCI standard-risk ALL and the UK study comparing prednisone (40 mg/m^2 per day) versus dexamethasone (6 or 6.5 mg/m^2 per day) during induction showed higher EFS rate in the dexamethasone arm mainly owing to lower CNS relapse [94, 95]. However, in the Japanese TCCSG study for non-high-risk ALL comparing prednisone (60 mg/m^2 per day during induction and 40 mg/m^2 per day during post-remission) and dexamethasone (8 mg/m^2 per day during induction and 6 mg/m^2 per day during post-remission) showed no difference in remission, EFS, OS, and CNS relapse rates [96]. In the AIEOP-BFM ALL 2000 trial, all the patients were randomized to

receive either 60 mg/m^2 per day of prednisone or 10 mg/m^2 per day of dexamethasone [97]. In this study, dexamethasone led to significant reduction in relapse risk, showing largest effect on extramedullary relapse, but was counterbalanced by significant higher induction-related death rate in the dexamethasone arm, thus led to no survival difference. However, subgroup analysis showed higher survival advantage of dexamethasone arm in T-ALL patients with good early response to prednisone prophase. Finally, in the COG trial for NCI high-risk B-ALL, dexamethasone (10 mg/m^2 per day) for 14 days showed better EFS rate in younger age (1–9 years old) patients who received high-dose methotrexate in second randomization compared to prednisone (60 mg/m^2 per day) for 28 days but showed no benefit for older patients (10 years old or older) with excess rate of osteonecrosis [98]. Although glucocorticoid is an essential key drug for ALL treatment, it is associated with adverse events, such as infection, osteonecrosis, psychosis, etc., and generally higher using dexamethasone [99]. Dexamethasone seems to be beneficial in the patients with lower risk of relapse (such as young patients or good early treatment responders), but caution is needed for use in higher dose (e.g., 10 mg/m^2 per day) for older patients.

Asparaginase is another essential key drug for ALL treatment, and there are currently three preparations available, *Escherichia coli* (*E.coli*), *Erwinia chrysanthemi*, and polyethylene glycosylated (PEG) *E. coli* derived. These three preparations have different cytotoxicity and half-lives (both, PEG > *E. coli* > *Erwinia*); therefore, optimizing dose and schedule is crucial to maintain asparagine depletion thus leading to obtain therapeutic effect [100]. In the contemporary ALL treatment in North America and Europe, PEG is favorably used because of longer half-life and potentially lower immunogenicity [9]. However, in countries where PEG is not available, native *E.coli* asparaginase is used. *Erwinia* is generally reserved for patients who develop hypersensitivity reactions [101]. Major adverse events of asparaginase are coagulopathy, acute pancreatitis, and hypersensitivity. The rate of thrombosis in pediatric ALL treatment is approximately 5%, and most of the events occur during induction phase [102, 103]. Attempts to prevent thrombotic events such as prophylactic antithrombin replacement and use of low-molecular heparin are frequently done as clinical practice; however, there is no clear evidence of efficacy in these interventions. Asparaginase-induced acute pancreatitis generally occurs during induction phase and could be fatal [104]. Re-treatment with asparaginase even with different preparations is not recommended because probability of recurrence is very high. The most critical and frequently observed asparaginase-related adverse event is hypersensitivity, which is observed in 20–30% of the cases throughout ALL treatment with native *E.coli* preparation (most often observed in re-induction phase). Intramuscular injection could reduce antibody production compared to intravenous injection; however, once hypersensitivity reaction occurs with either native or PEG *E.coli* preparations, it should be replaced with *Erwinia*. Even without clinical symptoms, antibody production could cause "silent inactivation" of administered asparaginase. In that sense, measurement of serum asparaginase activity would be a useful tool to monitor the therapeutic effect and alter asparaginase treatment, if necessary, especially during the post-remission phase [105].

Addition of anthracycline to three-drug induction (corticosteroid, vincristine, and asparaginase) is utilized in many study groups including the BFM. In some groups such as COG and the UK, anthracycline is not used for NCI standard-risk patients [3, 106]. Because use of anthracyclines could cause late cardiotoxic events even in patients with cumulative dose of less than 300 mg/m^2, anthracycline reduction or omitting is a preferable option for children with ALL [107]. However, the groups treating patients with three-drug induction are commonly using vincristine/dexamethasone pulse in maintenance phase instead. Therefore, reduction of certain elements should be considered in the balance of intensity of the total therapy.

Consolidation Therapy

Following remission induction, 6–8 months of intensive combination chemotherapy is administered aiming to consolidate remission status.

In the BFM regimen, an early intensification course (designated as protocol IB) follows directly after the four-drug induction (protocol IA), alternating with combination of cyclophosphamide, 6-mercaptopurine, cytarabine, and intrathecal therapies (IT). This strategy was introduced by Hansjörg Riehm and his colleagues in the 1970s adopting the Goldie-Coldman hypothesis (a mathematical model predicting the likelihood of mutations leading to drug resistance) that the best chance of cure would be to use all effective non-cross-resistant drugs in the early phase of the treatment before the leukemia cells acquire resistance [108]. COG applies early intensification strategy only for the NCI high-risk cases because they could not prove its efficacy over the less-intensive consolidation with vincristine, 6-mercaptopurine, and IT-methotrexate for the standard-risk patients in the CCG 105 study [109].

After the early consolidation course, interim maintenance course follows. BFM group introduced high-dose methotrexate (HD-MTX, 5 g/m^2/dose) administered over 24 h followed by folinic acid rescue combined with 6-mercaptopurine and IT-methotrexate (protocol M) to intensify this course since BFM86 study [110]. In contrast, CCG had developed another strategy designated Capizzi methotrexate (Capizzi-MTX), with lower, escalating doses of intravenous methotrexate of 100–300 mg/m^2 through short infusions without folinic acid rescue followed by asparaginase. In COG AALL0232 study, randomly assigned HD-MTX arm demonstrated superior outcome compared to Capizzi-MTX for NCI high-risk B-ALL [98]. However, in COG AALL0434 study for T-ALL patients, Capizzi-MTX was superior to HD-MTX [111]. Irrespective of different approaches of intensification, the aim is to accumulate higher methotrexate polyglutamates, the active metabolites of methotrexate, in leukemic cells, which is associated with higher antileukemic activity. Accumulation varies widely between ALL subtypes: low in *TCF3-PBX1*, T-cell, and *ETV6-RUNX1* ALL (thus, might benefit from higher dose of methotrexate) and high in hyperdiploid B-ALL cases [112].

Before entering maintenance therapy, a delayed intensification or re-induction course (designated as protocol II in BFM) mimicking BFM protocol I (IA and IB) is administered: prednisone is substituted by dexamethasone, daunorubicin by doxoru-

bicin, and 6-mercaptopurine by 6-thioguanine. Delayed intensification course was also introduced by Riehm, adopting the Norton-Simon hypothesis (a hypothesis that a tumor is composed of populations of faster-growing chemosensitive cells and slower-growing chemoresistant cells) that the initial regimen must be effective enough to eradicate the low residual chemosensitive leukemia and substitution of some agents with non-cross-resistant agents to eradicate the remaining chemoresistant leukemia [113]. Delayed intensification course has been proven to be an essential element of ALL therapy for all the patients regardless of risk group stratified.

However, for the patients with unfavorable risk (e.g., poor prednisone responders), BFM standard regimen yielded EFS rate of lower than 50% even with addition of high-dose cytarabine and ifosfamide in BFM86 study [110]. Therefore, BFM introduced multiple courses of short intensive block therapy derived from their relapsed ALL study in consolidation phase since the study of BFM90, although its benefit is unclear [114]. In contrast, CCG investigators chose to intensify their consolidation phase with known ALL key drugs with limited hematologic toxicities, such as vincristine, asparaginase, and methotrexate, thus established "augmented BFM" therapy, which significantly improved the outcome of unfavorable risk patients [115].

Maintenance Therapy

Historically, maintenance therapy was introduced to prolong remission period achieved after induction phase. However, the therapy which lasts 2 years or longer consisting of oral intake of daily 6-mercaptopurine and weekly methotrexate with or without pulses of vincristine and dexamethasone is still an essential element in the context of contemporary intensive chemotherapy. There are several trials that attempted to shorten the duration of maintenance, but all failed: BFM group randomized 24-month and 18-month total duration, and shorter duration arm showed inferior outcome; TCCSG L92-13 study, evaluating 1-year total duration therapy in all risk groups, ended up with 59% EFS rate in 5 years, which was inferior to the historical control [116, 117]. Interestingly, the retrospective revisiting analysis of L92-13 study by Kato et al. has discovered the subgroups that might benefit from short duration maintenance: female, *TCF3-PBX1*, and *ETV6-RUNX1* [118].

It is quite common to encounter patients with 6-mercaptopuirne intolerances causing severe myelosuppression that results in frequent treatment disruptions and sometimes in risk of life-threatening infections. Recent pharmacogenomics studies have uncovered its mechanisms in part. The most well studied is genetic polymorphisms of *thiopurine S-methyltransferase* (*TPMT*) gene. TPMT catalyzes S-methylation of thiopurines to inactive methylated metabolites. As a result, patients with heterozygous or homozygous *TPMT* deficiency, which accounts for approximately 10% of Caucasian population, are associated with 6-mercaptopurine intolerance [119]. However, mutant alleles of *TPMT* are rare in Asian population (1.6% in Japanese). Recent studies identified germline polymorphisms of *nucleoside diphosphate-linked moiety X-type motif 15*

(*NUDT15*) gene, found in one third of Japanese population, to be associated with 6-mercaptopurine intolerance as well [120, 121]. Decreased enzymatic activity of mutant NUDT15 leads to excess levels of intracellular thiopurine active metabolites, thus results in cytotoxicity. Polygenic dosing algorithm that incorporates these pharmacogenomics data would provide personalized thiopurine therapy in the near future.

2.4.3.3 CNS-Directed Therapy

Control and prevention of CNS leukemia is a key component of ALL therapy. Cranial irradiation (24 Gy) has dramatically improved survival rate of children with ALL in the 1960s and 1970s, but it was associated with an increased risk of secondary CNS tumors, various endocrinopathies, growth impairment, and neurocognitive effects [122, 123]. As a result, CNS irradiation dose is reduced to 12–18 Gy and limited to only patients with higher risk of CNS relapse in the contemporary ALL therapy. CNS and hematological relapses are competing events; therefore, intensification of both systemic chemotherapy (high-dose methotrexate, asparaginase, and dexamethasone) and intrathecal chemotherapy plays important roles to prevent CNS relapse. With these intensifications, St. Jude Children's Research Hospital has succeeded in eliminating cranial irradiation for all the children with ALL in their frontline therapy [8].

2.4.3.4 Hematopoietic Stem Cell Transplantation

Allogeneic hematopoietic stem cell transplantation (SCT) is still an option for children with very high risk of relapse, but its role continues to be limited in the context of contemporary treatment. Allo-SCT is no longer routinely recommended for children with Ph+ ALL due to the development of TKI-combined chemotherapy, and indication for infants with ALL is controversial [124]. For those receiving allo-SCT, post-transplant leukemia-free survival seems not to be affected by stem cell sources [125–127]. In terms of conditioning regimen, use of total body irradiation is generally recommended except for infants because of its higher therapeutic effect on preventing leukemia relapse [128, 129]. Finally, MRD status before SCT is a strong predictor of post-SCT relapse [126].

2.4.3.5 Special Subcategories

Down Syndrome ALL

Children with constitutive trisomy 21 or Down syndrome (DS) is associated with higher risk of developing B-ALL, but not during infancy, compared to non-DS children. Biologically, prevalence of common abnormalities in non-DS ALL, such as

ETV6-RUNX1, high hyperdiploidy, *BCR-ABL1*, or *MLL-AF4*, is low, and overexpression of CRLF2 is found in more than half the cases as previously described [64]. DS patients with ALL have higher cumulative incidence of relapse and treatment-related mortality, especially infection related, resulting in lower EFS and OS rates than non-DS ALL patients [130, 131]. Therefore, caution is needed not to reduce chemotherapy dosages excessively, but at the same time, careful management of infections is necessary throughout therapy including maintenance.

Adolescent and Young Adults with ALL

Historically, adolescents and young adults (AYA; age 15 years old or higher) with ALL had inferior EFS and OS rates compared to children. This is in part because of higher prevalence of unfavorable risk ALL (e.g., T-cell and Ph+) and lower prevalence of favorable risk ALL (e.g., *ETV6-RUNX1* and high hyperdiploid) in AYAs. However, recent clinical studies have shown feasibility and significant improved outcomes in AYAs with ALL treated with pediatric ALL protocol [132–134]. Hence, the consensus has been established that AYA patients should be treated based on pediatric ALL regimen.

2.5 Future Challenges

More than 80% of children with ALL are currently cured; however, this was accomplished by identification of optimal dosing and schedules of chemotherapeutic agents that have developed before the 1970s, not by identification of novel innovative agents. One of the major challenges in pediatric ALL would be to increase the cure rate of patient subsets with dismal prognosis that is unlikely to be improved with contemporary therapy. Owing to rapid progress in genomic analysis, there has been a tremendous increase in understanding of ALL biology in the past few years, and its application is currently ongoing. One of the most successful examples is the introduction of TKI in the treatment of Ph+ ALL, although optimal agents, dosing, and combination chemotherapy are still not determined [54]. There is substantial number of novel agents under evaluation: TKI (imatinib, dasatinib) for Ph-like ALL [58], epigenetic modifiers (DNA methyltransferase inhibitors, histone deacetylase inhibitors, DOT1L inhibitor) for *MLL*-rearranged ALL [135, 136], proteasome inhibitors (bortezomib) [137], monoclonal antibodies (rituximab, epratuzumab) [138], monoclonal antibodies conjugated with immunotoxins or chemotherapeutic drugs (moxetumomab, inotuzumab ozogamicin) [139], bispecific T-cell engager antibodies (blinatumomab) [140], chimeric antigen receptor (CAR) T-cell therapy [141], etc. It is expected that the genomic landscape of ALL would be fully unmasked in the near future and that the integration of discovered genomics into contemporary therapy would progress in order to realize precision medicine which would result in further improvement in the outcome of childhood ALL.

References

1. Conter V, Bartram CR, Valsecchi MG, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. Blood. 2010;115:3206–14.

2. Schrappe M, Valsecchi MG, Bartram CR, et al. Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. Blood. 2011;118:2077–84.

3. Vora A, Goulden N, Wade R, et al. Treatment reduction for children and young adults with low-risk acute lymphoblastic leukaemia defined by minimal residual disease (UKALL 2003): a randomised controlled trial. Lancet Oncol. 2013;14:199–209.

4. Veerman AJ, Kamps WA, van den Berg H, et al. Dexamethasone-based therapy for childhood acute lymphoblastic leukaemia: results of the prospective Dutch Childhood Oncology Group (DCOG) protocol ALL-9 (1997–2004). Lancet Oncol. 2009;10:957–66.

5. Domenech C, Suciu S, De Moerloose B, et al. Dexamethasone (6 mg/m^2/day) and prednisolone (60 mg/m^2/day) were equally effective as induction therapy for childhood acute lymphoblastic leukemia in the EORTC CLG 58951 randomized trial. Haematologica. 2014;99:1220–7.

6. Schmiegelow K, Forestier E, Hellebostad M, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. Leukemia. 2010;24:345–54.

7. Hunger SP, Lu X, Devidas M, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the Children's Oncology Group. J Clin Oncol. 2012;30:1663–9.

8. Pui C-H, Campana D, Pei D, et al. Treating childhood acute lymphoblastic leukemia without cranial irradiation. N Engl J Med. 2009;360:2730–41.

9. Place AE, Stevenson KE, Vrooman LM, et al. Intravenous pegylated asparaginase versus intramuscular native *Escherichia coli* L-asparaginase in newly diagnosed childhood acute lymphoblastic leukaemia (DFCI 05-001): a randomised, open-label phase 3 trial. Lancet Oncol. 2015;16:1677–90.

10. Yeoh AEJ, Ariffin H, Chai ELL, et al. Minimal residual disease-guided treatment deintensification for children with acute lymphoblastic leukemia: results from the Malaysia-Singapore Acute Lymphoblastic Leukemia 2003 Study. J Clin Oncol. 2012;30:2384–92.

11. Hasegawa D, Manabe A, Ohara A, et al. The utility of performing the initial lumbar puncture on day 8 in remission induction therapy for childhood acute lymphoblastic leukemia: TCCSG L99-15 study. Pediatr Blood Cancer. 2012;58:23–30.

12. Manabe A, Ohara A, Hasegawa D, et al. Significance of the complete clearance of peripheral blasts after 7 days of prednisolone treatment in children with acute lymphoblastic leukemia: the Tokyo Children's Cancer Study Group Study L99-15. Haematologica. 2008;93:1155–60.

13. Horibe K, Takimoto T, Tsuchida M, et al. Incidence and survival rates of hematological malignancies in Japanese children and adolescents (2006–2010): based on registry data from the Japanese Society of Pediatric Hematology. Int J Hematol. 2013;98:74–88.

14. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000;355:165–9.

15. Shah S, Schrader KA, Waanders E, et al. A recurrent germline PAX5 mutation confers susceptibility to pre-B cell acute lymphoblastic leukemia. Nat Genet. 2013;45:1226–31.

16. Zhang MY, Churpek JE, Keel SB, et al. Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. Nat Genet. 2015;47:180–5.

17. Papaemmanuil E, Hosking FJ, Vijayakrishnan J, et al. Loci on 7p12.2, 10q21.2 and 14q11.2 are associated with risk of childhood acute lymphoblastic leukemia. Nat Genet. 2009;41:1006–10.

18. Perez-Andreu V, Roberts KG, Harvey RC, et al. Inherited GATA3 variants are associated with Ph-like childhood acute lymphoblastic leukemia and risk of relapse. Nat Genet. 2013;45:1494–8.

19. Sherborne AL, Hosking FJ, Prasad RB, et al. Variation in CDKN2A at 9p21.3 influences childhood acute lymphoblastic leukemia risk. Nat Genet. 2010;42:492–4.
20. Treviño LR, Yang W, French D, et al. Germline genomic variants associated with childhood acute lymphoblastic leukemia. Nat Genet. 2009;41:1001–5.
21. Kendall G, Little MP, Wakeford R. Numbers and proportions of leukemias in young people and adults induced by radiation of natural origin. Leuk Res. 2011;35:1039–43.
22. Schüz J. Exposure to extremely low-frequency magnetic fields and the risk of childhood cancer: update of the epidemiological evidence. Prog Biophys Mol Biol. 2011;107:339–42.
23. Spector LG, Xie Y, Robison LL, et al. Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the Children's Oncology Group. Cancer Epidemiol Biomark Prev. 2005;14:651–5.
24. Greaves M. Infection, immune responses and the aetiology of childhood leukaemia. Nat Rev Cancer. 2006;6:193–203.
25. Kinlen LJ. Epidemiological evidence for an infective basis in childhood leukaemia. Br J Cancer. 1995;71:1–5.
26. Inaba H, Greaves M, Mulligan CG. Acute lymphoblastic leukaemia. Lancet. 2013;381:1943–55.
27. Kato M, Imamura T, Manabe A, et al. Prognostic impact of gained chromosomes in high-hyperdiploid childhood acute lymphoblastic leukaemia: a collaborative retrospective study of the Tokyo Children's Cancer Study Group and Japan Association of Childhood Leukaemia Study. Br J Haematol. 2014;166:295–8.
28. Harris MB, Shuster JJ, Carroll A, et al. Trisomy of leukemic cell chromosomes 4 and 10 identifies children with B-progenitor cell acute lymphoblastic leukemia with a very low risk of treatment failure: a Pediatric Oncology Group Study. Blood. 1992;79:3316–24.
29. Sutcliffe MJ, Shuster JJ, Sather HN, et al. High concordance from independent studies by the Children's Cancer Group (CCG) and Pediatric Oncology Group (POG) associating favorable prognosis with combined trisomies 4, 10, and 17 in children with NCI standard-risk B-precursor acute lymphoblastic leukemia: a Children's Oncology Group (COG) initiative. Leukemia. 2005;19:734–40.
30. Mulligan CG, Jeha S, Pei D, et al. Outcome of children with hypodiploid ALL treated with risk-directed therapy based on MRD levels. Blood. 2015;126:2896–9.
31. Nachman JB, Heerema NA, Sather H, et al. Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. Blood. 2007;110:1112–5.
32. Holmfeldt L, Wei L, Diaz-Flores E, et al. The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet. 2013;45:242–52.
33. Raimondi SC, Zhou Y, Mathew S, et al. Reassessment of the prognostic significance of hypodiploidy in pediatric patients with acute lymphoblastic leukemia. Cancer. 2003;98:2715–22.
34. Greaves MF, Wiemels J. Origins of chromosome translocations in childhood leukaemia. Nat Rev Cancer. 2003;3:639–49.
35. Gale KB, Ford AM, Repp R, et al. Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. Proc Natl Acad Sci U S A. 1997;94:13950–4.
36. Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. Blood. 2003;102:2321–33.
37. Zuna J, Madzo J, Krejci O, et al. ETV6/RUNX1 (TEL/AML1) is a frequent prenatal first hit in childhood leukemia. Blood. 2011;117:368–9.
38. Bhojwani D, Pei D, Sandlund JT, et al. ETV6-RUNX1-positive childhood acute lymphoblastic leukemia: improved outcome with contemporary therapy. Leukemia. 2012;26:265–70.
39. Uckun FM, Sensel MG, Sather HN, et al. Clinical significance of translocation t(1;19) in childhood acute lymphoblastic leukemia in the context of contemporary therapies: a report from the Children's Cancer Group. J Clin Oncol. 1998;16:527–35.
40. Jeha S, Pei D, Raimondi SC, et al. Increased risk for CNS relapse in pre-B cell leukemia with the t(1;19)/TCF3-PBX1. Leukemia. 2009;23:1406–9.
41. Inukai T, Hirose K, Inaba T, et al. Hypercalcemia in childhood acute lymphoblastic leukemia: frequent implication of parathyroid hormone-related peptide and E2A-HLF from translocation 17;19. Leukemia. 2007;21:288–96.

42. Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. Nat Rev Cancer. 2007;7:823–33.

43. Meyer C, Hofmann J, Burmeister T, et al. The MLL recombinome of acute leukemias in 2013. Leukemia. 2013a;27:2165–76.

44. Pieters R, Schrappe M, de Lorenzo P, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multi-centre randomised trial. Lancet. 2007;370:240–50.

45. Hilden JM, Dinndorf PA, Meerbaum SO, et al. Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. Blood. 2006;108:441–51.

46. Dreyer ZE, Hilden JM, Jones TL, et al. Intensified chemotherapy without SCT in infant ALL: results from COG P9407 (cohort 3). Pediatr Blood Cancer. 2015;62:419–26. doi:10.1002/pbc.25322.

47. Tomizawa D, Koh K, Sato T, et al. Outcome of risk-based therapy for infant acute lymphoblastic leukemia with or without an MLL gene rearrangement, with emphasis on late effects: a final report of two consecutive studies, MLL96 and MLL98, of the Japan Infant Leukemia Study Group. Leukemia. 2007;21:2258–63.

48. Koh K, Tomizawa D, Moriya Saito A, et al. Early use of allogeneic hematopoietic stem cell transplantation for infants with MLL gene-rearrangement-positive acute lymphoblastic leukemia. Leukemia. 2015;29:290–6.

49. Andersson AK, Ma J, Wang J, et al. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. Nat Genet. 2015;47:330–7.

50. Aricò M, Schrappe M, Hunger SP, et al. Clinical outcome of children with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia treated between 1995 and 2005. J Clin Oncol. 2010;28:4755–61.

51. Biondi A, Schrappe M, de Lorenzo P, et al. Imatinib after induction for treatment of children and adolescents with Philadelphia-chromosome-positive acute lymphoblastic leukaemia (EsPhALL): a randomised, open-label, intergroup study. Lancet Oncol. 2012;13:936–45.

52. Schultz KR, Carroll A, Heerema NA, et al. Long-term follow-up of imatinib in pediatric Philadelphia chromosome-positive acute lymphoblastic leukemia: Children's Oncology Group study AALL0031. Leukemia. 2014;28:1467–71.

53. Manabe A, Kawasaki H, Shimada H, et al. Imatinib use immediately before stem cell transplantation in children with Philadelphia chromosome-positive acute lymphoblastic leukemia: results from Japanese pediatric leukemia/lymphoma study group (JPLSG) study Ph(+) ALL04. Cancer Med. 2015;4:682–9.

54. Schultz KR, Bowman WP, Aledo A, et al. Improved early event-free survival with Imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a Children's Oncology Group Study. J Clin Oncol. 2009;27:5175–81.

55. Boer Den ML, van Slegtenhorst M, de Menezes RX, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol. 2009;10:125–34.

56. Mullighan CG, Su X, Zhang J, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 2009b;360:470–80.

57. Roberts KG, Morin RD, Zhang J, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell. 2012;22:153–66.

58. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med. 2014a;371:1005–15.

59. Moorman AV, Richards SM, Robinson HM, et al. Prognosis of children with acute lymphoblastic leukaemia (ALL) and intrachromosomal amplification of chromosome 21 (iAMP21). Blood. 2007;109:2327–30.

60. Harrison CJ, Moorman AV, Schwab C, et al. An international study of intrachromosomal amplification of chromosome 21 (iAMP21): cytogenetic characterization and outcome. Leukemia. 2014;28:1015–21.

61. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446:758–64.
62. Mullighan CG, Miller CB, Radtke I, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature. 2008a;453:110–4.
63. Cario G, Zimmermann M, Romey R, et al. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood. 2010;115:5393–7.
64. Mullighan CG, Collins-Underwood JR, Phillips LAA, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet. 2009a;41:1243–6.
65. Russell LJ, Capasso M, Vater I, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood. 2009;114:2688–98.
66. Mullighan CG, Zhang J, Harvey RC, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 2009c;106:9414–8.
67. Harvey RC, Mullighan CG, Chen I-M, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010;115:5312–21.
68. Coustan-Smith E, Mullighan CG, Onciu M, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncol. 2009;10:147–56.
69. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481:157–63.
70. Mullighan CG, Phillips LA, Su X, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science. 2008b;322:1377–80.
71. Hof J, Krentz S, van Schewick C, et al. Mutations and deletions of the TP53 gene predict nonresponse to treatment and poor outcome in first relapse of childhood acute lymphoblastic leukemia. J Clin Oncol. 2011;29:3185–93.
72. Meyer JA, Wang J, Hogan LE, et al. Relapse-specific mutations in NT5C2 in childhood acute lymphoblastic leukemia. Nat Genet. 2013b;45:290–4.
73. Mullighan CG, Zhang J, Kasper LH, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011;471:235–9.
74. Tzoneva G, Perez-Garcia A, Carpenter Z, et al. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. Nat Med. 2013;19:368–71.
75. Mahmoud HH, Rivera GK, Hancock ML, et al. Low leukocyte counts with blast cells in cerebrospinal fluid of children with newly diagnosed acute lymphoblastic leukemia. N Engl J Med. 1993;329:314–9.
76. Bürger B, Zimmermann M, Mann G, et al. Diagnostic cerebrospinal fluid examination in children with acute lymphoblastic leukemia: significance of low leukocyte counts with blasts or traumatic lumbar puncture. J Clin Oncol. 2003;21:184–8.
77. Dutch Childhood Oncology Group, Te Loo DMWM, Kamps WA, et al. Prognostic significance of blasts in the cerebrospinal fluid without pleiocytosis or a traumatic lumbar puncture in children with acute lymphoblastic leukemia: experience of the Dutch Childhood Oncology Group. J Clin Oncol. 2006;24:2332–6.
78. Pui C-H. Toward optimal central nervous system-directed treatment in childhood acute lymphoblastic leukemia. J Clin Oncol. 2003;21:179–81.
79. Cairo MS, Gerrard M, Sposto R, et al. Results of a randomized international study of high-risk central nervous system B non-Hodgkin lymphoma and B acute lymphoblastic leukemia in children and adolescents. Blood. 2007;109:2736–43.
80. Reiter A, Schrappe M, Tiemann M, et al. Improved treatment results in childhood B-cell neoplasms with tailored intensification of therapy: a report of the Berlin-Frankfurt-Münster Group Trial NHL-BFM 90. Blood. 1999;94:3294–306.
81. Tsurusawa M, Mori T, Kikuchi A, et al. Improved treatment results of children with B-cell non-Hodgkin lymphoma: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group B-NHL03 Study. Pediatr Blood Cancer. 2014;61:1215–21.

82. Nagayama J, Tomizawa D, Koh K, et al. Infants with acute lymphoblastic leukemia and a germline MLL gene are highly curable with use of chemotherapy alone: results from the Japan Infant Leukemia Study Group. Blood. 2006;107:4663–5.

83. Smith M, Arthur D, Camitta B, et al. Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. J Clin Oncol. 1996;14:18–24.

84. Schrappe M, Hunger SP, Pui C-H, et al. Outcomes after induction failure in childhood acute lymphoblastic leukemia. N Engl J Med. 2012;366:1371–81.

85. Möricke A, Reiter A, Zimmermann M, et al. Risk-adjusted therapy of acute lymphoblastic leukemia can decrease treatment burden and improve survival: treatment results of 2169 unselected pediatric and adolescent patients enrolled in the trial ALL-BFM 95. Blood. 2008;111:4477–89.

86. Gaynon PS, Desai AA, Bostrom BC, et al. Early response to therapy and outcome in childhood acute lymphoblastic leukemia: a review. Cancer. 1997;80:1717–26.

87. Coustan-Smith E, Behm FG, Sanchez J, et al. Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. Lancet. 1998;351:550–4.

88. van Dongen JJ, Seriu T, Panzer-Grümayer ER, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet. 1998;352:1731–8.

89. Patrick K, Wade R, Goulden N, et al. Outcome for children and young people with early T-cell precursor acute lymphoblastic leukaemia treated on a contemporary protocol, UKALL 2003. Br J Haematol. 2014;166:421–4.

90. Roberts KG, Pei D, Campana D, et al. Outcomes of children with BCR-ABL1-like acute lymphoblastic leukemia treated with risk-directed therapy based on the levels of minimal residual disease. J Clin Oncol. 2014b;32:3012–20.

91. Borowitz MJ, Devidas M, Hunger SP, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group Study. Blood. 2008;111:5477–85.

92. Faham M, Zheng J, Moorhead M, et al. Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. Blood. 2012;120:5173–80.

93. Farber S, Diamond LK. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid. N Engl J Med. 1948;238:787–93.

94. Bostrom BC, Sensel MR, Sather HN, et al. Dexamethasone versus prednisone and daily oral versus weekly intravenous mercaptopurine for patients with standard-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. Blood. 2003;101:3809–17.

95. Mitchell CD, Richards SM, Kinsey SE, et al. Benefit of dexamethasone compared with prednisolone for childhood acute lymphoblastic leukaemia: results of the UK Medical Research Council ALL97 randomized trial. Br J Haematol. 2005;129:734–45.

96. Igarashi S, Manabe A, Ohara A, et al. No advantage of dexamethasone over prednisolone for the outcome of standard- and intermediate-risk childhood acute lymphoblastic leukemia in the Tokyo Children's Cancer Study Group L95-14 protocol. J Clin Oncol. 2005;23:6489–98.

97. Möricke A, Zimmermann M, Valsecchi MG, et al. Dexamethasone vs prednisone in induction treatment of pediatric ALL: results of the randomized trial AIEOP-BFM ALL 2000. Blood. 2016;127:2101–12.

98. Larsen EC, Devidas M, Chen S, et al. Dexamethasone and high-dose methotrexate improve outcome for children and young adults with high-risk B-acute lymphoblastic leukemia: a report from Children's Oncology Group Study AALL0232. J Clin Oncol. 2016;34:2380–8.

99. Mattano LA, Sather HN, Trigg ME, Nachman JB. Osteonecrosis as a complication of treating acute lymphoblastic leukemia in children: a report from the Children's Cancer Group. J Clin Oncol. 2000;18:3262–72.

100. Asselin BL, Whitin JC, Coppola DJ, et al. Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol. 1993;11:1780–6.

101. Vrooman LM, Supko JG, Neuberg DS, et al. Erwinia asparaginase after allergy to *E. coli* asparaginase in children with acute lymphoblastic leukemia. Pediatr Blood Cancer. 2010;54:199–205.

102. Caruso V, Iacoviello L, Di Castelnuovo A, et al. Thrombotic complications in childhood acute lymphoblastic leukemia: a meta-analysis of 17 prospective studies comprising 1752 pediatric patients. Blood. 2006;108:2216–22.

103. Grace RF, Dahlberg SE, Neuberg D, et al. The frequency and management of asparaginase-related thrombosis in paediatric and adult patients with acute lymphoblastic leukaemia treated on Dana-Farber Cancer Institute consortium protocols. Br J Haematol. 2011;152:452–9.

104. Kearney SL, Dahlberg SE, Levy DE, et al. Clinical course and outcome in children with acute lymphoblastic leukemia and asparaginase-associated pancreatitis. Pediatr Blood Cancer. 2009;53:162–7.

105. Vrooman LM, Stevenson KE, Supko JG, et al. Postinduction dexamethasone and individualized dosing of *Escherichia coli* L-asparaginase each improve outcome of children and adolescents with newly diagnosed acute lymphoblastic leukemia: results from a randomized study—Dana-Farber Cancer Institute ALL Consortium Protocol 00-01. J Clin Oncol. 2013;31:1202–10.

106. Matloub Y, Bostrom BC, Hunger SP, et al. Escalating intravenous methotrexate improves event-free survival in children with standard-risk acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood. 2011;118:243–51.

107. Kremer LCM, van der Pal HJH, Offringa M, et al. Frequency and risk factors of subclinical cardiotoxicity after anthracycline therapy in children: a systematic review. Ann Oncol. 2002;13:819–29.

108. Riehm H, Gadner H, Welte K. The west-berlin therapy study of acute lymphoblastic leukemia in childhood—report after 6 years (author's transl). Klin Padiatr. 1977;189:89–102.

109. Tubergen DG, Gilchrist GS, O'Brien RT, et al. Improved outcome with delayed intensification for children with acute lymphoblastic leukemia and intermediate presenting features: a Children's Cancer Group phase III trial. J Clin Oncol. 1993;11:527–37.

110. Reiter A, Schrappe M, Ludwig WD, et al. Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood. 1994;84:3122–33.

111. Winter SS, Devidas M, Chen S, et al. Capizzi-style methotrexate with Pegasparagase (C-MTX) is superior to high-dose methotrexate (HDMTX) in T-lineage acute lymphoblastic leukemia (T-ALL): results from Children's Oncology Group (COG) AALL0434. Blood. 2015;126:794a.

112. Kager L, Cheok M, Yang W, et al. Folate pathway gene expression differs in subtypes of acute lymphoblastic leukemia and influences methotrexate pharmacodynamics. J Clin Invest. 2005;115:110–7.

113. Henze G, Langermann HJ, Brämswig J, et al. The BFM 76/79 acute lymphoblastic leukemia therapy study (author's transl). Klin Padiatr. 1981;193:145–54.

114. Schrappe M, Reiter A, Ludwig WD, et al. Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. Blood. 2000;95:3310–22.

115. Nachman JB, Sather HN, Sensel MG, et al. Augmented post-induction therapy for children with high-risk acute lymphoblastic leukemia and a slow response to initial therapy. N Engl J Med. 1998;338:1663–71.

116. Möricke A, Zimmermann M, Reiter A, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia. 2010;24:265–84.

117. Toyoda Y, Manabe A, Tsuchida M, et al. Six months of maintenance chemotherapy after intensified treatment for acute lymphoblastic leukemia of childhood. J Clin Oncol. 2000;18:1508–16.

118. Kato M, Ishimaru S, Seki M, et al. Long-term outcome of six-month maintenance chemotherapy for acute lymphoblastic leukemia in children. Leukemia. 2017;31(3):580–4.

119. Relling MV, Hancock ML, Boyett JM, et al. Prognostic importance of 6-mercaptopurine dose intensity in acute lymphoblastic leukemia. Blood. 1999;93:2817–23.

120. Moriyama T, Nishii R, Perez-Andreu V, et al. NUDT15 polymorphisms alter thiopurine metabolism and hematopoietic toxicity. Nat Genet. 2016;48:367–73.

121. Yang JJ, Landier W, Yang W, et al. Inherited NUDT15 variant is a genetic determinant of Mercaptopurine intolerance in children with acute lymphoblastic leukemia. J Clin Oncol. 2015;33:1235–42.
122. Krull KR, Brinkman TM, Li C, et al. Neurocognitive outcomes decades after treatment for childhood acute lymphoblastic leukemia: a report from the St Jude Lifetime Cohort Study. J Clin Oncol. 2013;31:4407–15.
123. Pui C-H, Cheng C, Leung W, et al. Extended follow-up of long-term survivors of childhood acute lymphoblastic leukemia. N Engl J Med. 2003;349:640–9.
124. Oliansky DM, Camitta B, Gaynon P, et al. The role of cytotoxic therapy with hematopoietic stem cell transplantation in the treatment of pediatric acute lymphoblastic leukemia: update of the 2005 evidence-based review. ASBMT position statement. Biol Blood Marrow Transplant. 2012;18:979–81.
125. Eapen M, Rubinstein P, Zhang M-J, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet. 2007;369:1947–54.
126. Leung W, Campana D, Yang J, et al. High success rate of hematopoietic cell transplantation regardless of donor source in children with very high-risk leukemia. Blood. 2011;118:223–30.
127. Peters C, Schrappe M, Stackelberg von A, et al. Stem-cell transplantation in children with acute lymphoblastic leukemia: a prospective international multicenter trial comparing sibling donors with matched unrelated donors-the ALL-SCT-BFM-2003 trial. J Clin Oncol. 2015;33:1265–74.
128. Bunin N, Aplenc R, Kamani N, et al. Randomized trial of busulfan vs total body irradiation containing conditioning regimens for children with acute lymphoblastic leukemia: a pediatric blood and marrow transplant consortium study. Bone Marrow Transplant. 2003;32:543–8.
129. Eapen M, Raetz E, Zhang M-J, et al. Outcomes after HLA-matched sibling transplantation or chemotherapy in children with B-precursor acute lymphoblastic leukemia in a second remission: a collaborative study of the Children's Oncology Group and the Center for International Blood and Marrow Transplant Research. Blood. 2006;107:4961–496.
130. Buitenkamp TD, Izraeli S, Zimmermann M, et al. Acute lymphoblastic leukemia in children with Down syndrome: a retrospective analysis from the Ponte di Legno Study Group. Blood. 2014;123:70–7.
131. Goto H, Inukai T, Inoue H, et al. Acute lymphoblastic leukemia and down syndrome: the collaborative study of the Tokyo Children's Cancer Study Group and the Kyushu Yamaguchi Children's Cancer Study Group. Int J Hematol. 2011;93:192–8.
132. Boissel N, Auclerc M-F, Lhéritier V, et al. Should adolescents with acute lymphoblastic leukemia be treated as old children or young adults? Comparison of the French FRALLE-93 and LALA-94 trials. J Clin Oncol. 2003;21:774–80.
133. Hayakawa F, Sakura T, Yujiri T, et al. Markedly improved outcomes and acceptable toxicity in adolescents and young adults with acute lymphoblastic leukemia following treatment with a pediatric protocol: a phase II study by the Japan Adult Leukemia Study Group. Blood Cancer J. 2014;4:e252.
134. Stock W, La M, Sanford B, et al. What determines the outcomes for adolescents and young adults with acute lymphoblastic leukemia treated on cooperative group protocols? A comparison of Children's Cancer Group and Cancer and Leukemia Group B studies. Blood. 2008;112:1646–54.
135. Daigle SR, Olhava EJ, Therkelsen CA, et al. Potent inhibition of DOT1L as treatment of MLL-fusion leukemia. Blood. 2013;122:1017–25.
136. Stumpel DJPM, Schneider P, van Roon EHJ, et al. Specific promoter methylation identifies different subgroups of MLL-rearranged infant acute lymphoblastic leukemia, influences clinical outcome, and provides therapeutic options. Blood. 2009;114:5490–8.
137. Messinger YH, Gaynon PS, Sposto R, et al. Bortezomib with chemotherapy is highly active in advanced B-precursor acute lymphoblastic leukemia: therapeutic advances in Childhood Leukemia & Lymphoma (TACL) study. Blood. 2012;120:285–90.
138. Raetz EA, Cairo MS, Borowitz MJ, et al. Chemoimmunotherapy reinduction with epratuzumab in children with acute lymphoblastic leukemia in marrow relapse: a Children's Oncology Group Pilot Study. J Clin Oncol. 2008;26:3756–62.

139. Kantarjian HM, Deangelo DJ, Stelljes M, et al. Inotuzumab Ozogamicin versus standard therapy for acute lymphoblastic leukemia. N Engl J Med. 2016;375:740–53.
140. Stackelberg von A, Locatelli F, Zugmaier G, et al. Phase I/phase II study of Blinatumomab in pediatric patients with relapsed/refractory acute lymphoblastic leukemia. J Clin Oncol. 2016;34:4381–9.
141. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med. 2014;371:1507–17.

Chapter 3
Acute Myeloid Leukemia

Souichi Adachi, Akitoshi Kinoshita, Daisuke Tomizawa,
Takashi Taga, and Hiroyuki Takahashi

Abstract Acute myeloid leukemia (AML) is a rare type of childhood cancer. With tremendous efforts by the collaborative study groups worldwide in the past decades, survival rates have currently reached approximately 70% in de novo AML and 80% in myeloid leukemia associated with Down syndrome and acute promyelocytic leukemia (APL). Advance in genomic analyses would contribute to further understanding of the pathobiology of AML, which is expected to result in development of better risk stratification, novel molecular targeted therapy, and finally to overcome the disease in the future.

3.1   Introduction

Acute myeloid leukemia (AML) in children accounts for about 25% of pediatric leukemia and affects approximately 180 patients annually in Japan [1]. The prognosis for pediatric AML has improved, and the long-term survival rate now approaches 70%. However, considering the fact that overall survival (OS) rates for pediatric cancer patients are now approaching 80%, there is considerable room for further improvement for refractory AML. Currently, for patients with newly diagnosed

S. Adachi, M.D., Ph.D. (✉)
Human Health Sciences, Kyoto University, Kyoto, Japan
e-mail: adachiso@kuhp.kyoto-u.ac.jp

A. Kinoshita, M.D., Ph.D.
Department of Pediatrics, St. Marianna University School of Medicine, Kanagawa, Japan

D. Tomizawa, M.D., Ph.D.
Children's Cancer Center, National Center for Child Health and Development, Tokyo, Japan

T. Taga, M.D., Ph.D.
Department of Pediatrics, Shiga University of Medical Science, Shiga, Japan

H. Takahashi, M.D., Ph.D.
Department of Pediatrics, Toho University, Tokyo, Japan

AML, clinical studies are conducted separately according to the three disease sub-types, de novo AML, acute promyelocytic leukemia (APL), and myeloid leukemia associated with Down syndrome (ML-DS).

In this chapter, diagnosis of pediatric AML and the current status of pediatric AML (de novo AML, APL, and ML-DS) are presented.

3.2 Diagnosis of Acute Myeloid Leukemia

Diagnosis of AML still relies on morphologic analysis of blood and bone marrow (BM) smears. Although diagnosis may be made from the blood smear alone, BM examination should always be performed. Occasionally, BM aspiration may be difficult in AML patients because of myelofibrosis, which is usually associated with acute megakaryoblastic leukemia (AMKL), or "packed marrow." In these cases, BM biopsy is required. Cellularity of BM is variable in patients with AML unlike acute lymphoblastic leukemia (ALL). AML associated with hypocellular marrow in children should be differentiated from AML secondary to Fanconi anemia.

For a diagnosis of AML, a marrow or blood blast count of 20% or more is required. The exception to this rule is the cases with t(15;17)(q22;q21)/*PML-RARA*, t(8;21)(q22;q22.1)/*RUNX1-RUNX1T1* (formerly known as *AML1-ETO*), inv(16) (p13.1q22) or t(16;16)(p13.1;q22)/*CBFB-MYH11*, and ML-DS [2]. Differentiation between AML and advanced myelodysplastic syndrome (MDS) may be difficult in children with low percentage of blasts. In these cases, it is recommended to repeat BM examination including biopsy. If the blast count increases in a few weeks, the case should be diagnosed as AML [3].

Several types of blast cells and/or abnormal cells may be found in AML. Myeloblasts tend to be larger than lymphoblasts, with round or irregular nuclei, fine chromatin, one or more nucleoli, abundant basophilic cytoplasms, numerous azurophilic granules, and/or Auer rods. Auer rods are the hallmark of AML, but its absence does not exclude its diagnosis. APL is characterized by the proliferation of abnormal promyelocytes with large and coarse azurophilic granules. Some of the promyelocytes contains bundles of Auer rods, called as "faggot cells." Cases with hypogranular APL variants are characterized by abnormal promyelocytes with very fine dust-like granules and lobulated nuclear shape. Monoblasts are large, often with a folded nucleus, fine chromatin, one or more large nucleoli, and abundant blue-gray cytoplasm, in which fine azurophilic granules and vacuoles may be present. Eosinophils with basophilic granules in the cytoplasm are characteristics of acute myelomonocytic leukemia with eosinophilia, which is often associated with recurrent chromosomal abnormalities on chromosome 16 [4]. Pure erythroid leukemia represents a proliferation of medium-to large-size immature erythroblasts with round nuclei, fine chromatin, and one or more nucleoli. Leukemic megakaryoblasts are highly polymorphic, ranging from small blasts with scant cytoplasm and fine-to-dense chromatin resembling lymphoblasts to large cells with abundant and basophilic cytoplasm, fine chromatin, and one or more nucleoli: small and large blasts may be present in the same patient.

They may be bi- or multinucleated, may show distinct blebs or pseudopods, and may form clusters.

AML is subclassified according to the French-American-British (FAB) classification. The FAB classification recognizes eight subtypes of AML. AML with myeloid differentiation is subclassified according to the extent of differentiation as M0 to be the most undifferentiated, following M1, M2, and M3. AML with monoblastic differentiation is subclassified by the degree of granulocytic differentiation as M4 or M5, in which promonocytes are also counted as blast equivalents. AML with a predominant erythroid population is subclassified as M6 and AML with proliferation of megakaryoblasts as M7. Although the FAB classification is based on morphology, the diagnosis of M0 (positive for myeloid markers such as MPO and/or CD13, CD33, CD117) or M7 (positive for platelet markers such as CD41 and/or CD42, CD61) has to be confirmed by immunophenotyping.

Cytochemistry is a quick and useful method to distinguish between AML and ALL. Detection of myeloperoxidase or Sudan Black B indicates myeloid differentiation, but its absence does not exclude myeloid lineage. Nonspecific esterase (NSE) indicates monocytic differentiation, but its absence also does not exclude monocytic lineage. Therefore, immunophenotyping using multicolor flow cytometry is now used to determine lineage affiliation. Although immunophenotyping is required to establish the diagnosis of M0, M7, and acute leukemia of ambiguous lineage, it does not substitute for morphological diagnosis.

Cytogenetic analysis is mandatory at diagnosis. Initial workup should also include evaluation of prognostically relevant genetic aberrations, including at least the following fusion genes: *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA*, *KMT2A* (*MLL*) rearrangements, and internal tandem duplication of *fms-related tyrosine kinase III* (*FLT3*-ITD). Fluorescent *in situ* hybridization (FISH) is necessary to identify *MLL* rearrangements including cryptic cases. It is recommended to store methanol/acetic-fixed cell pellets in cases of failure in cytogenetic analysis.

Although FAB classification has been widely accepted among hematologists and health care providers, concordance among observers tends to be relatively low. [5] More importantly, the FAB classification does not always correlate with biology or molecular genetics of AML that are clinically relevant. [6] The World Health Organization (WHO) classification incorporated cytogenetics and molecular genetic aberrations, both of which have its evidence for prognostic relevance, into the classification scheme of AML. This classification is applicable to pediatric patients as well as adults with AML. In the 2001 classification, the patients with t(15;17), t(8;21), inv(16) or t(16;16), or *MLL* translocations were diagnosed as "AML with recurrent cytogenetic abnormalities" [7]. Major revisions in 2008 were expansion of the AML with recurrent cytogenetic abnormalities category, which included *CCAAT/enhancer-binding protein alpha* (*CEBPA*) and *nucleophosmin* (*NPM1*) mutations as provisional entities, addition of a new category "AML with myelodysplasia-related changes (AML-MRC)," and a strict definition of acute leukemia of ambiguous lineage [8]. The AML-MRC category consists of the following three factors: AML arising from previous MDS or an MDS/myeloproliferative neoplasm, AML with specific MDS-related cytogenetic abnormalities, and/or AML with multilineage dysplasia. Although AML-MRC category has been known to occur

mainly in elderly patients, the recent nationwide Japanese study using a central review revealed that patients with AML-MRC are not rare among children [9].

The WHO classification has been recently updated in 2016 (Table 3.1). AML with t(6;9)(p23;q34.1)/*DEK-NUP214* (formerly known as *DEK-CAN*) and AML with t(1;22)(p13.3;q13.3)/*RBM15-MKL1* (*OTT-MAL*), both of which occur in pediatric patients, are categorized as AML with recurrent genetic abnormalities. *NPM1* mutations and biallelic *CEBPA* are also formally recognized as AML with recurrent genetic abnormalities. *BCR-ABL1* and mutated *RUNX1* are added to provisional entity. Since the clinical significance of multilineage dysplasia alone has been questioned, the presence of multilineage dysplasia alone is not classified as AML-MRC when a mutation of *NPM1* or biallelic mutation of *CEBPA* is present in the new classification [10, 11]. Moreover, the new classification removed the subcategory of acute erythroid leukemia, erythroid/myeloid type (previously defined as >50% BM erythroid precursors and >20% myeloblasts among nonerythroid cells). Myeloblasts are always counted as a percentage of total marrow cells, and the majority of such cases have <20% total blast cells and would be classified as MDS [2].

Table 3.1 WHO 2016 classification of acute myeloid leukemia (AML) and related neoplasms

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*
APL with *PML-RARA*
AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
AML with t(6;9)(p23;q34.1); *DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1*
AML with mutated *NPM1*
AML with biallelic mutations of *CEBPA*
Provisional entity: AML with *BCR-ABL1*, AML with mutated *RUNX1*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise categorized (NOS)
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome
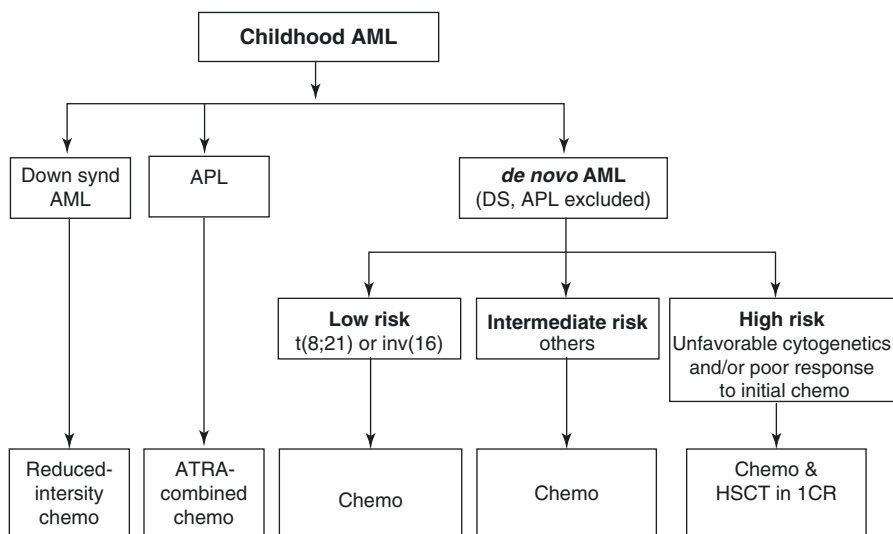
Fig. 3.1 Algorithm of treatment for children with acute myeloid leukemia. AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-transretinoic acid; CR, complete remission; DS, Down syndrome; HSCT, hematopoietic stem cell transplantation. [Adopted from Clinical Guideline for Pediatric Leukemia and Lymphoma (ver.3) Japanese Society of Pediatric Hematology and Oncology.]

3.3   Treatment of De Novo AML

The principle of AML treatment is based on "total cell kill" theory, to eradicate all the leukemic blasts that have expanded inside the patient's body. This is attempted by multi-agent chemotherapy with optional use of hematopoietic stem cell transplantation (SCT) for high risk of relapse. In addition, risk stratification, means of optimizing therapy by evaluating relapse risk with known prognostic factors, and supportive care have contributed to improve survival rate in children with AML.

Treatment algorithm of pediatric AML proposed in the guideline of the Japanese Society of Pediatric Hematology and Oncology is shown in Fig. 3.1. ML-DS and APL are treated separately with each disease-specific protocol. The rest of the children with AML (de novo AML) are treated with appropriate risk-stratified therapies.

3.3.1   Prognostic Factors

3.3.1.1   Biologic and Genetic Features

It is considered that AML occurs as a consequence of malignant transformation of an abnormal single myeloid progenitor cell via multi-step alterations. One model classified the different types of these mutations in two types: the Type I mutations including mutations that function as proliferation and survival signals (e.g., *FLT3*, *KIT*, *RAS*) and the Type II mutations including mutations that lead to differentiation arrest or enhanced self-renewal (e.g., *RUNX1-RUNX1T1*, *CBFB-MYH11*) [12]. The

leukemia stem-cell model proposes that the clonal origin of AML cells reside at the top of hierarchical organization, possessing unlimited self-renewal capacity and capability of propagating leukemia cells [13]. Recent genome-wide analyses are challenging to uncover full landscape of AML leukemogenesis. These novel technologies have found that AML contains fewer genetic alterations than do other malignancies. In particular, pediatric AML contains only 2–4 somatic copy-number alterations per single leukemia cell and no copy-number alterations in approximately one-third of cases, indicating that the development of AML may require fewer genetic alterations compared to other malignancies [14]. Although full picture of AML leukemogenesis is still unclear, disease-specific gene alterations regulate biological character of AML cells, thus have prognostic value, and would be good candidates for therapeutic target.

(a) Core-Binding Factor AML

AML with t(8;21) or either inv(16) or t(16;16) are called "core-binding factor (CBF)" AML and are associated with favorable prognosis. t(8;21) creates *RUNX1-RUNX1T1* fusion gene. The abnormal fusion protein binds DNA and interacts with CBFβ, but dominantly represses transcriptional activation through interactions with the nuclear corepressor complex. t(8;21)-AML comprises 12% of pediatric AML in the USA and Europe and higher prevalence in east Asia (nearly 30% of pediatric AML in Japan). It is clinically associated with FAB M2 morphology and extramedullary disease. Large international retrospective analysis of 838 patients with t(8;21)-AML by the International BFM Study Group (I-BFM-SG) showed additional del(9q) and +4 were associated with treatment failure [15]. Additionally, patients with t(8;21)-AML were likely to benefit from protocols that included high doses of anthracyclines, etoposide, and cytarabine.

inv(16) or t(16;16) creates *CBFB-MYH11* fusion gene. The rearrangement causes formation of abnormal CBFβ with impaired function, thus leading to improper differentiation of myeloid cells. AML with inv(16) accounts for 10% or less of pediatric AML. It is associated with myelomonocytic morphology with abnormal eosinophilia (FAB M4Eo).

The *KIT* gene, encoding a type III receptor tyrosine kinase, is mutated in nearly 20% of CBF-AML. Prognostic impact of mutated *KIT* in CBF-AML is controversial [16–18]; however, recent analysis among the Japanese AML-05 cohort showed significant poor prognosis of *KIT* exon 17 mutations in t(8;21)-AML; especially exon 17 D816V mutation was associated with higher relapse rate [19].

(b) *KMT2A* Gene Rearrangements

KMT2A (*MLL*) gene, located at chromosome band 11q23, encodes a histone methyltransferase that is involved in epigenetic regulation of blood cell development [20]. Rearrangements of *MLL* occur as a result of balanced chromosomal translocations that fuse the *MLL* gene to one of more than 70 known partner genes [21]. *MLL* rearrangement accounts for 15–20% of pediatric AML, and the most common is *MLL-AF9* derived from t(9;11)(p22;q23) which accounts for ~50% of the cases. International retrospective analysis of 756 children with *MLL*-rearranged AML by the I-BFM-SG identified large differences

in outcome according to *MLL* subtypes, showing significant poor survival rate of t(1;11)(q21;q23), t(6;11)(q27;q23), t(10;11)(p12;q23), and t(10;11)(p11.2;q23) cases [22]. Recent analysis of the Japanese AML-05 cohort showed clear difference in the outcome of *MLL-AF9* AML according to the expression of the *ecotropic viral integration site-1* (*EVI1*) gene, overexpression being a poor prognostic factor [23].

(c) Genetic Alterations in Non-Down Syndrome Acute Megakaryoblastic Leukemia
 AMKL (FAB M7) accounts for 10% of AML in non-Down syndrome (non-DS) children. Unlike DS AMKL with mutated *GATA1*, prognosis of non-DS AMKL is reported to be poor [24], but the outcome is moderately improving with modern intensive chemotherapy [25]. Heterogeneity of non-DS AMKL has been described in several studies. I-BFM-SG reported that non-DS AMKL could be classified in three groups: good risk group with 7p abnormalities; poor risk group with normal karyotypes, −7, 9p abnormalities including t(9;11)/*MLL-AF9*, −13/13q-, and −15; and intermediate risk group with others including t(1;22)/*RBM15-MKL1*, found in infants, and 11q23/*MLL* rearrangements other than *MLL-AF9* [26]. Recent genomic studies identified two novel recurrent genetic abnormalities in non-DS AMKL: *CBFA2T3-GLIS2* created by cryptic inv(16)(p13.3q24.3) and *NUP98-KDM5A* (formerly known as *NUP98-JARID1A*) by cryptic t(11;15)(15;q35) [27, 28]. *CBFA2T3-GLIS2* and *NUP98-KDM5A* comprise ~15 and ~10% of pediatric non-DS AMKL cases, respectively, and both confer poor prognosis [29].

(d) *FLT3*-ITD
 FLT3-ITD accounts for more than 20% of adult AML and 5–10% of pediatric AML and is associated with poor prognosis [30–32]. *FLT3*-ITD is most commonly observed in cytogenetically normal (CN)-AML, but with other translocations as well. It is considered that the presence of *FLT3*-ITD has no prognostic impact when present with favorable risk cytogenetics such as CBF-AML or t(15;17)/*PML-RARA* APL. Some study groups such as Children's Oncology Group (COG) include only *FLT3*-ITD with high allelic ratio in high-risk criteria.

(e) *NUP98-NSD1*
 Nucleoporin 98kD (*NUP98*) gene located on chromosome 11p15 fuses to more than 20 different partner genes. Among them, *NUP98-NSD1* created by cryptic t(5;11)(q35;p15.5) was recently identified by the Dutch group using array-based genomic tests [33]. *NUP98-NSD1* comprised 4.2% of pediatric AML and 16.1% of CN-AML. Clinically, this abnormality is associated with FAB M4 and M5 and the presence of *FLT3*-ITD and *WT1* mutations and confers poor prognosis.

(f) Others
 Monosomy 7 and monosomy 5 or 5q deletions account for ~5% of pediatric AML and are traditionally used as poor-risk factors in many studies [34, 35]. Monosomy 5 or 5q deletions are associated with complex karyotype (>3 chromosomal abnormalities, excluding recurrent changes). Other poor prognostic cytogenetics

include t(16;21)(p11;q22)/*FUS-ERG*, t(9;22)/*BCR-ABL1*, t(6;9)/*DEK-NUP214*, and inv(3) or t(3;3)/*RPN1-MECOM*, but are very rare in children.

With conventional cytogenetic analysis, approximately 20% of the cases fall into a CN-AML category with intermediate prognosis. However, development of genetic studies has revealed that CN-AML is a heterogeneous disease with various prognoses. Among them, *FLT3*-ITD and *NUP98-NSD1* are associated with unfavorable outcome as previously described, while biallelic mutations of *CEBPA* and *NPM1* are associated with favorable outcome [36–40].

3.3.1.2 Early Treatment Response

Risk stratification evaluating morphological treatment response in the bone marrow after the first course of chemotherapy is currently utilized in many pediatric AML study groups, which is predictive of final outcome.

Recently, measurement of minimal residual disease (MRD) targeting leukemic clone-specific features has been increasingly used for better risk stratification. Methods that are applicable to AML are flow cytometry detecting aberrant combinations of leukemic cell-surface antigen, polymerase chain reaction (PCR) amplification of leukemia-specific mutated gene transcripts, quantitative measurement of WT1 expression, etc. Detection of fusion transcripts by reverse transcript (RT)-PCR method has sensitivity of 10^{-4}–10^{-5}; however, it is applicable to only 50% of the cases. Moreover, it is well known that fusions such as *RUNX1-RUNX1T1* and *CBFB-MYH11* could persist in patients with long-term remission. Hence, flow-based MRD is more widely used because of its applicability (more than 90% of the cases) and its specificity, although the sensitivity is one log lower than the PCR-based method. In the AML02 study of St. Jude Children's Research Hospital, MRD of 1% or higher after initial induction course clearly segregated patients with high and low risk of relapse, and the relapse rate was only 17% for the patients whose MRD level was below 0.1% after the second induction [41]. However, both disease-free survival (DFS) and OS rates were low for poor prognostic patients [e.g., t(6;11), t(10;11), AMKL without t(1;22)] even with negative MRD (<0.1%) [42], suggesting the stronger impact of biological factors on outcome in AML.

3.3.2 AML Therapy

Therapy for AML is consisted of remission-induction and post-remission phases. In both phases, chemotherapy including two major key drugs, cytarabine and anthracyclines, is the mainstay of the treatment. Choice of post-remission therapy, especially indication of allogeneic SCT, is determined by risk of relapse evaluated by known prognostic factors.

3.3.2.1 Remission-Induction Phase

Remission-induction therapy aims to achieve morphological complete remission (CR), defined as less than 5% blasts in bone marrow with regeneration of normal hematopoiesis and no evidence of residual extramedullary disease. Time of evaluating remission differs by protocol, but most typical is after completing two courses, and 80–90% of the children achieve remission in contemporary therapy.

The first successful induction regimen was "3 + 7" established in 1980s by the Cancer and Leukemia Group B (CALGB) in the USA for adult AML, 7 days of cytarabine (100 mg/m^2) by continuous infusion plus 3 days of daunorubicin (45 mg/m^2), which resulted in 60–70% CR rate [43]. Until now, intensification of "3 + 7" has been attempted to improve the outcome of AML.

(a) Cytarabine Intensification
    CALGB 8321 study compared cytarabine dose of 200 mg/m^2/day with 100 mg/m^2/day in induction, but could not find clear advantage of higher cytarabine dose [44]. Still, several studies use cytarabine dose of 200 mg/m^2: ECM consisted of cytarabine (200 mg/m^2, 7 days)/mitoxantrone (5 mg/m^2, 5 days) combination following 5 days of etoposide (150 mg/m^2) which is a standard induction regimen in Japan since the early 1990s [45, 46]. Several studies have compared 100–200 mg/m^2 continuous infusion versus high dose (1–3 g/m^2/dose infused every 12 h for 3–7 days), but have not demonstrated superiority of high-dose cytarabine [47, 48]. Medical Research Council (MRC) studies in the United Kingdom (the UK) used different schedules of cytarabine in induction: 100 mg/m^2/dose intravenous push every 12 h [49]. In MRC AML 9 study, 10 days of cytarabine with daunorubicin/thioguanine was superior to 5-day cytarabine combination, which became the standard induction in the UK. Therefore, cytarabine schedule of either 100–200 mg/m^2 continuous infusion or 100 mg/m^2/dose intravenous push every 12 h is considered as standard in the contemporary induction regimen.

(b) Use of Different Anthracyclines and Their Dose Intensification
    There are several formulations of anthracyclines in clinical use: the most common are doxorubicin, daunorubicin, idarubicin (a daunorubicin derivative), and mitoxantrone (an anthracendione derivative). Doxorubicin is not used for AML treatment, because it showed higher mortality from infection and/or gastrointestinal toxicity compared to daunorubicin in the past CALGB study [43]. Among the pediatric studies, AML-BFM 93 compared 3 days of daunorubicin 60 mg/m^2 (ADE) and idarubicin 12 mg/m^2 (AIE): idarubicin showing faster day 15 blast clearance, but similar EFS and OS rates [50]. MRC AML 12 compared 3 days of daunorubicin 50 mg/m^2 (ADE) and mitoxantrone 12 mg/m^2 (MAE): better DFS and lower cumulative incidence of relapse were observed in MAE, but CR and OS rates were similar [51]. Recently, liposomal daunorubicin is drawing attention because of low accumulation to heart and high therapeutic index. AML BFM 2004 study compared 3 days of liposomal daunorubicin 80 mg/m^2 (ADxE) and idarubicin 12 mg/m^2 (AIE): although liposomal

daunorubicin was less toxic and more active in t(8;21)-AML, EFS, OS, and relapse incidence were similar between the two arms [52].

Adult studies demonstrated superior CR and OS rates in dose-escalated dauno-rubicin of 90 mg/m^2 for 3 days compared with conventional dose of 45 mg/m^2 in combination with cytarabine [53, 54]. However, this approach is not appropriate for children because of increased risk of developing late cardiotoxicity.

In conclusion, the use of either daunorubicin 60 mg/m^2 or idarubicin/mitoxantrone 10–12 mg/m^2 for 3 days is considered standard in the contemporary induction therapy for children with AML.

(c) Addition of Extra Agents to Cytarabine/Anthracycline Backbone

MRC AML 10 trial compared etoposide and thioguanine in combination with cytarabine/daunorubicin induction backbone, but no difference in outcome was observed [55]. Although no clear benefit has been demonstrated for adding extra agents to the standard induction backbone, etoposide is often used in many pediatric AML induction regimens.

One attractive additional agent is gemtuzumab ozogamicin (GO), a cali-cheamicin conjugated anti-CD33 monoclonal antibody. Because GO demonstrated reasonable activity as a single agent use for relapsed or refractory AML [56, 57], GO combination with standard induction backbone was evaluated in several groups as frontline AML therapy. One adult randomized trial by the Southwest Oncology Group (SWOG) showed no therapeutic efficacies, but higher fatal induction toxicity in GO arm, which led to withdrawal of the product in the US market [58]. However, MRC AML 15 trial in the UK showed benefit of adding GO 3 mg/m^2 to one of the three randomized induction regimens for patients with favorable cytogenetics [59], and French ALFA-0701 study showed improved outcome in adult AML patients by adding low fractionated-dose GO (3 mg/m^2/dose on days 1, 4, and 7) to 3 + 7 induction [60]. The pediatric study COG AAML0531 demonstrated improved EFS through reduction of relapse rate by adding 3 mg/m^2 of GO to the standard regimen [61].

3.3.2.2   Post-Remission Phase

Following remission-induction phase, post-remission therapy is administered aiming to consolidate remission status. It has been clarified in the early adult Eastern Cooperative Oncology Group (ECOG) study in 1983 that all patients eventually relapse without post-remission therapy [62].

(a) High-Dose Cytarabine Intensification

The effect of intensifying post-remission chemotherapy was demonstrated by adult CALGB study comparing three different doses of cytarabine in consolidation courses, showing significant improvement in OS for patients <60 years old when a high-dose cytarabine was given, especially those with favorable cytogenetics [63, 64]. In addition, CALGB investigators reported that the outcome of CBF-AML adults who were treated with multiple courses of high-dose

cytarabine was better than those who only received one course of high-dose cytarabine [65, 66]. Among the pediatric studies, BFM group showed that high-dose cytarabine with mitoxantrone (HAM regimen) following initial induction was beneficial for t(8;21)-AML [67, 68]. The Japanese pediatric AML trials have incorporated repetitive high-dose cytarabine cycles in consolidation and produced 70% or higher OS rate [46]. Thus, post-remission high-dose cytarabine is considered as standard approach in pediatric AML.

(b) Number of Treatment Courses

Optimal number of total treatment courses is an open question. MRC AML 12 trial compared a total of five courses versus four courses of chemotherapy, showing no survival benefit of fifth course of chemotherapy [51]. However, comparison of the two Japanese consecutive trials (AML99 and AML-05) demonstrated conflicting results by different cytogenetic group. While both EFS and OS did not differ in non-CBF-AML children between six and five courses with identical cumulative anthracycline dosage [69], significant worse EFS was observed in CBF-AML children who received five courses of chemotherapy with reduction of cumulative anthracycline dosage [70].

(c) Maintenance Therapy

Unlike chemotherapy for ALL, maintenance is unnecessary in the context of contemporary AML therapy with exception of APL. French Leucémie Aiguë Myéloblastique Enfant (LAME) 89 and 91 studies randomized children with AML to receive or not to receive maintenance therapy: strikingly, they not only demonstrated lack of benefit in DFS, but showed inferior OS in the maintenance group [71]. So far, BFM is the only group still incorporating maintenance phase in the frontline pediatric AML therapy [72].

(d) Central Nervous System-Directed Therapy

In AML, high leukocyte count, young age (<2 years), monoblastic or myelomonoblastic leukemia (FAB M4 or M5), and cytogenetics of t(8;21), inv(16), or chromosome 11 abnormalities are associated with central nervous system (CNS) leukemia and/or relapse [73]. Outcome of AML patients with CNS involvement is not poor, partly because they are more likely to have favorable cytogenetics thus with lower rate of systemic relapse. Current practice for treatment and prevention of CNS leukemia employs both intrathecal and systemic chemotherapy. Cranial irradiation is not generally used in AML, except the BFM group that continues to administer either 12 or 18Gy of cranial irradiation in AML BFM 2004 trial because of the result from AML BFM 87 trial that observed higher systemic relapse rate in nonirradiated patients [72].

(e) Hematopoietic Stem Cell Transplantation for AML

Since the 1980s, allogeneic SCT from matched sibling donor became widely applicable as post-remission therapy for children with AML. Most of the clinical studies conducted between the 1980s to the 1990s allocated patients to allo-SCT by "Mendelian or genetic randomization," that those with matched sibling donor to allo-SCT and those without to chemotherapy (or autologous SCT). In the Children's Cancer Group (CCG) 2891 study, allo-SCT had a significantly better DFS and OS than did chemotherapy or auto-SCT [74]. However, no

difference was observed in trials AML BFM 98, European Organization of Research and Treatment of Cancer (EORTC) Children's Leukemia Group (CLG) 58,921, and MRC AML 10 [75]. As risk stratification by cytogenetic abnormalities was introduced from the late 1990s, a consensus was built that no indication of allo-SCT in first CR for favorable cytogenetics such as t(8;21) and inv(16). Along with improved survival by intensive chemotherapy and increasing concern of late effects, allo-SCT is currently restricted to only high-risk cases in first CR.

Since the 2000s, allo-SCTs from alternative donors (e.g., unrelated bone marrow or cord blood donor, haploidentical donor) have been increasingly performed with comparable outcome [76, 77]. Current standard conditioning for pediatric AML is busulfan-based regimen without total body irradiation (TBI), because most of the studies showed identical or even inferior outcome with TBI conditioning for both adult and pediatric AML [78, 79]. In terms of reducing late effects, use of reduced intensity conditioning (RIC) is an attractive option for children undergoing allo-SCT [80–82]. Although there are some promising results, the role of RIC should be carefully evaluated in the context of clinical trial.

3.3.3   Novel Therapeutic Approach for AML

Although approximately 70% of the children with AML are eventually cured, 10% of the patients fail to achieve CR and 30% of the patients suffer from disease recurrence (Table 3.2) [46, 48, 51, 52, 61, 83–86]. One of the well-recognized reinduction regimens for these patients is FLAG, combination of fludarabine, cytarabine, and granulocyte colony-stimulating factor, with or without anthracycline [87]. However, further CR rate is 50–60% and OS rate is 30–40% at most [88]. Improvement in the outcome of frontline treatment also seems to have reached the ceiling with conventional therapeutic approach, since most of the interventions tested in various clinical trials demonstrated negative results or very small improvement. Therefore, development of novel therapeutic approaches is urgently needed to further improve the outcome of AML.

There is substantial number of novel agents under evaluation: new class of chemotherapy (clofarabine, vosaroxin, CPX-351), tyrosine kinase inhibitors (FLT3 inhibitors, KIT inhibitors, RAS pathway inhibitors, Polo-like kinase inhibitor, Aurora-kinase inhibitor), proteasome inhibitors (bortezomib, carfilzomib), epigenetic agents (methyltransferase inhibitors, histone deacetylases, DOT1L inhibitor), immunotherapy [SGN33A, bispecific T-cell engager (BiTE) antibodies, chimeric antigen receptor (CAR) T cells], etc. [89]. It is expected that the genomic landscape of AML would be fully unmasked in the near future and that the integration of discovered genomics into contemporary therapy would progress in order to realize precision medicine which would result in further improvement in the outcome of childhood AML.

Table 3.2 Results of the recently reported cooperative trials for pediatric AML

Study Group	Trial	Years	No. of patients	Age	%SCT in 1CR	EFS, % (year)	OS, % (year)	RR, %	Reference
Japanese childhood AML cooperative study	AML99	2000–2002	240	0–15	Allo 17% Auto 2%	61 (5)	75 (5)	32	[46]
BFM	AML-BFM 2004	2004–2010	521	0–18	NA	55 (5)	74 (5)	29	[52]
JPLSG	AML-05	2006–2010	443	0–18	12%	54 (3)	73 (3)	30	[70, 83]
St. Jude Children's Research Hospital	AML02	2002–2008	216	0–21	25%	63 (3)	71 (3)	21	[48]
AIEOP	AML2002/01	2002–2011	482	0–18	Allo 29% Auto 21%	55 (8)	67 (8)	24	[84]
COG	AAML0531 (GO arm)	2006–2010	1022	0–29	NA	53 (3)	69 (3)	32	[61]
NOPHO	AML 2004	2004–2009	151	0–15	15%	57 (3)	69 (3)	30	[85, 86]
MRC	AML12	1995–2002	564	0–15	11%	54 (10)	63 (10)	32	[51]

BFM Berlin-Frankfurt-Münster, *JPLSG* Japanese Pediatric Leukemia/Lymphoma Study Group, *AIEOP* Associazione Italiana Ematologia Oncologia Pediatrica, *COG* Children's Oncology Group, *NOPHO* Nordic Society of Paediatric Hematology and Oncology, *MRC* Medical Research Council *SCT* hematopoietic stem cell transplantation, *EFS* event-free survival, *OS* overall survival, *RR* cumulative incidence of relapse, *GO* gemtuzumab ozogamicin, *NA* not available

3.4 Myeloid Leukemia Associated with Down Syndrome

Down syndrome (DS) results from trisomy 21 and occurs in 1 in 700–1000 births [90, 91]. Although the incidence of neoplasms in DS does not differ significantly from that in the general population, the distribution of malignancies is different [92]. Patients with DS show a unique spectrum of malignancies, with a 10- to 20-fold higher risk of acute leukemia and a lower incidence of solid tumors [92, 93]. The most frequent form of leukemia during childhood, both with and without DS, is ALL, and the incidence of ALL in children with DS is approximately 20-fold higher than that in non-DS children [92]. However, the most marked increase in incidence in DS infants is AMKL, known as "myeloid leukemia associated with DS (ML-DS)." The development of ML-DS is closely linked to a preceding temporary form of neonatal leukemia called transient abnormal myelopoiesis (TAM). TAM and ML-DS are classified as an independent category as myeloid proliferations of Down syndrome in recent WHO classification [2, 94, 95]. Both generally manifest as megakaryoblastic phenotype,

with TAM occurring at birth or within days of birth and being resolved in 1–2 months. About 1–2% of children with DS develop AML during the first 4 years of life. MDS often precedes AMKL and can last for several months. The clinical course of patients of MDS in DS appears to be relatively indolent, presents initially with a period of thrombocytopenia, and lacks significant increase of blasts. Findings from recent clinical and laboratory studies revealed that quantitative alterations of *RUNX1*, *ETS2*, and *ERG* genes (all located at 4-Mb region of chromosome 21) that resulted from constitutional trisomy 21 up-regulate *GATA1* mutations and cause TAM [96] and that additional genetic alterations including those in epigenetic regulators and signaling molecules are involved in the progression from TAM to ML-DS [97]. Children with DS above the age of 5 with AML or MDS have different biology to ML-DS, such as non-megakaryoblastic phenotype and no *GATA1* mutation of blasts, and should be considered not as "typical ML-DS," but as "conventional" AML or MDS.

ML-DS has unique characteristics of higher sensitivity to chemotherapeutic agents. *In vitro* studies showed that ML-DS blasts were significantly more sensitive to chemotherapeutic drugs than non-DS AML cells [98]. ML-DS blasts are especially sensitive to cytarabine, possibly because of the effect of *GATA1* mutations and trisomy 21 on the levels of cytarabine-metabolizing enzymes [99]. Before the 1990s, most patients with ML-DS received suboptimal therapy, resulting in poor outcomes. In 1992, high rates of EFS with intensive AML treatment were reported from the Pediatric Oncology Group (POG) [100]. After recognition of the favorable outcome of ML-DS patients treated with the AML protocol, recruitment to collaborative studies for ML-DS patients increased, but it became apparent that treatment-related toxicity was high in most series [101–103]. Since then, several collaborative groups have adapted their AML protocols for ML-DS by reducing the dosage of chemotherapeutic agents [103]. As a result, in recent clinical studies in several countries, ML-DS children were treated separately and less intensively than non-DS AML children. Recent clinical trials for ML-DS are summarized in Table 3.3 [101–109]. In Europe, the ML-DS 2006 study by the I-BFM-SG, which was based on the reduced intensity arm of the BFM 98 protocol with further elimination of etoposide in consolidation courses, was conducted. This protocol consists of four courses including high-dose cytarabine. The US AAML0431 study, conducted from 2007 to 2011 by the COG, which also included high-dose cytarabine, showed excellent outcomes [110]. The Japanese trials for ML-DS had investigated less intensive chemotherapy compared with those conducted in Western countries, and treatment outcomes were comparable [108, 109, 111]. Toronto group reported long-term results of an ultralow dose cytarabine-based regimens suggested that at least some population of ML-DS could be cured by minimum intensive therapy [106]. Predicting prognostic factors had been examined by several studies; however, no universal factors had been found to date [107, 109, 112]. On the other hand, it is reported that relapsed/refractory cases are rarely salvageable, with very limited role of allo-SCT [113]. In terms of treatment outcome, ML-DS is a heterogeneous disease and risk-oriented therapy is a reasonable strategy, so that finding an accurate method, perhaps MRD, for identifying a subgroup with poor prognosis is urgently needed. Moreover, new therapeutic approaches for relapsed/refractory cases using

Table 3.3 Outcome of recent clinical trials for myeloid leukemia with Down syndrome

Study	Years	N	Cumulative dose of agents (mg/m^2) DRB (^{a}THP)	Ara-C	ETP	TRM, %	EFS, % (year)	OS, % (year)	Reference
AML BFM 98	1998–2003	67	220–240	23–29,000	950	5	89 (3)	91 (3)	[50, 101]
NOPHO AML 93	1988–2002	41	300	48,600	1600	5	85 (8)	NA	[104]
MRC AML10/12	1988–2002	46	670	10,600	0	15	74 (5)	74 (5)	[103]
CCG 2861/2891	1989–1999	160	320	15,800	1600	4	77 (6)	79 (6)	[102]
COG A2971	1999–2003	132	320	27,200	0	3	79 (5)	84 (5)	[105]
Toronto, LD-Ara-C	1990–2003	34	0	7400	0	0	67 (5)	77 (5)	[106]
AML99 DS	2000–2004	72	250^a	3500	2250	1	83 (4)	84 (4)	[107]
JCCLSG 9805DS	1998–2006	24	190^a	12,600	200	12.5	83 (5)	88 (5)	[108]
JPLSG AML-D05	2008–2010	72	SR: 250^a HR: 170^a	SR: 3500 HR: 12,800	SR: 1350 HR: 1050	1	83 (3)	88 (3)	[109]

BFM Berlin-Frankfurt-Münster, *NOPHO* Nordic Society of Paediatric Hematology and Oncology, *MRC* Medical Research Council, *CCG* Children's Cancer Group, *COG* Children's Oncology Group, *JCCLSG* Japanese Children's Cancer and Leukemia Study Group, *JPLSG* Japanese Pediatric Leukemia/Lymphoma Study Group
DRB daunorubicin, *THP* pirarubicin, *Ara-C* cytarabine, *ETP* etoposide, *TRM* treatment-related mortality, *EFS* event-free survival, *OS* overall survival, *LD-Ara-C* low-dose cytarabine, *SR* standard risk, *HR* high risk

new drugs such as Wee 1 inhibitor [114], Aurora-kinase inhibitor [115], and histone deacetylase inhibitors [116] will be also needed.

3.5 Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a distinct subtype of AML characterized by t(15;17) translocation attributed to *PML/RARA* fusion transcript. Since the life-threatening complications, disseminated intravascular coagulation (DIC), often develop initially in patients with APL, the disease must be managed as an oncologic emergency, and rapid and appropriate diagnosis and simultaneous commencement of remission induction therapy are of great importance [117]. Introduction of all-trans retinoic acid (ATRA) as a differentiating agent combined with conventional chemotherapy made this subtype as one of the most curable leukemia subtype.

The diagnosis of APL is first suspected by the existence of clinical or laboratory findings of DIC. APL cells express annexin II on their cell surfaces that engage plasminogen activator and its substrate plasminogen, resulting in excess production of plasmin. Because plasmin degrades both fibrinogen and fibrin, hemorrhagic symptom caused by hyperfibrinolysis is the main finding in APL-related DIC. In laboratory tests, therefore, hypofibrinogenemia and elevated fibrinogen/fibrin degradation product (FDP), rather than prolongation of coagulation time, are remarkable. Morphologically, APL cells represent characteristic patterns with varying size and shapes of nucleus, numerous cytoplasmic granules, and bizarre Auer rods. Especially, the bundle of Auer rods, a "faggot cell," is highly specific in APL. Cytoplasmic granules and Auer rods are also recognized by peroxidase staining of leukemic cells. APL cells possess distinct patterns in immunophenotype; myeloid-associated maturation markers such as MPO, CD13, and CD33 are frequently positive with a characteristic feature of CD34 and HLA-DR negativity [118]. Because the retardation of induction therapy sometimes leads to fatal hemorrhagic complications, ATRA should be administered immediately without waiting for the results of cytogenetic or genetic examinations.

Remission induction therapy for APL consists of ATRA and anthracyclines. The treatment efficacy is first recognized by the resolution of DIC, that is, the decrease of plasma FDP value [119]. However, because anthracycline treatment induces apoptosis of APL cells resulting in reactivation of fibrinolytic system, hemorrhagic complications may deteriorate during anthracycline administration. In this condition, appropriate antihemostatic treatment and platelet transfusion are indispensable. Another severe complication during induction therapy is APL-differentiation syndrome (APL-DS) that is caused by chemical mediators produced by differentiated leukemic promyelocytes [120]. APL-DS is characterized mainly by fever, fluid retention, and respiratory distress, occurring especially with hyperleukocytosis. Although glucocorticoid therapy and transient cessation of ATRA are effective, mechanical ventilation is sometimes required in severe cases.

Consolidation and maintenance therapies consist of ATRA and anthracycline with or without cytarabine and ATRA with or without oral cytotoxic agents, respectively. With these treatment modalities, the EFS and OS rates in childhood APL reached around 80%–90%, respectively [121–127]. Table 3.4 shows recent treatment outcome of childhood APL conducted by multicenter collaborative study groups. In Japan, a nationwide prospective study, AML-P05, was conducted by the JPLSG between 2006 and 2011. The main aim of this study was to evaluate an efficacy of treatment using reduced intensity of consolidation therapy with minimized cumulative doses of anthracyclines. The 3-year EFS and OS rates in this study were 83.6% and 90.7%, respectively, which were comparable to that of preceding childhood APL studies. Therefore, it was concluded that a single administration of anthracycline in each consolidation phase seemed sufficient in the treatment of childhood APL.

Monitoring MRD by detecting *PML/RARA* fusion transcript has high predictive value of disease outcome and is a useful tool to guide therapeutic options in APL [128]. Actually, persistence or reemergence of MRD almost always results in overt

Table 3.4 Outcome of recent clinical trials for childhood acute promyelocytic leukemia

Group	Study	N	Anthracyclines (mg)[a]	EFS, % (year)	OS, % (year)	Reference
European APL	APL93/2000	84	411	NA	Children: 80.4 (5) Adolescents: 93.6 (5)	[121]
AIEOP	AIDA0493	107	600	76 (10)	86 (10)	[127]
PETHEMA	LPA96/99	66	600–760	77 (5)	87 (5)	[125]
BFM	AML93–2004	81	330	73 (5)	89 (5)	[50, 101, 122]
North American	C9710	56	415	53 (3)	87 (3)	[124]
COG	AAML0631	102	275–325	NA	NA	[124]
Japanese childhood AML cooperative study	AML99-M3	58	282	91.4 (7)	93.1 (7)	[123]
JPLSG	AML-P05	43	246	83.6 (3)	90.7 (3)	[126]

AIEOP Associazione Italiana Ematologia Oncologia Pediatrica, *PETHEMA* Programa de Estudio y Tratamiento de las Hemopatías Malignas, *BFM* Berlin-Frankfurt-Münster, *COG* Children's Oncology Group, *JPLSG* Japanese Pediatric Leukemia/Lymphoma Study Group
EFS event free survival, *OS* overall survival
aThe cumulative anthracycline dose was converted to doxorubicin equivalent with the following ratios: 1:0.83 for daunorubicin, 1:5 for idarubicin, 1:4 for mitoxantrone, 1:0.6 for pirarubicin, and 1:0.2 for aclarubicin according to the JPLSG criteria of anthracycline equivalents

hematological relapse, and early intervention in this situation (molecular relapse) will significantly improve the patients' outcome [129]. Therefore, MRD positivity at the end of consolidation therapy necessitates alternative salvage therapy.

Recently, the second class of APL differentiating agent, arsenic trioxide (ATO), is introduced [130]. Unlike ATRA, which facilitates the terminal differentiation of leukemic promyelocytes by binding PML-RARA oncogenic transcription factor, ATO induces myeloid differentiation by degrading PML-RARA protein. Therefore, ATRA and ATO were considered to possess synergistic effect against APL cells. At first, ATO was introduced in adult patients with relapsed APL and resulted in high second remission rates without affecting the rate of severe adverse events, and the efficacy of ATO were also demonstrated in relapsed childhood APL. Very recently, two European groups reported significantly higher rates of EFS among adult patients with non-high-risk APL (i.e., initial WBC count $<10 \times 10^9$/L) treated with ATRA and ATO only, which was comparable to those who received conventional ATRA-combined chemotherapy [131, 132]. They concluded that APL would be curable only with differentiating agents, without giving cytotoxic agents like anthracyclines, at least in non-high risk APL patients.

At present, ATO is beginning to be introduced in prospective studies for the treatment of newly diagnosed childhood APL to test the efficacy and the safety of this drug in children [89]. In the ongoing AML-P13 trial for childhood APL in Japan, all three consolidation phases are replaced by ATO courses to further minimize the cumulative dose of anthracycline and to maximize the effect of treatment. These studies may shift the treatment paradigm for childhood APL in the near future.

References

1. Horibe K, Takimoto T, Tsuchida M, et al. Incidence and survival rates of hematological malignancies in Japanese children and adolescents (2006-2010): based on registry data from the Japanese Society of Pediatric Hematology. Int J Hematol. 2013;98:74–88.
2. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127:2391–405.
3. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. Blood. 2012;120:3187–205.
4. Arthur DC, Bloomfield CD. Association of partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute non-lymphocytic leukemia. Blood. 1983;62:931.
5. Argyle JC, Benjamin DR, Lampkin B, Hammond D. Acute nonlymphocytic leukemias of childhood. Inter-observer variability and problems in the use of the FAB classification. Cancer. 1989;63:295–301.
6. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. J Clin Oncol. 2003;21:4642–9.
7. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100:2292–302.
8. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937–51.
9. Kinoshita A, Miyachi H, Matsushita H, et al. Acute myeloid leukaemia with myelodysplastic features in children: a report of Japanese Paediatric Leukaemia/Lymphoma Study Group. Br J Haematol. 2014;167:80–6.
10. Bacher U, Schnittger S, Macijewski K, et al. Multilineage dysplasia does not influence prognosis in CEBPA-mutated AML, supporting the WHO proposal to classify these patients as a unique entity. Blood. 2012;119:4719–22.
11. Falini B, Macijewski K, Weiss T, et al. Multilineage dysplasia has no impact on biologic, clinicopathologic, and prognostic features of AML with mutated nucleophosmin (NPM1). Blood. 2010;115:3776–86.
12. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;100:1532–42.
13. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3:730–7.
14. Radtke I, Mullighan CG, Ishii M, et al. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc Natl Acad Sci U S A. 2009;106:12944–9.
15. Klein K, Kaspers G, Harrison CJ, et al. Clinical impact of additional cytogenetic aberrations, cKIT and RAS mutations, and treatment elements in pediatric t(8;21)-AML: results from an International Retrospective Study by the International Berlin-Frankfurt-Münster Study Group. J Clin Oncol. 2015;33:4247–58.

16. Goemans BF, Zwaan CM, Miller M, et al. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia. 2005;19:1536–42.

17. Pollard JA, Alonzo TA, Gerbing RB, et al. Prevalence and prognostic significance of KIT mutations in pediatric patients with core binding factor AML enrolled on serial pediatric cooperative trials for de novo AML. Blood. 2010;115:2372–9.

18. Shimada A, Ichikawa H, Taki T, et al. Low frequency of KIT gene mutation in pediatric acute myeloid leukemia with inv(16)(p13q22): a study of the Japanese Childhood AML Cooperative Study Group. Int J Hematol. 2007;86:289–90.

19. Tokumasu M, Murata C, Shimada A, et al. Adverse prognostic impact of KIT mutations in childhood CBF-AML: the results of the Japanese Pediatric Leukemia/Lymphoma Study Group AML-05 trial. Leukemia. 2015;29:2438–41.

20. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet. 2002;30:41–7.

21. Meyer C, Hofmann J, Burmeister T, et al. The MLL recombinome of acute leukemias in 2013. Leukemia. 2013;27:2165–76.

22. Balgobind BV, Raimondi SC, Harbott J, et al. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. Blood. 2009;114:2489–96.

23. Matsuo H, Kajihara M, Tomizawa D, et al. EVI1 overexpression is a poor prognostic factor in pediatric patients with mixed lineage leukemia-AF9 rearranged acute myeloid leukemia. Haematologica. 2014;99:e225–7.

24. Reinhardt D, Diekamp S, Langebrake C, et al. Acute megakaryoblastic leukemia in children and adolescents, excluding Down's syndrome: improved outcome with intensified induction treatment. Leukemia. 2005;19:1495–6.

25. Schweitzer J, Zimmermann M, Rasche M, et al. Improved outcome of pediatric patients with acute megakaryoblastic leukemia in the AML-BFM 04 trial. Ann Hematol. 2015;94:1327–36.

26. Inaba H, Zhou Y, Abla O, et al. Heterogeneous cytogenetic subgroups and outcomes in childhood acute megakaryoblastic leukemia: a retrospective international study. Blood. 2015;126:1575–84.

27. de Rooij JDE, Hollink IHIM, Arentsen-Peters STCJM, et al. NUP98/JARID1A is a novel recurrent abnormality in pediatric acute megakaryoblastic leukemia with a distinct HOX gene expression pattern. Leukemia. 2013;27:2280–8.

28. Gruber TA, Larson Gedman A, Zhang J, et al. An Inv(16)(p13.3q24.3)-encoded CBFA2T3-GLIS2 fusion protein defines an aggressive subtype of pediatric acute megakaryoblastic leukemia. Cancer Cell. 2012;22:683–97.

29. de Rooij JDE, Masetti R, van den Heuvel-Eibrink MM, et al. Recurrent abnormalities can be used for risk group stratification in pediatric AMKL: a retrospective intergroup study. Blood. 2016;127:3424–30.

30. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98:1752–9.

31. Meshinchi S, Woods WG, Stirewalt DL, et al. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood. 2001;97:89–94.

32. Zwaan CM, Meshinchi S, Radich JP, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood. 2003;102:2387–94.

33. Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood. 2011a;118:3645–56.

34. Hasle H, Alonzo TA, Auvrignon A, et al. Monosomy 7 and deletion 7q in children and adolescents with acute myeloid leukemia: an international retrospective study. Blood. 2007;109:4641–7.

35. Johnston DL, Alonzo TA, Gerbing RB, et al. Outcome of pediatric patients with acute myeloid leukemia (AML) and −5/5q- abnormalities from five pediatric AML treatment protocols: a report from the Children's Oncology Group. Pediatr Blood Cancer. 2013;60:2073–8.

36. Brown P, McIntyre E, Rau R, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007;110:979–85.

37. Ho PA, Alonzo TA, Gerbing RB, et al. Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood. 2009;113:6558–66.

38. Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, et al. Characterization of CEBPA mutations and promoter hypermethylation in pediatric acute myeloid leukemia. Haematologica. 2011b;96:384–92.

39. Hollink IHIM, Zwaan CM, Zimmermann M, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia. 2009;23:262–70.

40. Shiba N, Funato M, Ohki K, et al. Mutations of the GATA2 and CEBPA genes in paediatric acute myeloid leukaemia. Br J Haematol. 2013;164:142–5.

41. Inaba H, Coustan-Smith E, Cao X, et al. Comparative analysis of different approaches to measure treatment response in acute myeloid leukemia. J Clin Oncol. 2012;30:3625–32.

42. Karol SE, Coustan-Smith E, Cao X, et al. Prognostic factors in children with acute myeloid leukaemia and excellent response to remission induction therapy. Br J Haematol. 2015;168:94–101.

43. Yates J, Glidewell O, Wiernik P, et al. Cytosine arabinoside with daunorubicin or adriamycin for therapy of acute myelocytic leukemia: a CALGB study. Blood. 1982;60:454–62.

44. Dillman RO, Davis RB, Green MR, et al. A comparative study of two different doses of cytarabine for acute myeloid leukemia: a phase III trial of Cancer and Leukemia Group B. Blood. 1991;78:2520–6.

45. Tomizawa D, Tabuchi K, Kinoshita A, et al. Repetitive cycles of high-dose cytarabine are effective for childhood acute myeloid leukemia: long-term outcome of the children with AML treated on two consecutive trials of Tokyo Children's Cancer Study Group. Pediatr Blood Cancer. 2007;49:127–32.

46. Tsukimoto I, Tawa A, Horibe K, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2009;27:4007–13.

47. Becton D, Dahl GV, Ravindranath Y, et al. Randomized use of cyclosporin A (CsA) to modulate P-glycoprotein in children with AML in remission: Pediatric Oncology Group Study 9421. Blood. 2006;107:1315–24.

48. Rubnitz JE, Inaba H, Dahl G, et al. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. Lancet Oncol. 2010;11:543–52.

49. Gibson BES, Wheatley K, Hann IM, et al. Treatment strategy and long-term results in paediatric patients treated in consecutive UK AML trials. Leukemia. 2005;19:2130–8.

50. Creutzig U, Ritter J, Zimmermann M, et al. Idarubicin improves blast cell clearance during induction therapy in children with AML: results of study AML-BFM 93. AML-BFM Study Group. Leukemia. 2001a;15:348–54.

51. Gibson BES, Webb DKH, Howman AJ, et al. Results of a randomized trial in children with Acute Myeloid Leukaemia: medical research council AML12 trial. Br J Haematol. 2011;155:366–76.

52. Creutzig U, Zimmermann M, Bourquin J-P, et al. Randomized trial comparing liposomal daunorubicin with idarubicin as induction for pediatric acute myeloid leukemia: results from Study AML-BFM 2004. Blood. 2013;122:37–43.

53. Fernandez HF, Sun Z, Yao X, et al. Anthracycline dose intensification in acute myeloid leukemia. N Engl J Med. 2009;361:1249–59.

54. Lowenberg B, Ossenkoppele GJ, van Putten W, et al. High-dose daunorubicin in older patients with acute myeloid leukemia. N Engl J Med. 2009;361:1235–48.

55. Stevens RF, Hann IM, Wheatley K, Gray RG. Marked improvements in outcome with chemotherapy alone in paediatric acute myeloid leukemia: results of the United Kingdom Medical Research Council's 10th AML trial. MRC Childhood Leukaemia Working Party. Br J Haematol. 1998;101:130–40.

56. Arceci RJ, Sande J, Lange B, et al. Safety and efficacy of gemtuzumab ozogamicin in pediatric patients with advanced CD33+ acute myeloid leukemia. Blood. 2005;106:1183–8.

57. Zwaan CM, Reinhardt D, Zimmerman M, et al. Salvage treatment for children with refractory first or second relapse of acute myeloid leukaemia with gemtuzumab ozogamicin: results of a phase II study. Br J Haematol. 2010;148:768–76.

58. Petersdorf SH, Kopecky KJ, Slovak M, et al. A phase 3 study of gemtuzumab ozogamicin during induction and postconsolidation therapy in younger patients with acute myeloid leukemia. Blood. 2013;121:4854–60.

59. Burnett AK, Hills RK, Milligan D, et al. Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. J Clin Oncol. 2011;29:369–77.

60. Castaigne S, Pautas C, Terré C, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. Lancet. 2012;379:1508–16.

61. Gamis AS, Alonzo TA, Meshinchi S, et al. Gemtuzumab ozogamicin in children and adolescents with de novo acute myeloid leukemia improves event-free survival by reducing relapse risk: results from the randomized phase III Children's Oncology Group trial AAML0531. J Clin Oncol. 2014;32:3021–32.

62. Cassileth PA, Harrington DP, Hines JD, et al. Maintenance chemotherapy prolongs remission duration in adult acute nonlymphocytic leukemia. J Clin Oncol. 1988;6:583–7.

63. Bloomfield CD, Lawrence D, Byrd JC, et al. Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. Cancer Res. 1998;58:4173–9.

64. Mayer RJ, Davis RB, Schiffer CA, et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. N Engl J Med. 1994;331:896–903.

65. Byrd JC, Dodge RK, Carroll A, et al. Patients with t(8;21)(q22;q22) and acute myeloid leukemia have superior failure-free and overall survival when repetitive cycles of high-dose cytarabine are administered. J Clin Oncol. 1999;17:3767–75.

66. Byrd JC, Ruppert AS, Mrózek K, et al. Repetitive cycles of high-dose cytarabine benefit patients with acute myeloid leukemia and inv(16)(p13q22) or t(16;16)(p13;q22): results from CALGB 8461. J Clin Oncol. 2004;22:1087–94.

67. Creutzig U, Ritter J, Zimmermann M, et al. Improved treatment results in high-risk pediatric acute myeloid leukemia patients after intensification with high-dose cytarabine and mitoxantrone: results of Study Acute Myeloid Leukemia-Berlin-Frankfurt-Münster 93. J Clin Oncol. 2001b;19:2705–13.

68. Creutzig U, Zimmermann M, Bourquin J-P, et al. Second induction with high-dose cytarabine and mitoxantrone: different impact on pediatric AML patients with t(8;21) and with inv(16). Blood. 2011;118:5409–15.

69. Hasegawa D, Tawa A, Tomizawa D, et al. Attempts to optimize post-induction treatment in childhood acute myeloid leukemia without core binding factors: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG). Blood. 2012;120:3545a.

70. Tomizawa D, Tawa A, Watanabe T, et al. Excess treatment reduction including anthracyclines results in higher incidence of relapse in core binding factor acute myeloid leukemia in children. Leukemia. 2013a;27:2413–6.

71. Perel Y, Auvrignon A, LeBlanc T, et al. Impact of addition of maintenance therapy to intensive induction and consolidation chemotherapy for childhood acute myeloblastic leukemia:

results of a prospective randomized trial, LAME 89/91. Leucámie Aiqüe Myéloïde Enfant. J Clin Oncol. 2002;20:2774–82.

72. Creutzig U, Zimmermann M, Ritter J, et al. Treatment strategies and long-term results in paediatric patients treated in four consecutive AML-BFM trials. Leukemia. 2005b;19:2030–42.

73. Pui C-H, Howard SC. Current management and challenges of malignant disease in the CNS in paediatric leukaemia. Lancet Oncol. 2008;9:257–68.

74. Woods WG, Neudorf S, Gold S, et al. A comparison of allogeneic bone marrow transplantation, autologous bone marrow transplantation, and aggressive chemotherapy in children with acute myeloid leukemia in remission. Blood. 2001;97:56–62.

75. Niewerth D, Creutzig U, Bierings MB, Kaspers GJL. A review on allogeneic stem cell transplantation for newly diagnosed pediatric acute myeloid leukemia. Blood. 2010;116:2205–14.

76. Leung W, Campana D, Yang J, et al. High success rate of hematopoietic cell transplantation regardless of donor source in children with very high-risk leukemia. Blood. 2011;118:223–30.

77. Michel G, Rocha V, Chevret S, et al. Unrelated cord blood transplantation for childhood acute myeloid leukemia: a Eurocord Group analysis. Blood. 2003;102:4290–7.

78. Copelan EA, Hamilton BK, Avalos B, et al. Better leukemia-free and overall survival in AML in first remission following cyclophosphamide in combination with busulfan compared with TBI. Blood. 2013;122:3863–70.

79. de Berranger E, Cousien A, Petit A, et al. Impact on long-term OS of conditioning regimen in allogeneic BMT for children with AML in first CR: TBI+CY versus BU+CY: a report from the Société Française de Greffe de Moelle et de Thérapie Cellulaire. Bone Marrow Transplant. 2014;49:382–8.

80. Bitan M, He W, Zhang M-J, et al. Transplantation for children with acute myeloid leukemia: a comparison of outcomes with reduced intensity and myeloablative regimens. Blood. 2014;123:1615–20.

81. Ishida H, Adachi S, Hasegawa D, et al. Comparison of a fludarabine and melphalan combination-based reduced toxicity conditioning with myeloablative conditioning by radiation and/or busulfan in acute myeloid leukemia in Japanese children and adolescents. Pediatr Blood Cancer. 2014;21:2141–7.

82. Nagler A, Rocha V, Labopin M, et al. Allogeneic hematopoietic stem-cell transplantation for acute myeloid leukemia in remission: comparison of intravenous busulfan plus cyclophosphamide (Cy) versus total-body irradiation plus Cy as conditioning regimen—a report from the acute leukemia working party of the European Group for Blood and Marrow Transplantation. J Clin Oncol. 2013;31:3549–56.

83. Tomizawa D, Tawa A, Watanabe T, et al. Appropriate dose reduction in induction therapy is essential for the treatment of infants with acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group. Int J Hematol. 2013b;98:578–88.

84. Pession A, Masetti R, Rizzari C, et al. Results of the AIEOP AML 2002/01 multicenter prospective trial for the treatment of children with acute myeloid leukemia. Blood. 2013;122:170–8.

85. Abrahamsson J, Forestier E, Heldrup J, et al. Response-guided induction therapy in pediatric acute myeloid leukemia with excellent remission rate. J Clin Oncol. 2011;29:310–5.

86. Hasle H, Abrahamsson J, Forestier E, et al. Gemtuzumab ozogamicin as postconsolidation therapy does not prevent relapse in children with AML: results from NOPHO-AML 2004. Blood. 2012;120:978–84.

87. Kaspers GJL, Zimmermann M, Reinhardt D, et al. Improved outcome in pediatric relapsed acute myeloid leukemia: results of a randomized trial on liposomal daunorubicin by the International BFM Study Group. J Clin Oncol. 2013;31:599–607.

88. Nakayama H, Tabuchi K, Tawa A, et al. Outcome of children with relapsed acute myeloid leukemia following initial therapy under the AML99 protocol. Int J Hematol. 2014;100:171–9.

89. Zwaan CM, Kolb EA, Reinhardt D, et al. Collaborative efforts driving progress in pediatric acute myeloid leukemia. J Clin Oncol. 2015;33:2949–62.
90. Parker SE, Mai CT, Canfield MA, et al. Updated national birth prevalence estimates for selected birth defects in the United States, 2004-2006. Birth Defects Res A Clin Mol Teratol. 2010;88:1008–16.
91. Roizen NJ, Patterson D. Down's syndrome. Lancet. 2003;361:1281–9.
92. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000;355:165–9.
93. Fong CT, Brodeur GM. Down's syndrome and leukemia: epidemiology, genetics, cytogenetics and mechanisms of leukemogenesis. Cancer Genet Cytogenet. 1987;28:55–76.
94. Lange BJ, Kobrinsky N, Barnard DR, et al. Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. Blood. 1998;91:608–15.
95. Roy A, Roberts I, Vyas P. Biology and management of transient abnormal myelopoiesis (TAM) in children with Down syndrome. Semin Fetal Neonatal Med. 2012;17:196–201.
96. Banno K, Omori S, Hirata K, et al. Systematic cellular disease models reveal synergistic interaction of Trisomy 21 and GATA1 mutations in hematopoietic abnormalities. Cell Rep. 2016;15:1228–41.
97. Yoshida K, Toki T, Okuno Y, et al. The landscape of somatic mutations in Down syndrome-related myeloid disorders. Nat Genet. 2013;45:1293–9.
98. Zwaan CM, Kaspers GJL, Pieters R, et al. Different drug sensitivity profiles of acute myeloid and lymphoblastic leukemia and normal peripheral blood mononuclear cells in children with and without Down syndrome. Blood. 2002;99:245–51.
99. Ge Y, Stout ML, Tatman DA, et al. GATA1, cytidine deaminase, and the high cure rate of Down syndrome children with acute megakaryocytic leukemia. J Natl Cancer Inst. 2005;97:226–31.
100. Ravindranath Y, Abella E, Krischer JP, et al. Acute myeloid leukemia (AML) in Down's syndrome is highly responsive to chemotherapy: experience on Pediatric Oncology Group AML Study 8498. Blood. 1992;80:2210–4.
101. Creutzig U, Reinhardt D, Diekamp S, et al. AML patients with Down syndrome have a high cure rate with AML-BFM therapy with reduced dose intensity. Leukemia. 2005a;19:1355–60.
102. Gamis AS, Woods WG, Alonzo TA, et al. Increased age at diagnosis has a significantly negative effect on outcome in children with Down syndrome and acute myeloid leukemia: a report from the Children's Cancer Group Study 2891. J Clin Oncol. 2003;21:3415–22.
103. Rao A, Hills RK, Stiller C, et al. Treatment for myeloid leukaemia of Down syndrome: population-based experience in the UK and results from the Medical Research Council AML 10 and AML 12 trials. Br J Haematol. 2006;132:576–83.
104. Abildgaard L, Ellebaek E, Gustafsson G, et al. Optimal treatment intensity in children with Down syndrome and myeloid leukaemia: data from 56 children treated on NOPHO-AML protocols and a review of the literature. Ann Hematol. 2006;85:275–80.
105. Sorrell AD, Alonzo TA, Hilden JM, et al. Favorable survival maintained in children who have myeloid leukemia associated with Down syndrome using reduced-dose chemotherapy on Children's Oncology Group trial A2971: a report from the Children's Oncology Group. Cancer. 2012;118:4806–14.
106. Al-Ahmari A, Shah N, Sung L, et al. Long-term results of an ultra low-dose cytarabine-based regimen for the treatment of acute megakaryoblastic leukaemia in children with Down syndrome. Br J Haematol. 2006;133:646–8.
107. Kudo K, Kojima S, Tabuchi K, et al. Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with Down syndrome and acute myeloid leukemia: the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2007;25:5442–7.
108. Taga T, Shimomura Y, Horikoshi Y, et al. Continuous and high-dose cytarabine combined chemotherapy in children with down syndrome and acute myeloid leukemia: Report from the

Japanese children's cancer and leukemia study group (JCCLSG) AML 9805 down study. Pediatr Blood Cancer. 2011;57:36–40.

109. Taga T, Watanabe T, Tomizawa D, et al. Preserved high probability of overall survival with significant reduction of chemotherapy for myeloid leukemia in Down syndrome: a nation-wide prospective study in Japan. Pediatr Blood Cancer. 2016;63:248–54.

110. Taub JW, Berman JN, Hitzler JK, et al. Improvement in treatment outcome and identification of a new prognostic parameter in Down Syndrome Acute Myeloid Leukemia (DS-AML): Results of the Children's Oncology Group (COG) Phase III AAML0431 Trial. Blood. 2014;124:278.

111. Kudo K, Hama A, Kojima S, et al. Mosaic Down syndrome-associated acute myeloid leukemia does not require high-dose cytarabine treatment for induction and consolidation therapy. Int J Hematol. 2010;91:630–5.

112. Blink M, Zimmermann M, Neuhoff von C, et al. Normal karyotype is a poor prognostic factor in myeloid leukemia of Down syndrome: a retrospective, international study. Haematologica. 2014;99:299–307.

113. Taga T, Saito AM, Kudo K, et al. Clinical characteristics and outcome of refractory/relapsed myeloid leukemia in children with Down syndrome. Blood. 2012;120:1810–5.

114. Caldwell JT, Edwards H, Buck SA, et al. Targeting the wee1 kinase for treatment of pediatric Down syndrome acute myeloid leukemia. Pediatr Blood Cancer. 2014;61:1767–73.

115. Wen Q, Goldenson B, Silver SJ, et al. Identification of regulators of polyploidization presents therapeutic targets for treatment of AMKL. Cell. 2012;150:575–89.

116. Stankov MV, Khatib El M, Kumar Thakur B, et al. Histone deacetylase inhibitors induce apoptosis in myeloid leukemia by suppressing autophagy. Leukemia. 2014;28:577–88.

117. Sanz MA, Grimwade D, Tallman MS, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2009;113:1875–91.

118. Kaleem Z, Crawford E, Pathan MH, et al. Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. Arch Pathol Lab Med. 2003;127:42–8.

119. Tallman MS, Lefèbvre P, Baine RM, et al. Effects of all-trans retinoic acid or chemotherapy on the molecular regulation of systemic blood coagulation and fibrinolysis in patients with acute promyelocytic leukemia. J Thromb Haemost. 2004;2:1341–50.

120. de la Serna J, Montesinos P, Vellenga E, et al. Causes and prognostic factors of remission induction failure in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and idarubicin. Blood. 2008;111:3395–402.

121. Bally C, Fadlallah J, Leverger G, et al. Outcome of acute promyelocytic leukemia (APL) in children and adolescents: an analysis in two consecutive trials of the European APL Group. J Clin Oncol. 2012;30:1641–6.

122. Creutzig U, Zimmermann M, Dworzak M, et al. Favourable outcome of patients with child-hood acute promyelocytic leukaemia after treatment with reduced cumulative anthracycline doses. Br J Haematol. 2010;149:399–409.

123. Imaizumi M, Tawa A, Hanada R, et al. Prospective study of a therapeutic regimen with all-trans retinoic acid and anthracyclines in combination of cytarabine in children with acute promyelocytic leukaemia: the Japanese childhood acute myeloid leukaemia cooperative study. Br J Haematol. 2010;152:89–98.

124. Kutny MA, Gregory J, Feusner JH. Treatment of paediatric APL: how does the therapeutic approach differ from adults? Best Pract Res Clin Haematol. 2014;27:69–78.

125. Ortega JJ, Madero L, Martín G, et al. Treatment with all-trans retinoic acid and anthracycline monochemotherapy for children with acute promyelocytic leukemia: a multicenter study by the PETHEMA Group. J Clin Oncol. 2005;23:7632–40.

126. Takahashi H, Watanabe T, Kinoshita A, et al. High event-free survival rate with minimum-dose-anthracycline treatment in childhood acute promyelocytic leukaemia: a nationwide pro-spective study by the Japanese Paediatric Leukaemia/Lymphoma Study Group. Br J Haematol. 2016;174:437–43.

127. Testi AM, Biondi A, Coco Lo F, et al. GIMEMA-AIEOPAIDA protocol for the treatment of newly diagnosed acute promyelocytic leukemia (APL) in children. Blood. 2005;106:447–53.

128. Diverio D, Rossi V, Avvisati G, et al. Early Detection of Relapse by Prospective Reverse Transcriptase-Polymerase Chain Reaction Analysis of the PML/RARα Fusion Gene in Patients With Acute Promyelocytic Leukemia Enrolled in the GIMEMA-AIEOP Multicenter "AIDA" Trial. Blood. 1998;92:784–9.

129. Esteve J, Escoda L, Martin G, et al. Outcome of patients with acute promyelocytic leukemia failing to front-line treatment with all-trans retinoic acid and anthracycline-based chemotherapy (PETHEMA protocols LPA96 and LPA99): benefit of an early intervention. Leukemia. 2007;21:446–52.

130. Sanz MA, Fenaux P, Coco FL. Arsenic trioxide in the treatment of acute promyelocytic leukemia. A review of current evidence. Haematologica. 2005;90(9):1231–5.

131. Burnett AK, Russell NH, Hills RK, et al. Arsenic trioxide and all-trans retinoic acid treatment for acute promyelocytic leukaemia in all risk groups (AML17): results of a randomised, controlled, phase 3 trial. Lancet Oncol. 2015;16:1295–305.

132. Lo Coco F, Avvisati G, Vignetti M, et al. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. N Engl J Med. 2013;369:111–21.

Chapter 4
Myelodysplastic Syndrome (MDS) and Juvenile Myelomonocytic Leukemia (JMML)

Daisuke Hasegawa and Atsushi Manabe

Abstract Myelodysplastic syndrome (MDS) is a group of clonal hematopoietic stem cell disorders and is uncommon in children comparing to adults. According to the proportion of blasts in the bone marrow (BM) and peripheral blood, it is divided into low-grade MDS and advanced MDS. Low-grade MDS often shows hypocellular BM, which makes differentiation from aplastic anemia and inherited bone marrow failure syndrome challenging. Treatment strategy for low-grade MDS should be determined based on the severity of cytopenias, karyotypes, and donor availability. In advanced MDS, two-thirds have chromosomal aberrations such as monosomy 7, and acute myeloid leukemia (AML) is the major differential diagnosis. Allogeneic hematopoietic cell transplantation (HCT) is the mainstay of treatment for children with advanced MDS; however, relapse and transplant-related mortality are obstacle to cure.

Juvenile myelomonocytic leukemia (JMML) is an aggressive myeloid neoplasm of early childhood characterized by excessive proliferation of monocytic and granulocytic cells. The hypersensitivity of myeloid progenitor cells to GM-CSF is regarded as a hallmark of JMML and is caused by mutations in genes regulating the RAS signaling pathway (i.e., *NRAS*, *KRAS*, *NF1*, *PTPN11*, and *CBL*). Although allogeneic HCT is the treatment of choice for most patients with JMML, a subset of patients harboring mutations in *CBL* or *RAS* can be managed without HCT.

4.1 Introduction

Myelodysplastic syndrome (MDS) is a term defined as a group of clonal hematopoietic stem cell disorders characterized by cytopenia, dysplasia in any one of the myeloid lineages, ineffective hematopoiesis, and a varying risk of progression to

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D. Hasegawa • A. Manabe (✉)
Department of Pediatrics, St. Luke's International Hospital,
9-1, Akashi-cho, Chuo-ku, Tokyo 104-8560, Japan
e-mail: hase-dai@umin.net; manabe-luke@umin.ac.jp

acute myeloid leukemia (AML). MDS is uncommon in children comparing to adults, and several differences between MDS in children and adults have been identified, such as the rarity of refractory anemia with ringed sideroblasts (RARS) and MDS associated with del(5q) chromosome [1]. Pediatric MDS can be evolved from inherited bone marrow failure syndromes (IBMFS) such as Fanconi anemia (FA). Importantly, the therapeutic aim in children with MDS is cure, not palliation.

Considering these differences, a pediatric approach to the diagnosis and management of MDS and myeloproliferative diseases (MPD) was proposed in 2003 [1] and was incorporated into the fourth edition of the World Health Organization (WHO) classification in 2008 [2]. This classification mentioned unique characteristics of pediatric MDS without increased blasts, i.e., low-grade MDS. For example, neutropenia and thrombocytopenia are more likely to be the presenting manifestations in children in contrast to adults who often present with isolated anemia [3]. Children with low-grade MDS frequently show hypocellular marrow [3]. Consequently, "refractory cytopenia of childhood (RCC)" was introduced as a provisional entity in the 2008 WHO classification [2]. It is recommended that children who meet the criteria for refractory cytopenia with multilineage dysplasia (RCMD) be considered as RCC until the prognostic significance of a multilineage dysplasia is clarified in children [2]. However, the significance of the RCC classification remains to be established so far and still causes controversies [3].

Pediatric MDS with increased blasts, i.e., advanced MDS, includes not only refractory anemia with excess blasts (RAEB) but RAEB in transformation (RAEB-T) in the WHO classification [1, 2].

Juvenile myelomonocytic leukemia (JMML) was established as a single entity of the disease in late 1997 [4]. It may resemble chronic myelomonocytic leukemia (CMML) in adults; however, the hypersensitivity of myeloid progeny cells to granulocyte colony-stimulating factor (GM-CSF), which is a hallmark of the disease, clearly distinguishes JMML from CMML in adults [5]. The WHO classification also followed this concept in 2000 [2].

In this chapter, we will describe MDS first and JMML in a second part so that the readers may understand better.

4.2   Pediatric MDS

4.2.1   Epidemiology

Pediatric MDS is an uncommon disorder as mentioned above. Combined population-based data from Denmark and British Columbia in Canada indicated an annual incidence of MDS of 1.8 per million children aged 0–14 years, which corresponds to 4% of all hematological malignancies in children [6]. In a retrospective study of MDS and JMML diagnosed between 1990 and 1997, the frequencies of pediatric

MDS and JMML were estimated as 7.7% of childhood leukemia in Japan with a high proportion of therapy-related cases (23%) [7].

From February 2009 to October 2013, 1000 children who were suspected of having MDS, MPD, and bone marrow (BM) failure were prospectively registered into the Japanese Society of Pediatric Hematology/Oncology database and underwent the central review of morphology and histology [3]. Of those, 339 and 24 children were diagnosed with de novo low-grade MDS and RAEB, respectively (Fig. 4.1). This indicated that the annual incidence of de novo MDS in Japan was 78 cases, more than 90% of which were classified as low-grade MDS. Disease registry data between 2006 and 2010 showed that the frequencies of MDS/MPD in Japan were 8.0% of leukemia and crude incidence rate was 0.3 cases per 100.000 populations at risk [8].

Irrespective of subtype, MDS is diagnosed in all age groups with median age at diagnosis ranging from 6 to 12 years from previous studies [9–14]. Boys and girls are equally affected.

Total (2009.2 – 2013.10)	1,000
Bone marrow failure	570
AA	137
RCC	236
RCMD	103
Hepatitis-related	38
PNH	3
IBMFS	53
Advanced MDS / AML	97
RAEB	24
Therapy-related	21
AA-related	1
IBMFS-related	5
De novo AML	32
Down syndrome-related	14
MPN	116
Anemia	68
Thrombocytopenia	51
Neutropenia	26
Others	72
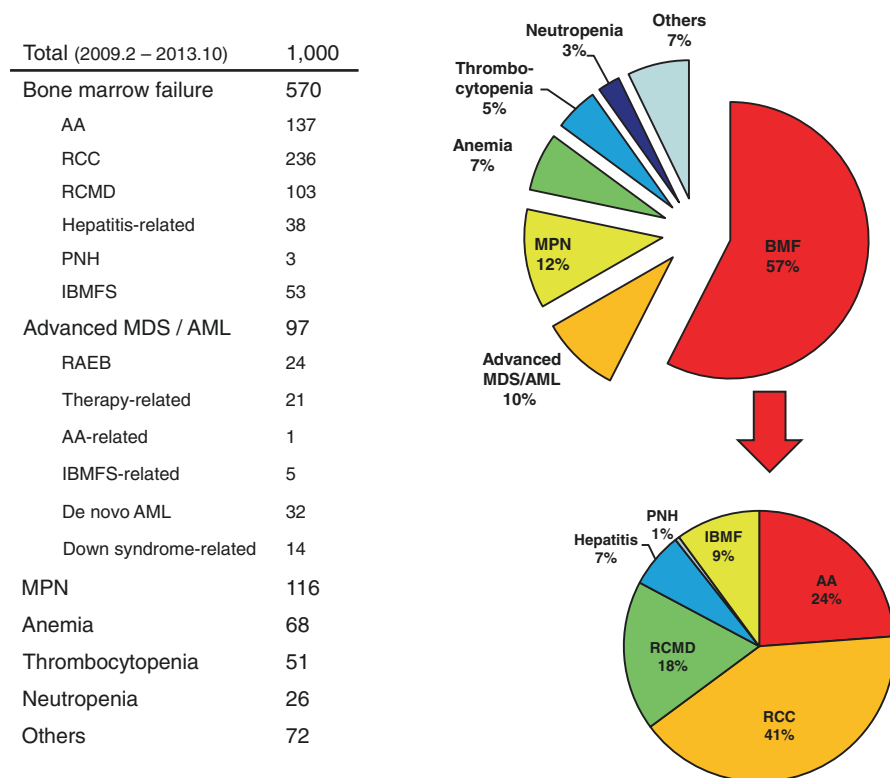
Fig. 4.1 Distribution of patients with myelodysplastic syndrome, myeloproliferative neoplasms, and bone marrow failure in Japan (Reference [3]). One thousand patients were prospectively underwent the central review of morphology and histology from February 2009 to October 2013. This data was provided by the courtesy of Dr. Asahito Hama

4.2.2   Classification

Historically, The French–American–British (FAB) cooperative group produced the first classification of MDS in 1982, based on experiences from adult patients, which divided MDS into five subgroups: refractory anemia (RA), RARS, RAEB, RAEB-T, and CMML [15]. Distinction between these subtypes was largely based on the proportion of blasts in the peripheral blood (PB) and bone marrow (BM) and the degree of monocytosis in the PB. The FAB classification became an important tool of communication about MDS and had a prognostic impact. In 2001, the WHO classification of hematological malignancies incorporates both morphology and cytogenetic changes [16]. The 2001 WHO classification lowered the threshold for distinguishing AML from MDS, from 30 to 20% blasts in the BM. Although JMML was recognized as a separate entity in the WHO classification, both the FAB and the WHO classifications were based on the review of adult cases and did not acknowledge the differences between MDS in children and adults. For example, RARS and the unique 5q-syndrome were extremely rare in children. The significance of multilineage dysplasia and a blast threshold of 20% for distinction between MDS and AML were unknown in children.

Considering these issues, a pediatric approach to the diagnosis and management of MDS and MPD was proposed in 2003 [1]. It divided MDS/MPD in children into three main groups; JMML, myeloid proliferations related to Down syndrome, and MDS (Table 4.1). MDS was further subdivided into refractory cytopenia (RC), RAEB, and RAEB-T. This classification of pediatric MPD/MDS was incorporated into the fourth edition of the WHO classification in 2008 [2]. RCC is defined as a provisional entity characterized by persistent cytopenia with <5% blasts in the BM, <2% blasts in the PB, and dysplastic changes in two or three lineages or exceeding 10% in one single lineage [2]. RAEB is applied to children with MDS in whom there are 2–19% blasts in the PB or 5–19% blasts in the BM, similarly to adult MDS [2]. However, the prognostic significance of subdivision of RAEB into RAEB-1 and RAEB-2 has not been shown in children so far [17]. Children with 20–29% blasts

Table 4.1 Diagnostic categories of myelodysplastic and myeloproliferative diseases in children (Reference [1, 2])

Myelodysplastic/Myeloproliferative disease		
Juvenile myelomonocytic leukemia (JMML)		
Myeloid proliferations related to Down syndrome (DS)		
Transient abnormal myelopoiesis (TAM)		
Myeloid leukemia of DS (ML-DS)		
Myelodysplastic syndrome (MDS)	(PB blasts)	(BM blasts)
Refractory cytopenia (RC)	<2%	<5%
Refractory anemia with excess blasts (RAEB)*	2–19%	5–19%
RAEB in transformation (RAEB-T)	20–29%	20–29%

in the PB and/or BM and myelodysplasia often have slowly progressive disease and behave more like MDS than AML; therefore, the subtype of RAEB-T is retained in the 2008 WHO classification of childhood MDS [2]. Children with recurrent cytogenetic abnormalities such as t(8;21)(q22;q22), inv(16)(p13.1q22) or t(16;16) (p13.1;q22), or t(15;17)(q22;q12) should be considered as AML regardless of blast percentage.

MDS that arises in a previously healthy child are referred to as "primary MDS" and that developing in a child with a known predisposing condition are referred to as "secondary MDS". Secondary MDS is seen in patients who previously received chemo- or radiation therapy (therapy-related MDS) [18], in patients with IBMFS [19], following acquired aplastic anemia [20], and as familial MDS [21].

MDS associated with Down syndrome has been reported to account for 20–25% of cases of childhood MDS in the past; however, myeloid leukemia in Down syndrome with blasts less than 20% is now thought as distinct entity from other subtypes of childhood MDS and classified as "myeloid leukemia of Down syndrome" regardless of blast percentage [2].

4.2.3 Clinical and Laboratory Characteristics

Children with MDS usually present with symptoms related to cytopenias such as bleeding tendency, infection, and pallor. Some children have no clinical symptoms and can be diagnosed incidentally during a routine work-up. Cytopenia is generally milder in MDS than aplastic anemia (AA). The length of persistent cytopenia should be at least 1 month. Leukocytosis and organomegaly are generally not features of MDS. Table 4.2 shows minimal diagnostic criteria for pediatric MDS [1, 2].

4.2.4 Morphology and Histology

BM can be hypo-, normo-, or hypercellular in pediatric MDS. In children with low-grade MDS, cellularity was reduced in about half of patients [9, 10]. Since hematopoietic cells in hypocellular low-grade MDS are often distributed in a

Table 4.2 Minimal diagnostic criteria for MDS (Reference [1, 2])

At least two of the following:
1. Sustained unexplained cytopenia (neutropenia, thrombocytopenia, or anemia)
2. At least bilineage morphologic myelodysplasia
3. Acquired clonal cytogenetic abnormality in hematopoietic cells
4. Increased blasts ($\geqq 5\%$)
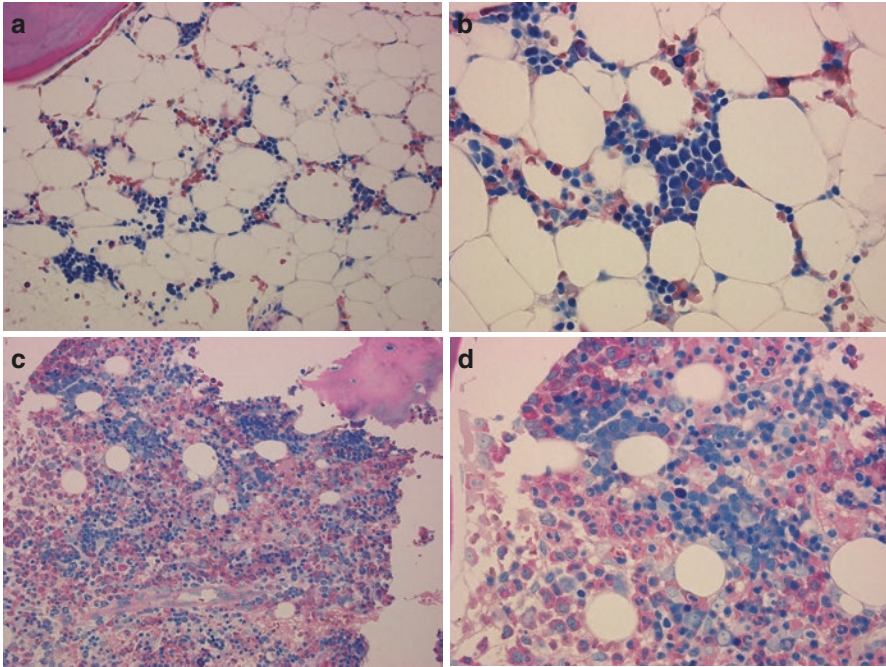
Fig. 4.2 Representative pictures of bone marrow (BM) biopsies obtained from patients with pediatric low-grade MDS (AS-D naphthol chloroacetate esterase stain with Giemsa staining), (**a**, **b**) show BM specimens of hypocellular refractory cytopenia of childhood, whereas (**c**, **d**) show those of hypercellular refractory cytopenia with multilineage dysplasia (**c**, **d**). (**a**) Patchy distribution of erythropoiesis accompanied by sparsely distributed granulopoiesis, in an otherwise adipocytic BM. (**b**) Immature erythroid precursors form one or several islands. (**c**) Immature erythropoiesis and granulopoiesis are distributed diffusely. (**d**) Left-shifted erythroid and myeloid cells are increased. These pictures were provided by the courtesy of Dr. Masafumi Ito

patchy pattern (Fig. 4.2a), information obtained from aspiration cytology is limited [22, 23]. Therefore, BM biopsies, which mirror the topography and cellularity of the local hematopoiesis, should be performed at least twice in order to diagnose hypoplastic BM disorders in children [2, 22, 23]. Patchy erythropoiesis with impaired maturation accompanied by sparsely distributed granulopoiesis, in an otherwise adipocytic BM is a characteristic of hypocellular low-grade MDS (Fig. 4.2b) [2, 22, 23]. Since megakaryocytes are markedly decreased or absent, immunohistochemistry is mandatory for the detection of micromegakaryocytes. A subset of children with low-grade MDS shows normo- or hypercellular BM, in which morphological and histological findings are reminiscent of advanced MDS, i.e., hematopoietic cells are diffusely distributed and dysplasia may be prominent (Fig. 4.2c, d).

Both PB and BM show characteristic dysplastic features such as megaloblastic changes of erythroid cells, psudo-Pelger-Huet anomaly of granulocytes, and micromegakaryocytes (Table 4.3) [24, 25]. Although dysplasia is a pathognomonic feature of MDS, dysplastic feature constitutes only one aspect of the morphologic diagnosis and can be observed in various non-clonal disorders in children (Table 4.4).

Table 4.3 Myelodysplastic features found in childhood MDS (Reference [24])

Erythroid series	
Nuclear lobulation	Presence of erythroblasts with lobulated nuclei (kidney-shaped, bilobulated, multilobulated, bizarre irregular nuclear profile)
Multinuclearity	Two or more distinctly separated nuclei of the same or of different sizes
Megaloblastoid changes	At least 1.5 times the size of a normal poly- or orthochromatic erythroblast with coarse condensation of chromatin and an increased nuclear-to-cytoplasmic ratio or orthochromatic erythroblasts with decreased nuclear-to-cytoplasmic ratio and at least double the size of a normal erythrocyte of the same maturational state
Cytoplasmic granules or inclusions	Presence of granules or nuclear fragments that can be definitely differentiated from ribosomal RNA
Myeloid series	
Pseudo-Pelger-Huet anomaly	Mature granulocytes with either a centrally located round to ovoid nucleus (monolobated type) or two round nuclei of similar size connected by a slender chromatin bridge (bilobated type)
Bizarre nuclear shape	Abnormal nuclear shape, including irregularly lobulated nuclei of segmented granulocytes with chromatin clumping or large twisted bands, large bands or metamyelocytes, multinuclearity (two distinctly separated neutrophilic bands or segmented nuclei)
A- or hypogranularity	Abnormal, neutrophil or azurophil granules have to be markedly or completely absent, and the cytoplasm of mature neutrophilic granulocytes has to stain pale blue/gray or translucent in the Romanowsky–Giemsa stain. All maturation stages except blast cells should be affected.
Nuclear/cytoplasmic (N/C) asynchrony	Mature neutrophilic granulocytes and metamyelocytes with basophilic cytoplasm and myelocytes with neutrophilic cytoplasm
Megakaryocytic series	
Micromegakaryocyte	Mononucleated megakaryocyte with a size comparable to that of a promyelocyte or less, lacking features of a blast cell
Small binucleated megakaryocyte	Small megakaryocyte with the size of a micromegakaryocyte or slightly larger, with two round well-separated nuclei.
Megakaryocyte with small round separated nuclei	Megakaryocytes of any size with multiple, at least three, round separated nuclei
Megakaryocytes with nonlobated round nucleus	Megakaryocytes of normal or reduced size with a nonlobated round nucleus and a mature granular cytoplasm

Table 4.4 Disorders with cytopenia, hypoplastic bone marrow, and dysplastic features

• *Non-hematological disorders*
– Rheumatic disease
– Congenital immunodeficiency (e.g., Wiskott-Aldrich syndrome)
– Metabolic disorders (e.g., mevalonate kinase deficiency)
– Pearson syndrome
– Drug-induced (e.g., valproic acid)
– Infection(e.g., *cytomegalovirus*, human parvovirus)
– Nutritional disorder(e.g., deficiency of vitamin B12 or folate)
• *Hematological disorders*
– Aplastic anemia
– Inherited bone marrow failure syndrome (e.g., Fanconi anemia, dyskeratosis congenita)
– Paroxysmal nocturnal hemoglobinuria
– Hemolytic anemia
– Hemophagocytic lymphohistiocytosis

4.2.5 Cytogenetics

The frequencies of abnormal karyotype vary according to the subtype. In low-grade MDS, two-thirds of patients have normal karyotype [10, 22]. Monosomy 7 and other chromosomal abnormalities including trisomy 8 were seen in 10% and 10–20% of patients with low-grade MDS, respectively [10, 22].

In contrast, only a third of patients with advanced MDS have normal karyotype, and two-thirds have chromosomal aberrations, in which monosomy 7 is the most common and accounts for 20–30% of patients [12–14, 26]. The outcome for patients with monosomy 7 is not worse than that of other children with MDS [17, 26]. Structural complex abnormalities defined as ≥3 chromosomal aberrations including at least one structural aberration are associated with a very poor outcome [12, 26]. Favorable cytogenetic aberrations defined by the prognostic scoring system in adult MDS such as -Y, del(11q), del(20q), and del(5q) [27] are rarely seen in children, and their prognostic significances remain to be established in pediatric MDS [17].

4.2.6 Pathobiology

Intrinsic defects in hematopoietic stem cell caused by acquired cytogenetic and genetic abnormalities are thought as hallmark features of adult MDS. Recently, molecular basis has been comprehensively addressed in a large cohort of MDS in adults [28]. About 90% of adult patients with MDS carry at least one oncogenic mutation, and two-thirds of them are found in individuals with a normal karyotype. Driver mutant genes in MDS include those of RNA splicing machinery (*SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*), DNA methylation (*TET2*, *DNMT3A*, and *IDH1/2*), chromatin modification (*ASXL1* and *EZH2*), transcription regulation (*RUNX1*),

DNA repair (*TP53*), signal transduction (*CBL*, *NRAS*, and *KRAS*), and cohesin complex (*STAG2*) [25]. Although mutations in *CBL*, *DNMT3A*, *CSF3R*, *CALR*, and spliceosome genes have been reported to be rare in pediatric MDS so far [29–32], the landscape of molecular pathogenesis in pediatric MDS is still largely unknown. Epigenetic alterations such as hypermethylation of *CDKN2B* gene occur in children with MDS at a similar frequency to adults [33].

Considering that 10–25% of children with MDS have a known constitutional disorder [6, 7], inherited abnormalities predisposing to MDS/AML might play an important role in pathogenesis in pediatric MDS. Familial MDS/AML syndromes are resulted from deficiencies of the hematopoietic transcription factors *CEBPA*, *RUNX1*, and *GATA2* [21]. Of these genes, germ line *GATA2* mutations are found to occur in 7% of children with primary MDS [34]. Mutation carriers were older at diagnosis and more likely to present with monosomy 7 and advanced disease.

Given that immunosuppressive therapy (IST) is effective in some children with low-grade MDS as well as MDS in adults or AA [35–37], a part of these disorders might share a common pathogenesis, that is, T-cell–mediated inhibition of hematopoiesis. Indeed, T-cell oligoclonality was identified in 40% of RCC patients [38]. Minor paroxysmal nocturnal hemoglobinuria clones, which are thought as a predictor of favorable response to IST, were also detected in 41% of RCC patients [39]. These findings suggest that pathogenesis attributes to activated-T cells in a subset of children with low-grade MDS.

4.2.7 Differential Diagnosis

In children, cytopenias with decrease of BM cellularity might be caused by a various underlying disorders (Table 4.4) [22]. Of those, AA and IBMFS are the most common and important differential diagnoses of low-grade MDS; however, the clinical and histopathological distinction between these three disorders is challenging. Since IBMFS, such as FA and dyskeratosis congenita (DC), show overlapping morphological features with low-grade MDS and AA and children without any phenotypic features can be diagnosed with IBMFS, IBMFS have to be excluded by careful past medical and family history and thorough physical examination. In fact, FA and DC were found in patients with hypo- or normocellular RCC with a prevalence of 14% and 2%, respectively [40]. Several laboratory studies, such as chromosome breakage test and telomere length assay, should be considered in differential diagnosis of hypoplastic BM disorders.

AML is the major differential diagnosis of advanced MDS [24]. Blast percentage and morphological finding in a single specimen are insufficient to differentiate MDS from AML, and clinical features and cytogenetic findings should be taken into account in differential diagnosis. For example, monosomy 7 is suggestive of MDS, whereas significant organomegaly and hyperleukocytosis are suggestive of AML [13]. However, a small subset of children may have borderline features with BM blasts of 20–30% and no cytogenetic abnormalities, and reevaluation of the BM examination after 2 weeks is recommended.

4.2.8   Management and Treatment

Since MDS is clonal hematopoietic stem cell disorder and residual "healthy" stem cells are limited, hematopoietic cell transplantation (HCT) is the mainstay of management for children with MDS. Other therapeutic strategies such as hematopoietic growth factors, immunomodulatory drugs, or low-dose chemotherapy are adopted as clinical practice in adult MDS who are not candidate for HCT, but these approaches are generally not indicated in children. Azacitidine, a hypomethylating agent, has demonstrated clinical efficacy that prolongs survival in adult with MDS [41]. A retrospective analysis suggested that azacitidine was also safe and effective in some children with MDS [42]. However, because most children who benefited from azacitidine showed stable disease, the role of azacitidine in childhood MDS may be providing time for donor search before HCT or a less toxic option in palliative situations.

HCT for children with advanced MDS is a still great challenge because of high risk of relapse and transplant-related mortality (TRM). Myeloablative conditioning regimen consisting of busulfan, cyclophosphamide, and melphalan followed by HCT resulted in a 63% overall survival [12]. The 5-year cumulative incidence of TRM and relapse is 21% each. Age at HCT greater than 12 years, interval between diagnosis and HCT longer than 4 months, and occurrence of acute or extensive chronic graft-versus-host disease (GVHD) were associated with increased TRM, whereas more advanced disease was associated with the risk of relapse [12]. Treatment outcome of children with MDS underwent cord blood transplantation seemed suboptimal [43]. Because of lack of firm evidence due to paucity of patients, appropriate conditioning regimen, GVHD prophylaxis, and stem cell source for children with advanced MDS remain to be established. In addition, the significance of pre-transplant intensive chemotherapy also has yet to be elucidated. A Japanese study adopted induction therapy with etoposide, cytarabine, and mitoxantrone prior to HCT in 16 children with advanced MDS and found that complete remission rate was 81%, and no toxic deaths occurred; however, whether this strategy improved overall outcome was unclear [14]. A European study indicated that intensive chemotherapy before HCT did not show any difference in relapse, treatment-related mortality, or survival [12]. It is interesting to note that patients with very advanced MDS (blasts ≥30%) may benefit from pre-transplant intensive chemotherapy [12].

Clinical courses of low-grade MDS are heterogeneous: approximately a third of patient remained stable for long periods without IST or HCT, whereas a subset of patients suffered from progressive disease [10]. Monosomy 7 significantly correlated with disease progression and patients whose disease progressed before HCT fared significantly worse [9]; therefore, those with unfavorable karyotype such as monosomy 7 and complex karyotype are encouraged to be transplanted as soon as possible. Those who suffer from transfusion dependency or severe neutropenia may also be candidates for HCT if suitable donor is available. Because TRM is the

major cause of treatment failure in children with low-grade MDS [44], reduced-intensity conditioning (RIC) regimen is an attractive option [45, 46]; however, it remains to be established whether RIC is an appropriate regimen for children with low-grade MDS with multilineage dysplasia, hypercellularity, or unfavorable karyotype.

If children with low-grade MDS without unfavorable karyotype require therapeutic intervention but suitable donor is unavailable, IST may be an advisable option. IST consisting of antithymocyte globulin (ATG) and cyclosporine has proven to be effective in 40 to 60% of children with low-grade MDS [10, 11, 36, 37, 47]. Some patients with chromosomal abnormalities or multilineage dysplasia were also reported to respond to IST [10, 11, 36, 37, 47]. However, patients who received IST remain at risk of clonal evolution and relapse, and the long-term failure-free survival rate was estimated as only 40–50% [10, 47]. As reliable biomarkers that can predict response have not yet identified, candidates for IST should be selected with utmost caution.

The outcome of children with therapy-related MDS is very poor. The survival of patients who receive only chemotherapy is dismal, and even HCT offers a cure only in 20–30% of patients [18, 48]. Compared with the outcomes of HCT in patients with therapy-related MDS, the results of secondary MDS/AML after AA appear to be favorable with a 41% overall survival rate [49]. Because TRM was the major cause of treatment failure, less toxic preparative regimens may be desirable in patients who develop secondary MDS/AML after AA. It is difficult to discuss the outcome of secondary MDS/AML arising from IBMFS because of its heterogeneity. Treatment with HCT in children with FA who have acute leukemia is challenging because of TRM and a high relapse rate. Data from the Center for International Blood and Marrow Transplant Research indicated that FA patients with MDS/AML had significantly worse 5-year survival than did patients with cytogenetic abnormalities alone (43% vs. 67%) [50]. In order to improve the outcome after HCT in children with FA and MDS/AML, low-dose chemotherapy prior to HCT or addition of high-dose cytarabine to fludarabine-based conditioning regimen has been attempted [51, 52].

4.3 Juvenile Myelomonocytic Leukemia

4.3.1 Epidemiology

JMML has an incidence of one in 1.2 million children per year and accounts for 3% of all hematologic malignancy in children [53]. The incidence of MDS and JMML in Japan was already described. The age at diagnosis is 2 years, ranging from 0.1 to 11, and males are predominating as a male to female ratio of 2:3 [4]. The reason why the disease affects more boys has not been elucidated.

4.3.2 Classification

The entity of JMM was established in 1997 [4]; however, diagnosis of JMML is not so simple, and a variety of clinical and laboratory findings may make diagnosis even more difficult. The clinical findings of JMML sometimes mimic human herpesvirus infections, leukocyte adhesion deficiency, infantile malignant osteopetrosis, hemophagocytic lymphohistiocytosis, and Wiskott-Aldrich syndrome [54]. Also, several important molecular findings have been identified, and they have been incorporated into the diagnostic criteria of JMML. Table 4.5 is a most recently issued diagnostic criteria proposed by Locatelli and Niemeyer [55], and it may be wise to realize that the criteria will be always changing in the future according to new findings to come.

4.3.3 Clinical and Laboratory Characteristics

Clinical presentations of JMML include fever, lymphadenopathy, skin rash, abdominal distention due to splenomegaly and hepatomegaly, purpura, and anemia. Overall, the children with JMML look very sick. Some patients have neurofibromatosis type 1(NF1) phenotype such as café au lait spots. White blood cells normally

Table 4.5 Updated clinical and laboratory diagnostic criteria of JMML (Reference [55])

I. Clinical and hematologic features (all 4 features mandatory)
•   Peripheral blood monocyte count $>1 \times 10^9$/L
•   Blast percentage in peripheral blood and bone marrow <20%
•   Splenomegaly
•   Absence of Philadelphia chromosome (*BCR/ABL* rearrangement)
II. Oncogenetic studies (1 finding is sufficient)
•   Somatic mutation in *PTPN11*[a] or *KRAS*[a] or *NRAS*[a,b]
•   Clinical diagnosis of NF1 or germ line *NF1* mutation
•   Germ line *CBL* mutation and loss of heterozygosity of *CBL*[c]
III. Only for those patients (10% of the whole number) without any oncogenetic parameter, besides the clinical and hematologic features listed under I, at least 2 of the following criteria have to be fulfilled:
•   Monosomy 7 or any other chromosomal abnormality
•   HbF increased for age
•   Myeloid precursors on peripheral blood smear
•   Spontaneous growth or GM-CSF hypersensitivity in colony assay
•   Hyperphosphorylation of STAT5

[a]Germ line mutations (indicating Noonan syndrome) need to be excluded
Diagnosis of JMML but spontaneous regression of myeloproliferation may be noted in:
[b]Few patients with *NRAS* mutation and normal HbF
[c]Patients with *CBL* germ line mutation and loss of heterozygosity

exceed 10×10^9/L, and monocyte count also exceeds 1×10^9/L. Monocytes are dysplastic and mimic band neutrophils on peripheral blood smear (Fig. 4.3). In addition, the peripheral smears have very important specific findings such as leukocytosis without hiatus leukemicus, myeloid dysplasia including pseudo-Pelger anomaly and hypersegmentation, thrombocytopenia with giant platelets, and dysplastic erythrocytes with erythroblasts. Bone marrow aspirates show hypercellularity without occasional blasts, which do not exceed 20%. Interestingly, increase in monocytes is not apparent in the bone marrow. The rearrangement of *BCR/ABL*, that is, Philadelphia chromosome, is not identified.

Karyotype of JMML is not specific. In fact, more than half of patients have normal karyotype, and the most common abnormality is monosomy 7, followed by a variety of abnormalities including trisomy 8 and translocations (Table 4.6) [56].
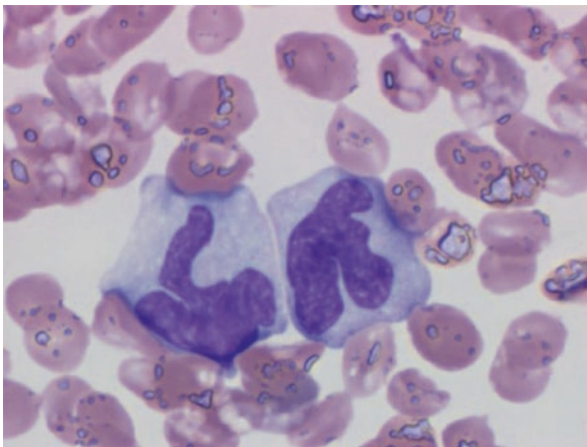
Fig. 4.3 Dysplastic monocytes in JMML on peripheral blood smear

Table 4.6 Karyotype of JMML ($n = 75$) (Reference [56])

Normal					54
Monosomy 7					11
Others	+8				1
	-Y				1
	+X,+11				1
	+9,+13,+19,+21				1
	+1,+8,add(8)(p11),+13,-15,-16				1
	add(6)(q25)				1
	add(10)(p13)				1
	t(3;18)(q25;q21)				1
	t(7;11)(p15;p15)				1
	t(9;12)(p22;q24)				1
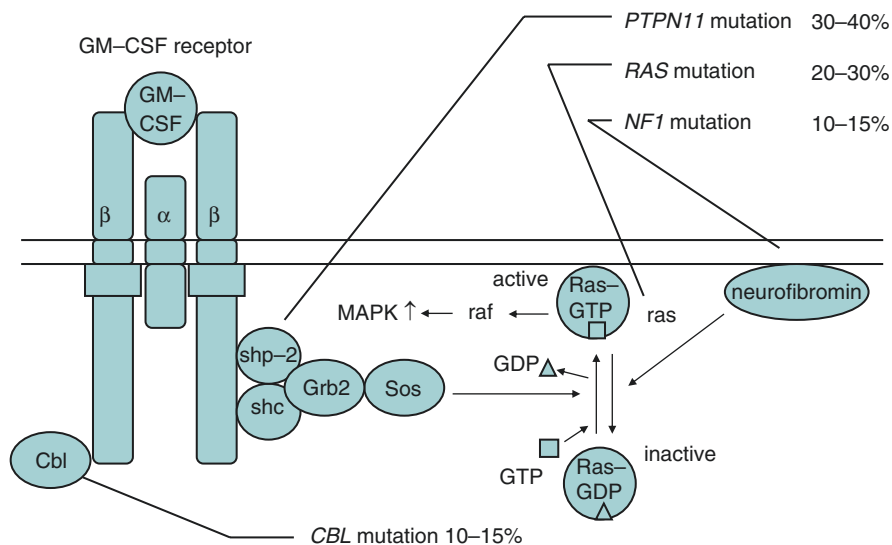
Fig. 4.4 Signal transduction in the downstream of GM-CSF receptor β chain

The hypersensitivity of myeloid progeny cells to GM-CSF was regarded as a hallmark of JMML [5], but the assay has not been easily standardized, and now molecular techniques are used more frequently. Some reports showed that viral infections such as HHV6, CMV, and EBV might cause GM-CSF hypersensitivity [57–59]. The hypersensitivity to GM-CSF is caused by deregulation of downstream signals of GM-CSF receptor β chain, and the aberrations of RAS pathways have been identified as mostly responsive for these changings. *RAS* was identified as a first player [60] and *PTPN11* followed [61]. In fact, NF1 is accompanied by neurofibromin, which directly intervene with RAS pathway (Fig. 4.4). Finally, *CBL* mutations were identified [62], and now molecular abnormalities can be identified either somatic or germ line in 90% of children with JMML. The detection of these molecular abnormalities is essential in diagnosis of JMML (Table 4.5). Hyperphosphorylation of STAT5 occurs in response to GM-CSF. It has been shown that this reaction may be unique in JMML and could be utilized as a diagnostic tool when molecular abnormalities are not identified [63, 64]. Thus, identification of molecular abnormalities has replaced colony assays, and hyperphosphorylation of STAT5 may also become alternative to gene mutations in some situations in the near future (Table 4.5).

4.3.4   Pathobiology

As described above, deregulated RAS signaling is thought to be a hallmark of JMML. Deregulated RAS signals cause proliferation of JMML cells, and *NF1*-knockout mouse exhibits JMML-like disease [65]. Abnormal RAS pathways are not only documented in JMML but also identified in congenital diseases including

Noonan syndrome, Costello syndrome, and cardio-facio-cutaneous syndrome (CFC syndrome). These are caused by germ line mutations of RAS-associated genes in complex ways, and they are now collectively categorized as RASopathies. For example, Noonan syndrome is caused not only by *PTPN11* mutation [66] but also by *KRAS* mutation [67], whereas Costello syndrome is caused by *HRAS* mutation, and CFC syndrome is caused by *KRAS* and *BRAF* [68, 69]. In these RASopathies, only Noonan syndrome presents as JMML early in infancy, but JMML features spontaneously disappear without treatment. Thus, whether gene mutations are identified only in germ line or in somatic, it is crucially important not only to diagnose the disease but also to guide the patient's management. In extreme case, somatic mosaicism of *NRAS* mutations was reported in patients with JMML [70].

As mentioned above, patients with Noonan syndrome sometimes present pictures of JMML with spontaneous remission; however, the story of *CBL* mutations is somewhat different. Patients with germ line *CBL* mutations present congenital anomalies which mimic NF1 and Noonan syndrome. If patients acquire loss of heterozygosity (LOH) on another allele, they start showing JMML pictures, which will spontaneously regress. Some of these children may have vasculitis later in life [62]. Thus, it is difficult to optimize treatment for each type of JMML, and germ line testing is eminently important even if somatic mutation is already identified.

Recently, second hit mutations were identified. Sakaguchi et al. conducted whole exome sequencing and identified *SETBP1* and *JAK3* as secondary mutations [71]. Patients who had secondary mutations had a worse outcome, and they conclude that those with secondary hits had more aggressive disease. More recently, Caye et al. showed that even mutated RAS pathway genes were overlapped in 17% of cases, and also those with overlap displayed worse outcome [72]. This challenged the dogmatic concept that RAS pathway mutations were exclusive. Also, Steglitz et al. did extensive characterization of gene mutations and showed that patients with more alterations had a worse outcome doing extensive characterization [73].

Epigenetic studies were previously done for tumor suppressor genes such as *CDKN2B* and *CDKN2A*, and hypermethylation of these genes was also examined in JMML. The result was that promoters of these genes were hypermethylated in a small subset of patients [33]. Recently, Olk-Batz et al. conducted more comprehensive analysis of hypermethylation using mass spectrometry and found that *BMP4*, *CALCA*, *CDKN2B*, and *RARB* were frequently hypermethylated, and those who with higher degree of hypermethylation had a worse outcome [74].

4.3.5 Differential Diagnosis

As described above, clinical findings of JMML sometimes mimic human herpesvirus infections, leukocyte-adhesion deficiency, infantile malignant osteopetrosis, hemophagocytic lymphohistiocytosis, and Wiskott-Aldrich syndrome. New criteria of the disease may help diagnosis of JMML (Table 4.5), and definitive diagnosis of JMML should be based on molecular techniques, preferably conducted by National Center for Pediatric Hematology-Oncology.

RAS-associated ALPS-like disease (RALD) is a recently identified entity, and it is caused by somatic *RAS* mutations [75]. Patients with RALD have a clinical picture, which mimics ALPS, and they sometimes show severe autoimmune hemolytic anemia. RALD is difficult to distinguish from JMML even with new JMML criteria.

4.3.6   Management and Treatment

In the past, patients with JMML had a dismal course, and allogeneic HCT was thought to be an only curative treatment for a long time [76, 77]. However, as molecular characterization of the disease developed, the treatment selection became more and more complicated. First of all, some patients with somatic *RAS* mutations survive long without HCT [78]. Also, patients with RALD show a similar clinical feature of JMML. Next, patients with germ line *CBL* mutations normally do not progress to aggressive disease and can be managed without HCT.

However, patients with somatic *PTPN11* mutation and NF1 are recommended for HCT as soon as possible. In fact, those with somatic *PTPN11* are prone to relapse even after HSCT. Maybe patients with *KRAS* and most patients with *NRAS* are also indicated for HCT. Previously identified prognostic factors such as older age, low platelet count and high HbF could still be utilized to guide treatment [4]. Yoshida et al. reported that the somatic *PTPN11* was associated with older age, increased HbF, and worse outcome after HCT [79]. Regarding natural course of the disease, Niemeyer et al. reported that most patients died because of massive infiltration of cells into vital organs such as lungs. Honda et al. showed that blastic crisis occurred in some patients, and the disease progressed to acute leukemia as an end-stage disease [80]. Complex karyotype including monosomy 7 was associated with emergence of blastic crisis in the study.

Regarding HCT, the report of EWOG-MDS repeatedly showed that the use of conditioning regimen of busulfan, cyclophosphamide, and melphalan, and rapid tapering of immunosuppressants were keys for successful HCT [81]. As for donor selections, cord blood was also used successfully in addition of the bone marrow [82]. In Japan, alternative regimen consisting of busulfan, fludarabine, and melphalan was used, and the result was encouraging [83]. Some approaches have been tried to improve the outcome of patients with aggressive disease. However, the significance of chemotherapy before HCT, splenectomy, or irradiation to spleen before HCT has not been supported by far.

The problem of HCT is that some patients still relapse after HCT. Some prognostic factors such as clinical feature and molecular findings have been identified, and innovative approach should be utilized in the future. One of them is a hypomethylating agent. 5-Azacitidine was tested as off-label use in Europe, and some patients showed a positive result [84]. The drug is now investigated as phase 2 study as a window therapy before HCT. Based on the molecular pathogenesis of the disease, the drugs, which interfere with RAS/MAPK pathways should be established in the future.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003;17:277–82.
2. Baumann I, Niemeyer CM, Bennett JM. Childhood myelodysplastic syndrome. In: Swerdlow SH, et al., editors. World Health Organization classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: IARC Press; 2008. p. 104–7.
3. Hasegawa D. The current perspective of low-grade myelodysplastic syndrome in children. Int J Hematol. 2016;103(4):360–4.
4. Niemeyer CM, Arico M, Basso G, et al. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. Blood. 1997;89(10):3534–43.
5. Emanuel PD, Bates LJ, Castleberry RP, Gualtieri RJ, Zuckerman KS. Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. Blood. 1991;77(5):925–9.
6. Hasle H, Wadsworth LD, Massing BG, McBride M, Schultz KR. A population-based study of childhood myelodysplastic syndrome in British Columbia, Canada. Br J Haematol. 1999;106:1027–32.
7. Sasaki H, Manabe A, Kojima S, Tsuchida M, Hayashi Y, Ikuta K, Okamura I, Koike K, Ohara A, Ishii E, Komada Y, Hibi S, Nakahata T. Myelodysplastic syndrome in childhood: a retrospective study of 189 patients in Japan. Leukemia. 2001;15:1713–20.
8. Horibe K, Saito AM, Takimoto T, Tsuchida M, Manabe A, Shima M, Ohara A, Mizutani S. Incidence and survival rates of hematological malignancies in Japanese children and adolescents (2006-2010): based on registry data from the Japanese Society of Pediatric Hematology. Int J Hematol. 2013;98(1):74–88.
9. Kardos G, Baumann I, Passmore SJ, Locatelli F, Hasle H, Schultz KR, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. Blood. 2003;102:1997–2003.
10. Hasegawa D, Chen X, Hirabayashi S, Ishida Y, Watanabe S, Zaike Y, et al. Clinical characteristics and treatment outcome in 65 cases with refractory cytopenia of childhood defined according to the WHO 2008 classification. Br J Haematol. 2014;166:758–66.
11. Hama A, Takahashi Y, Muramatsu H, Ito M, Narita A, Kosaka Y, et al. Comparison of long-term outcomes between children with aplastic anemia and refractory cytopenia of childhood who received immunosuppressive therapy with antithymocyte globulin and cyclosporine. Haematologica. 2015;100:1426–33.
12. Strahm B, Nöllke P, Zecca M, Korthof ET, Bierings M, Furlan I, Sedlacek P, Chybicka A, Schmugge M, Bordon V, Peters C, O'Marcaigh A, de Heredia CD, Bergstraesser E, Moerloose BD, van den Heuvel-Eibrink MM, Starý J, Trebo M, Wojcik D, Niemeyer CM. Locatelli F, EWOG-MDS study group. Hematopoietic stem cell transplantation for advanced myelodysplastic syndrome in children: results of the EWOG-MDS 98 study. Leukemia. 2011;25(3):455–62.
13. Hasle H, Niemeyer CM. Advances in the prognostication and management of advanced MDS in children. Br J Haematol. 2011;154(2):185–95.
14. Kikuchi A, Hasegawa D, Ohtsuka Y, Hamamoto K, Kojima S, Okamura J, Nakahata T, Manabe A, Japanese Paediatric Myelodysplastic Syndrome (MDS) Study Group. Outcome of children with refractory anaemia with excess of blast (RAEB) and RAEB in transformation (RAEB-T) in the Japanese MDS99 study. Br J Haematol. 2012;158(5):657–61.

15. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposals for the classification of the myelodysplastic syndromes. Br J Haematol. 1982;51:189–99.

16. Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. World Health Organization classification of tumours. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001.

17. Hasle H, Baumann I, Bergsträsser E, Fenu S, Fischer A, Kardos G, Kerndrup G, Locatelli F, Rogge T, Schultz KR, Starý J, Trebo M, van den Heuvel-Eibrink MM, Harbott J, Nöllke P, Niemeyer CM, European Working Group on Childhood MDS. The International Prognostic Scoring System (IPSS) for childhood myelodysplastic syndrome (MDS) and juvenile myelo-monocytic leukemia (JMML). Leukemia. 2004;18(12):2008–14.

18. Tsurusawa M, Manabe A, Hayashi Y, Akiyama Y, Kigasawa H, Inada H, Noguchi Y, Sawai N, Kobayashi R, Nagatoshi Y, Kawakami K, Kojima S, Nakahata T, MDS Committee of the Japanese Society of Pediatric Hematology. Therapy-related myelodysplastic syndrome in childhood: a retrospective study of 36 patients in Japan. Leuk Res. 2005;29(6): 625–32.

19. Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. Blood Rev. 2010;24(3):101–22.

20. Ohara A, Kojima S, Hamajima N, Tsuchida M, Imashuku S, Ohta S, Sasaki H, Okamura J, Sugita K, Kigasawa H, Kiriyama Y, Akatsuka J, Tsukimoto I. Myelodysplastic syndrome and acute myelogenous leukemia as a late clonal complication in children with acquired aplastic anemia. Blood. 1997;90:1009–13.

21. Babushok DV, Bessler M. Genetic predisposition syndromes: when should they be considered in the work-up of MDS? Best Pract Res Clin Haematol. 2015;28(1):55–68.

22. Niemeyer CM, Baumann I. Classification of childhood aplastic anemia and myelodysplastic syndrome. Hematology Am Soc Hematol Educ Program. 2011;2011:84–9.

23. Baumann I, Führer M, Behrendt S, Campr V, Csomor J, Furlan I, et al. Morphological differentiation of severe aplastic anaemia from hypocellular refractory cytopenia of childhood: reproducibility of histopathological diagnostic criteria. Histopathology. 2012;61:10–7.

24. Cantù Rajnoldi A, Fenu S, Kerndrup G, van Wering ER, Niemeyer CM, Baumann I, European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). Ann Hematol. 2005;84(7):429–33.

25. Cazzola M, Della Porta MG, Malcovati L. The genetic basis of myelodysplasia and its clinical relevance. Blood. 2013;122(25):4021–34.

26. Göhring G, Michalova K, Beverloo HB, Betts D, Harbott J, Haas OA, Kerndrup G, Sainati L, Bergstraesser E, Hasle H, Stary J, Trebo M, van den Heuvel-Eibrink MM, Zecca M, van Wering ER, Fischer A, Noellke P, Strahm B, Locatelli F, Niemeyer CM, Schlegelberger B. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. Blood. 2010;116(19):3766–9.

27. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. Blood. 2012;120(12):2454–65.

28. Haferlach T, Nagata Y, Grossmann V, Okuno Y, Bacher U, Nagae G, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. Leukemia. 2014;28:241–7.

29. Shiba N, Kato M, Park MJ, Sanada M, Ito E, Fukushima K, Sako M, Arakawa H, Ogawa S, Hayashi Y. CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome. Leukemia. 2010;24(5):1090–2.

30. Shiba N, Taki T, Park MJ, Shimada A, Sotomatsu M, Adachi S, Tawa A, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Arakawa H, Hayashi Y. DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia. Br J Haematol. 2012;156(3):413–4.

31. Sano H, Ohki K, Park MJ, Shiba N, Hara Y, Sotomatsu M, Tomizawa D, Taga T, Kiyokawa N, Tawa A, Horibe K, Adachi S, Hayashi Y. CSF3R and CALR mutations in paediatric myeloid disorders and the association of CSF3R mutations with translocations, including t(8; 21). Br J Haematol. 2015;170(3):391–7.

32. Hirabayashi S, Flotho C, Moetter J, Heuser M, Hasle H, Gruhn B, et al. Spliceosomal gene aberrations are rare, coexist with oncogenic mutations, and are unlikely to exert a driver effect in childhood MDS and JMML. Blood. 2012;119:e96–9.

33. Hasegawa D, Manabe A, Kubota T, Kawasaki H, Hirose I, Ohtsuka Y, Tsuruta T, Ebihara Y, Goto Y, Zhao XY, Sakashita K, Koike K, Isomura M, Kojima S, Hoshika A, Tsuji K, Nakahata T. Methylation status of the p15 and p16 genes in paediatric myelodysplastic syndrome and juvenile myelomonocytic leukaemia. Br J Haematol. 2005;128(6):805–12.

34. Wlodarski MW, Hirabayashi S, Pastor V, Starý J, Hasle H, Masetti R, Dworzak M, Schmugge M, van den Heuvel-Eibrink M, Ussowicz M, De Moerloose B, Catala A, Smith OP, Sedlacek P, Lankester AC, Zecca M, Bordon V, Matthes-Martin S, Abrahamsson J, Kühl JS, Sykora KW, Albert MH, Przychodzien B, Maciejewski JP, Schwarz S, Göhring G, Schlegelberger B, Cseh A, Noellke P, Yoshimi A, Locatelli F, Baumann I, Strahm B, Niemeyer CM; EWOG-MDS. Prevalence, clinical characteristics, and prognosis of GATA2-related myelodysplastic syndromes in children and adolescents. Blood. 2016;127(11):1387–97.

35. Sloand EM, Rezvani K. The role of the immune system in myelodysplasia: implications for therapy. Semin Hematol. 2008;45:39–48.

36. Yoshimi A, Baumann I, Fuhrer M, Bergsträsser E, Göbel U, Sykora KW, et al. Imunosuppressive therapy with anti-thymocyte globulin and cyclosporine A in selected children with hypoplastic refractory cytopenia. Haematologica. 2007;92:397–400.

37. Hasegawa D, Manabe A, Yagasaki H, Ohtsuka Y, Inoue M, Kikuchi A, et al. Treatment of children with refractory anemia: the Japanese Childhood MDS Study Group Trial (MDS99). Pediatr Blood Cancer. 2009;53:1011–5.

38. Aalbers AM, van den Heuvel-Eibrink MM, Baumann I, Beverloo HB, Driessen GJ, Dworzak M, et al. T-cell receptor Vβ skewing frequently occurs in refractory cytopenia of childhood and is associated with an expansion of effector cytotoxic T cells: a prospective study by EWOG-MDS. Blood Cancer J. 2014;4:e209.

39. Aalbers AM, van der Velden VH, Yoshimi A, Fischer A, Noellke P, Zwaan CM, et al. The clinical relevance of minor paroxysmal nocturnal hemoglobinuria clones in refractory cytopenia of childhood: a prospective study by EWOG-MDS. Leukemia. 2014;28:189–92.

40. Yoshimi A, Niemeyer C, Baumann I, Schwarz-Furlan S, Schindler D, Ebell W, et al. High incidence of Fanconi anaemia in patients with a morphological picture consistent with refractory cytopenia of childhood. Br J Haematol. 2013;160:109–11.

41. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, Schoch R, Gattermann N, Sanz G, List A, Gore SD, Seymour JF, Bennett JM, Byrd J, Backstrom J, Zimmerman L, McKenzie D, Beach C, Silverman LR. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol. 2009;10:223–32.

42. Cseh AM, Niemeyer CM, Yoshimi A, Catala A, Frühwald MC, Hasle H, van den Heuvel-Eibrink MM, Lauten M, De Moerloose B, Smith OP, Bernig T, Gruhn B, Kulozik AE, Metzler M, Olcay L, Suttorp M, Furlan I, Strahm B, Flotho C. Therapy with low-dose azacitidine for MDS in children and young adults: a retrospective analysis of the EWOG-MDS study group. Br J Haematol. 2016;172(6):930–6.

43. Madureira AB, Eapen M, Locatelli F, Teira P, Zhang MJ, Davies SM, Picardi A, Woolfrey A, Chan KW, Socié G, Vora A, Bertrand Y, Sales-Bonfim CM, Gluckman E, Niemeyer C, Rocha V, Eurocord-European Blood and Marrow Transplant Group, Center of International Blood and Marrow Transplant Registry, European Working Group on childhood MDS. Analysis of risk factors influencing outcome in children with myelodysplastic syndrome after unrelated cord blood transplantation. Leukemia. 2011;25(3):449–54.

44. Starý J, Locatelli F, Niemeyer CM. Stem cell transplantation for aplastic anemia and myelo-dysplastic syndrome. Bone Marrow Transplant. 2005;35(Suppl 1):S13–6.
45. Strahm B, Locatelli F, Bader P, Ehlert K, Kremens B, Zintl F, et al. Reduced intensity conditioning in unrelated donor transplantation for refractory cytopenia in childhood. Bone Marrow Transplant. 2007;40:329–33.
46. Inagaki J, Fukano R, Kurauchi K, Noguchi M, Tanioka S, Okamura J. Hematopoietic stem cell transplantation in children with refractory cytopenia of childhood: single-center experience using high-dose cytarabine containing myeloablative and aplastic anemia oriented reduced-intensity conditioning regimens. Biol Blood Marrow Transplant. 2015;21:565–9.
47. Yoshimi A, van den Heuvel-Eibrink MM, Baumann I, Schwarz S, Simonitsch-Klupp I, de Paepe P, et al. Comparison of horse and rabbit antithymocyte globulin in immunosuppressive therapy for refractory cytopenia of childhood. Haematologica. 2014;99:656–63.
48. Aguilera DG, Vaklavas C, Tsimberidou AM, Wen S, Medeiros LJ, Corey SJ. Pediatric therapy-related myelodysplastic syndrome/acute myeloid leukemia: the MD Anderson Cancer Center experience. J Pediatr Hematol Oncol. 2009;31(11):803–11.
49. Yoshimi A, Strahm B, Baumann I, Furlan I, Schwarz S, Teigler-Schlegel A, Walther JU, Schlegelberger B, Göhring G, Nöllke P, Führer M, Niemeyer CM. Hematopoietic stem cell transplantation in children and young adults with secondary myelodysplastic syndrome and acute myelogenous leukemia after aplastic anemia. Biol Blood Marrow Transplant. 2014;20(3):425–9.
50. Ayas M, Saber W, Davies SM, Harris RE, Hale GA, Socie G, LeRademacher J, Thakar M, Deeg HJ, Al-Seraihy A, Battiwalla M, Camitta BM, Olsson R, Bajwa RS, Bonfim CM, Pasquini R, Macmillan ML, George B, Copelan EA, Wirk B, Al Jefri A, Fasth AL, Guinan EC, Horn BN, Lewis VA, Slavin S, Stepensky P, Bierings M, Gale RP. Allogeneic hematopoietic cell transplantation for Fanconi anemia in patients with pretransplantation cytogenetic abnormalities, myelodysplastic syndrome, or acute leukemia. J Clin Oncol. 2013;31(13):1669–76.
51. Beier R, Maecker-Kolhoff B, Sykora KW, Chao M, Kratz C, Sauer MG. Minimal antileukaemic treatment followed by reduced-intensity conditioning in three consecutive children with Fanconi anaemia and AML. Bone Marrow Transplant. 2015;50(3):463–4.
52. Aoki T, Koh K, Ikeda Y, Sekinaka Y, Akiyama K, Mori M, Arakawa Y, Hanada R. Addition of high-dose cytarabine to fludarabine-based conditioning for hematopoietic stem cell transplantation for treating Fanconi anemia patients with advanced myeloid malignancy: a single-center experience and literature review. Biol Blood Marrow Transplant. 2016;22:1725–8.
53. Hasle H, Kerndrup G, Jacobsen BB. Childhood myelodysplastic syndrome in Denmark: incidence and predisposing conditions. Leukemia. 1995;9(9):1569–72.
54. Yoshimi A, Kamachi Y, Imai K, et al. Wiskott-Aldrich syndrome presenting with a clinical picture mimicking juvenile myelomonocytic leukaemia. Pediatr Blood Cancer. 2013;60(5):836–41.
55. Locatelli F, Niemeyer CM. How I treat juvenile myelomonocytic leukemia. Blood. 2015;125(7):1083–90.
56. Yoshida N, Hirabayashi S, Watanabe S, et al. A prospective registration of children with juvenile myelomonocytic leukemia: follow-up of 75 patients (in Japanese). Jpn J Clin Hematol. 2011;52:1853–8.
57. Lorenzana A, Lyons H, Sawaf H, Higgins M, Carrigan D, Emanuel PD. Human herpesvirus 6 infection mimicking juvenile myelomonocytic leukemia in an infant. J Pediatr Hematol Oncol. 2002;24(2):136–41.
58. Moritake H, Ikeda T, Manabe A, Kamimura S, Nunoi H. Cytomegalovirus infection mimicking juvenile myelomonocytic leukemia showing hypersensitivity to granulocyte-macrophage colony stimulating factor. Pediatr Blood Cancer. 2009;53(7):1324–6.
59. Manabe A, Yoshimasu T, Ebihara Y, Yagasaki H, Wada M, Ishikawa K, Hara J, Koike K, Moritake H, Park Y-D, Tsuji K, Nakahata T. Viral infections in juvenile myelomonocytic leukemia: Prevalence and clinical implications. J Pediatr Hematol Oncol. 2004;26(10):636–41.

60. Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. Blood. 1994;83(8):2248–54.
61. Tartaglia M, Niemeyer CM, Fragale A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003;34(2):148–50.
62. Niemeyer CM, Kang MW, Shin DH, et al. Germ line CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. Nat Genet. 2010;42(9): 794–800.
63. Kotecha N, Flores NJ, Irish JM, et al. Single-cell profiling identifies aberrant STAT5 activation in myeloid malignancies with specific clinical and biologic correlates. Cancer Cell. 2008;14(4):335–43.
64. Hasegawa D, Bugarin C, Giordan M, et al. Validation of flow cytometric phospho-STAT5 as a diagnostic tool for juvenile myelomonocytic leukemia. Blood Cancer J. 2013;3:e160.
65. Bollag G, Clapp DW, Shih S, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet. 1996;12:144–8.
66. Tartaglia M, Mehler EL, Goldberg R, et al. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet. 2001;29:465–8.
67. Schubbert S, Zenker M, Rowe SL, et al. Germline KRAS mutations cause Noonan syndrome. Nat Genet. 2006;38:331–6.
68. Aoki Y, Niihori T, Kawame H, et al. Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nat Genet. 2005;37:1038–40.
69. Niihori T, Aoki Y, Narumi Y, et al. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. Nat Genet. 2006;38:294–6.
70. Doisaki S, Muramatsu H, Shimada A, et al. Somatic mosaicism for oncogenic NRAS mutations in juvenile myelomonocytic leukemia. Blood. 2012;120:1485–8.
71. Sakaguchi H, Okuno Y, Muramatsu H, et al. Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. Nat Genet. 2013;45(8):937–41.
72. Caye A, Strullu M, Guidez F, et al. Juvenile myelomonocytic leukemia displays mutations in components of the RAS pathway and the PRC2 network. Nat Genet. 2015;47:1334–40.
73. Stieglitz E, Taylor-Weiner AN, Chang TY, et al. The genomic landscape of juvenile myelomonocytic leukemia. Nat Genet. 2015;47:1326–33.
74. Olk-Batz C, Poetsch AR, Nöllke P, et al. European Working Group of Myelodysplastic Syndromes in Childhood (EWOG-MDS). Aberrant DNA methylation characterizes juvenile myelomonocytic leukemia with poor outcome. Blood. 2011;117(18):4871–80.
75. Takagi M, Shinoda K, Piao J, et al. Autoimmune lymphoproliferative syndrome-like disease with somatic KRAS mutation. Blood. 2011;117:2887–90.
76. Locatelli F, Niemeyer C, Angelucci E, et al. Allogeneic bone marrow transplantation for chronic myelomonocytic leukemia in childhood: a report from the European Working Group on Myelodysplastic Syndrome in Childhood. J Clin Oncol. 1997;15(2):566–73.
77. Manabe A, Okamura J, Yumura-Yagi K, et al. MDS Committee of the Japanese Society of Pediatric Hematology. Allogeneic hematopoietic stem cell transplantation for 27 children with juvenile myelomonocytic leukemia diagnosed based on the criteria of the International JMML Working Group. Leukemia. 2002;16(4):645–9.
78. Matsuda K, Shimada A, Yoshida N, et al. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. Blood. 2007;109(12):5477–80.
79. Yoshida N, Yagasaki H, Xu Y, et al. Correlation of clinical features with the mutational status of GM-CSF signaling pathway-related genes in juvenile myelomonocytic leukemia. Pediatr Res. 2009;65(3):334–40.
80. Honda Y, Tsuchida M, Zaike Y, Masunaga A, Yoshimi A, Kojima S, Ito M, Kikuchi A, Nakahara T, Manabe A. Clinical characteristics of 15 children with juvenile myelomonocytic leukemia who developed blast crisis: MDS Committee of Japanese Society of Pediatric Hematology/Oncology. Br J Haematol. 2014;165:682–7.

81. Locatelli F, Nöllke P, Zecca M, et al. European Working Group on Childhood MDS, European Blood and Marrow Transplantation Group. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. Blood. 2005;105(1):410–9.
82. Locatelli F, Crotta A, Ruggeri A, et al. Analysis of risk factors influencing outcomes after cord blood transplantation in children with juvenile myelomonocytic leukemia: a EUROCORD, EBMT, EWOG-MDS CIBMTR study. Blood. 2013;122(12):2135–41.
83. Yabe M, Ohtsuka Y, Watanabe K, et al. Transplantation for juvenile myelomonocytic leukemia: A retrospective study of 30 children treated with a regimen of busulfan, fludarabine, and melphalan. Int J Hematol. 2015;101:184–90.
84. Furlan I, Batz C, Flotho C, et al. Intriguing response to azacitidine in a patient with juvenile myelomonocytic leukemia and monosomy 7. Blood. 2009;113(12):2867–8.

Chapter 5
Neutropenia (In Infancy and Childhood)

Masao Kobayashi, Yoko Mizoguchi, Shuhei Karakawa, Satoshi Okada, and Hiroshi Kawaguchi

Abstract Neutropenia is defined as a decrease in the number of circulating neutrophils in the peripheral blood with absolute neutrophil count less than 1000–1500/μL. Chronic neutropenia in pediatric patients is divided into three groups. Extrinsic factors, such as antibodies, some drugs, and nutritional deficiencies, lead to excessive destruction of neutrophils. Autoimmune neutropenia is a benign form of neutropenia shown in infancy to early childhood. Spontaneous recovery of neutropenia usually occurs within a few months to a few years. Acquired disorders of myeloid and stem cells present hypoplasia of myeloid cells. Congenital neutropenia is intrinsic defects in granulocytes or their progenitors and includes a heterogenous group of disorders. More than ten responsible gene mutations have been identified in congenital neutropenia. Most common congenital neutropenia is due to the gene mutation of neutrophil elastase. The hallmark of profound neutropenia is increased susceptibility to bacterial infections, cutaneous cellulitis, deep tissue abscesses, pneumonia, and septicemia. Almost patients with congenital neutropenia have been responded to administration of G-CSF. However, long-term use of G-CSF has the risk of the development of MDS/AML, suggesting the necessity of the careful follow-up. Hematopoietic stem cell transplantation should be considered for the curable treatment in severe congenital neutropenia.

5.1   Definition and Classification

Neutropenia is defined as a decrease in the number of circulating neutrophils in the peripheral blood. Normal neutrophil levels should be varied for age, race, and other factors. Neutrophils predominate at birth, but gradually decrease in the first few

M. Kobayashi, M.D., Ph.D. (✉) • Y. Mizoguchi, M.D., Ph.D • S. Karakawa, M.D., Ph.D.
S. Okada, M.D., Ph.D • H. Kawaguchi, M.D., Ph.D.
Department of Pediatrics, Hiroshima University Hospital,
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
e-mail: masak@hiroshima-u.ac.jp

days of life. During infancy, neutrophils constitute of 20–30% of circulating white blood cells (WBC). Near same level of neutrophils and lymphocyte is found in the peripheral blood at the age of 5–6 years. Approximately 60–70% of neutrophils are observed in the circulating WBC during adolescent. The lower limit for normal absolute neutrophil counts (ANC) is 1000/μL in infants between 2 weeks and 1 year of age and that is 1500/μL for children older than 1 year of age. Neutropenia can be transient or chronic that persists beyond 6 months.

Neutropenia in pediatric patients is classified into three causes, neutropenia caused by extrinsic to marrow myeloid cells, acquired disorders of myeloid and stem cells, and intrinsic disorders of proliferation and differentiation of myeloid and stem cells. The former causes include infections; drug-induced, immune neutron, and reticuloendothelial sequestration; bone marrow replacement; cancer chemotherapy; or radiation therapy to bone marrow.

The second causes consist of aplastic anemia, vitamin B12 or folate deficiency, acute and chronic leukemia, myelodysplasia, paroxysmal nocturnal hemoglobinuria, and chronic idiopathic neutropenia. The third causes are intrinsic disorders presented in Table 5.1 [1, 2].

Table 5.1 The classification of congenital neutropenias

Disease	Mutated gene	Inheritance	Associated features
1. Severe congenital neutropenias			
SCN1 (elastase deficiency)	*ELANE*	AD	Susceptibility to myelodysplastic syndrome/acute myelogenous leukemia
SCN2 (GFI1 deficiency)	*GFI1*		B/T lymphopenia
SCN3 (Kostmann disease)	*HAX1*	AR	Cognitive and neurological defects, susceptibility to myelodysplastic syndrome/acute myelogenous leukemia
SCN4 (G6PC3 deficiency)	*G6PC3*	AR	Structural heart defects, urogenital abnormalities, inner ear deafness, venous angiectasis of trunks and limbs
SCN5 (VPS45 deficiency)	*VPS45*	AR	Extramedullary hematopoiesis, bone marrow fibrosis, nephromegaly
2. Glycogen storage disease type 1b	*G6PT1*	AR	Fasting hypoglycemia, lactic acidosis, hyperlipidemia, hepatomegaly
3. Cyclic neutropenia	*ELANE*	AD	Oscillations of other leukocytes and platelets
4. X-linked neutropenia/myelodysplasia	*WAS*	XL, gain of function	Monocytopenia

Table 5.1 (continued)

Disease	Mutated gene	Inheritance	Associated features
5. P14/LAMTOR2 deficiency	*ROBLD3/LAMTOR2*	AR	Hypogammaglobulinemia, partial albinism, growth failure
6. Barth syndrome	Tafazzin (*TAZ*)	XL	Cardiomyopathy, myopathy, growth retardation
7. Cohen syndrome	*COHI*	AR	Retinopathy, developmental delay, facial dysmorphisms
8. Clericuzio syndrome poikiloderma with neutropnia	*C16ORF57*	AR	Poikiloderma, myelodysplastic syndrome
9. JAGN1 deficiency	*JAGN1*	AR	Some with a bone phenotype
10. 3-Methylglutaconic aciduria	*CLPB*	AR	Microcephaly, hypoglycemia, hypotonia, ataxia, seizures, cataracts, IUGR
11. G-CSF receptor deficiency	*CSFR3*	AR	Poor response to G-CSF

5.2 Clinical Manifestations of Neutropenia

The hallmark of neutropenia is increased susceptibility to bacterial infections. The most types of pyogenic infections observed in patients with neutropenia are cutaneous cellulitis, superficial or deep cutaneous abscesses, lymphadenopathy, upper and/or lower respiratory tract infections, and septicemia. Stomatitis, gingivitis, and periodontitis are often chronic problems, resulting in the loss of teeth. Perianal and perirectal inflammation and otitis media occur as well. Susceptibility to bacterial infection even in patients with severe neutropenia can be quite variable, depending on the underlying pathophysiology.

The representative disorders presenting neutropenia in infancy and childhood are described as follows.

5.3 Neutropenia Caused by Intrinsic Defects of Neutrophils or Their Progenitors (Congenital Neutropenia)

The current classification of congenital neutropenia is listed in Table 5.1 with the modification of the proposal of International Union of Immunological Societies Expert Committee presented in 2015 [2]. Severe congenital neutropenia (SCN) includes a heterogenous group of disorders with different responsible gene mutations and is divided into five disorders, SCN1–5. SCN1 is autosomal dominant or sporadic patterns of inheritance, and in this group of patients, most of them (60–80%) have diverse mutations in the neutrophil elastase gene (*ELANE*) [3, 4]. SCN3

is known as Kostmann disease following recessive pattern of inheritance. Its underlying genetic defect is due to homozygous or compound heterozygous mutations in the *HAX1* gene. *HAX1* mutations may be associated with neurologic deficits [5, 6]. Most patients with SCN have experienced frequent episodes of bacterial infections. Prior to the development of granulocyte colony-stimulating factor (G-CSF), patients died of infections and/or their complications. The administration of G-CSF succeeds in the increase of ANC and the decrease of infectious episodes in more than 95% of SCN patients. However, patients with SCN requiring the long-term use of high-dose G-CSF are at risk for developing myelodysplasia/acute myelogenous leukemia [7]. Hematopoietic stem cell transplantation should be considered for the curable treatment in SCN patients.

Cyclic neutropenia is a rare autosomal dominant disorder and presents in infancy or childhood. The disorder is characterized by regular, periodic oscillation, with the ANC ranging from normal to less than 200/μL, mirrored by reciprocal cycling of monocytes. The mean oscillatory period of the cycle is 21 days. During the neutropenic period, almost all patients suffer from malaise, fever, stomatitis, gingivitis, periodontitis, or pharyngitis, lymph node enlargement, and occasionally pneumonia. Gene sequencing showed mutations in the gene for *ELANE*, similar to SCN1 [3, 4]. It has been speculated that neutropenia results from activation of apoptotic pathway by mutant forms of *ELANE*. Approximately 5–10% of patients developed fatal overwhelming infection before the availability of G-CSF.

5.4 Increased Destruction of Neutrophils (Acquired Neutropenia)

Immune-mediated neutropenia is usually associated with the presence of antineutrophil antibodies, resulting in the excessive destruction of neutrophils.

Alloimmune neonatal neutropenia occurs after transplacental transfer of maternal alloantibodies. Maternal antibodies are produced by the incompatibility of neutrophil antigens between mother and babies, similar mechanism of Rh hemolytic disease. Infants may be asymptomatic or they may have infections, such as pyoderma, omphalitis, and pneumonia. Neutropenia usually resolves by 3 months of age with the disappearance of circulating antibodies. Treatment consists of supportive care and the administration of appropriate antibiotics with or without G-CSF.

Primary autoimmune neutropenia (AIN) is a benign form of neutropenia shown in infancy to early childhood. Patients usually have moderate to severe neutropenia with ANC < 500/μL. The median age of presentation is 8 to 10 months ranging 3–30 months. Approximately 90% of children show benign infections, such as pyoderma, otitis media, lymphadenopathy, and upper and lower respiratory tract infections with no life-threatening and responsive-to-standard antibiotics. The ANC varies from 0 to 500/μL, and monocytosis is common. The bone marrow picture reveals the myeloid hyperplasia with marked reduction of segmented neutrons due to their destruction by antibodies. Antineutrophil antibodies are often detected in

serum by immunofluorescence test using flow cytometry [8]. However, the test occasionally has false-negative or false-positive results. The careful diagnosis should be necessary with seeing patients' clinical course. Spontaneous recovery of neutron usually occurs within a few months to a few years. The median age at recovery is 30 months ranging 7–72 months. The specific treatment is not generally necessary because severe infection is rare. The administration of appropriate antibiotics with or without low-dose G-CSF is recommended in severe infections. Prophylactic use of sulfamethoxazole-trimethoprim is useful for patients suffering from frequent infections and/or recurrence of otitis media [9].

References

1. Walkovich KJ, Newburger PE. Leukopenia. In: Kliegman RM, Stanton BF, Schor NF, St. Games III JW, Behrman RE, editors. Nelson textbook of pediatrics. 20th edn. Philadelphia: Elsevier; 2016. p. 1047–53.
2. Picard C, Al-Herz W, Bousfiha A, et al. Primary immunodeficiency diseases: an update on the classification from the international Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. J Clin Immunol. 2015;35:696–726.
3. Horwitz M, Benson KF, Person RE, et al. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. Nat Genet. 1999;23:433–6.
4. Dale DC, Person RE, Bolyard AA, et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. Blood. 2000;96:2317–22.
5. Klein C, Grudzien M, Appaswamy G, et al. HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). Nat Genet. 2007;39:86–92.
6. Ishikawa N, Okada S, Miki M, et al. Neurodevelopmental abnormalities associated with severe congenital neutropenia due to the R86X mutation in the HA1 gene. J Med Genet. 2008;45:802–7.
7. Rosenberg PS, Alter BP, Bolyard AA, et al. The incidence of leukemia and mortality from sepsis in patients with severe congenital neutropenia receiving long-term G-CSF therapy. Blood. 2006;107:4628–35.
8. Kobayashi M, Nakamura K, Kawaguchi H, et al. Significance of the detection of antineutrophil antibodies in children with chronic neutropenia. Blood. 2002;99:4366–9.
9. Kobayashi M, Sato T, Kawaguchi H, et al. Efficacy of prophylactic use of trimethoprim-sulfamethoxazole in autoimmune neutron in infancy. J Pediatr Hematol Oncol. 2003;25: 553–7.

Part III
Red Blood Cell Disorders

Chapter 6
Childhood Aplastic Anemia

Hiroshi Yagasaki

Abstract Recent advances in genomics have enabled better identification of inherited aplastic anemia (AA), which contribute to better outcomes in these patients. Of note, development of the next-generation sequencing has identified minor clones and novel somatic mutations in AA. As a result, clonal hematopoiesis in AA has become widely recognized. The mechanism of disease progression from AA to myelodysplastic syndrome/acute myelogenous leukemia has been partially revealed.

The World Health Organization classification (2008) introduced the provisional entity of refractory cytopenia of childhood (RCC); however, the subsequent findings suggest that AA and RCC have a common clinical and molecular-pathological nature.

The excellent prognosis for children with severe AA reflects the efficacy of the current treatment algorithm including bone marrow transplantation (BMT) and immunosuppressive therapy (IST) based on disease severity and donor availability. In contrast, the choice of haploidentical donor BMT (haplo-BMT) or cord blood transplantation for refractory cases and how to treat non-severe AA remain controversial issues. Thrombopoietin-receptor agonist is a promising molecule to recover multilineage hematopoiesis. The US Food and Drug Administration and the European Medicines Agency approved eltrombopag for adults with severe AA who have failed to respond to the first IST. However, the optimal dose and dosage in children with SAA has yet to be determined. Clonal evolution following long-term use is another serious concern.

H. Yagasaki
Department of Pediatrics, Nihon University School of Medicine,
30-1 Ohyaguchi-kamicho, Itabashi-ku, Tokyo, Japan
e-mail: yagasaki.hiroshi@nihon-u.ac.jp

6.1   Introduction

Childhood aplastic anemia (AA) is a rare disease characterized by bone marrow (BM) aplasia and pancytopenia. Although typical AA is easy to diagnose, non-severe AA (nSAA) is sometimes difficult to differentiate from other diseases. Although the pathogenesis of AA is immune-mediated in most patients, recent advances in genomics have enabled better identification of inherited AAs, collectively called inherited bone marrow failure syndrome (IBFMS), which may contribute to better outcomes in these patients. Recent reports of excellent prognosis for children with severe AA (SAA) reflect the efficacy of the current treatment algorithm including stem cell transplantation (SCT) and immunosuppressive therapy (IST) based on disease severity and donor availability. In the past decade, several controversial and unresolved issues have been addressed by prospective studies, including two nationwide studies in Japan (AA-92 and AA-97).

6.2   Recent Findings in the Pathogenesis of AA

The pathogenesis of idiopathic AA (IAA) has been approached from the perspective of autoimmunity such as autoantibodies and T-cell receptor skewing [1, 2]. In the last decade, extensive investigations have been performed in the field of genomics. Development of the next-generation sequencing has enabled the identification of minor clones and novel mutations. Thus, clonal hematopoiesis in AA has become widely recognized. The mechanism of disease progression from AA to myelodysplastic syndrome/acute myelogenous leukemia (MDS/AML) has been partially revealed [3].

6.2.1   Genetic Predisposition

The yearly incidence of AA differs between East Asian and Western countries. Genetic background as well as environmental conditions may contribute to this difference. Genes involved in the apoptosis of CD34-positive hematopoietic stem cells, T-cell activation, and several cytokines—including IL-6, TNF-α, and TGF-β1—and specific HLA alleles have been proposed as likely candidates. In a Chinese population, HLA-A 02:01, HLA-A 02:06, HLA-B 13:01, HLA-DRB 107:01, HLA-DRB1 09:01, HLA-DRB1 15:01, and HLA-DQB1 06:02 have been reported as risk alleles [4]. However, controversial results have been reported for HLA-DR 15:01 in a Japanese population [5].

A novel insight into the genetic basis of AA has been provided by another study [6]. Loss of heterozygosity of chromosome 6p has been reported in a substantial proportion of AA patients. The missing genes were biased to particular HLA alleles (A 02:01, 02:06, 31:01, and B 40:02). This phenomenon represents "escape

hematopoiesis" which is thought to be mediated by cytotoxic T cells that target the autoantigens expressed on hematopoietic stem cells.

6.2.2   Clonal Evolution

Previous studies showed that clonal cytogenetic abnormalities developed with a cumulative incidence of 22% at 8 years after the diagnosis of childhood AA [7]. Monosomy 7 and trisomy 8 were repeatedly found. The incidence of clonal evolution was previously thought to be independent of the response to IST; however, recent reports have demonstrated that patients with very severe AA (vSAA) who fail to respond to IST and receive long-term granulocyte colony-stimulating factor (G-CSF) are significantly likely to acquire monosomy 7 [8]. Indeed, the evolution of MDS/AML has decreased dramatically since the use of G-CSF has been restricted in children with vSAA for the past two decades [9]. A randomized study in Europe that used antithymocyte globulin (ATG) and cyclosporin (CSA) with or without G-CSF showed that the incidences of MDS/AML in AA patients who did and did not receive G-CSF were 10.9% and 5.8%, respectively [10]. A significantly higher hazard (1.9) of MDS/AML was associated with the use of G-CSF. Conversely, trisomy 8 often presents in patients with AA and persists after IST despite hematological responses. However, the persistence of this condition has not been associated with an increased risk of malignant transformation.

A new sequencing technology has recently identified clonal hematopoiesis in AA arising early in disease, even in young patients [11]. The study showed the presence of myeloid malignancy-related genes such as *DNMT3A*, *BCOR*, and *ASXL1* in 19% of patients ($n = 150$). Somatic mutations in patients with a disease duration >6 months were associated with a risk of malignant transformation of 40%. A small-scale study showed that somatic mutations were found in 16/22 AA patients (73%) with a median disease duration of 1 year, in which 18 patients with pediatric-onset AA were included [12]. Although *PIGA* is the most frequently mutated, two mutations in *STAT5B* and *CAMK2G* were also identified, which show recurrent mutations in MDS. In another recent international study ($n = 439$), clonal hematopoiesis was evaluated by the presence of somatic mutation, 6p uniparental disomy, and copy number variation such as monosomy 7 and del(13) [13]. A minor clone was found in 47% of patients. The major somatic mutations were identified in *PIGA*, *BCOR*, *BCORL1*, *DNMT3A*, and *ASXL1*. Mutations in *PIGA*, *BCOR*, and *BCORL1* correlated with a better response to IST and a higher rate of progression-free survival. In contrast, minor clones with *DNMT3A*, *ASXL1*, *RUNX-1*, *JAK-2*, and *JAK-3* mutations tended to increase in prevalence and size over time, and this subgroup was associated with worse outcomes.

According to a recent Chinese database, 19 of 802 patients with IAA developed MDS/AML, while 21 patients (two with concurrent MDS) developed paroxysmal nocturnal hemoglobinuria (PNH) [14]. The cumulative incidence of clonal evolutions at 5 years was 3.7% (incidences of MDS/AML and PNH were 1.7% and 2.1%, respectively). Multivariate analysis revealed that patient age, disease severity, and

number of days of G-CSF therapy were risk factors for the evolution to MDS/ AML. The relative risk in vSAA was approximately seven times higher than that in SAA and nSAA.

PNH is another clonal disorder associated with AA, which is caused by a somatic mutation of *PIGA* on the X chromosome. Using highly sensitive flow cytometric analysis, minor PNH clones have been found in 41–70% of children with AA [14, 15]. Serious complications of PNH, such as hemolysis and thrombosis, are matters of concern in adolescent patients with long disease duration although such symptomatic PNH is rarely observed in young children [16].

6.2.3 Bone Marrow Microenvironment

Mesenchymal stem cell (MSC) or BM stromal cells constitute an essential component of the BM hematopoietic microenvironment, which are considered to have immunomodulatory properties as well as an ability to support hematopoiesis. Microarray expression analysis using SAA-MSC suggests that *CXCL12* might be associated with alterations in the BM microenvironment [17]. In addition, knockdown of *CXCL12*, *HGF*, *IL-18R1*, *FGF18*, or *RRM2* expression compelled control MSCs to behave like SAA-MSCs, with decreased survival and differentiation potential. Another study showed that low FGF2 expression in MSCs and low FGF2 protein level in BM may be involved in the pathogenesis of AA [18]. However, no definitive conclusion has been reached as to whether an insufficiency in the BM microenvironment is strongly associated with hematopoietic impairment.

6.3 Diagnosis

6.3.1 Differential Diagnosis, Classification, and Grading of Severity in Childhood AA

Several heterogeneous conditions in children present with pancytopenia (Table 6.1). To make a diagnosis of AA, it is essential to rule out underlying diseases. In particular, transient pancytopenia due to viral infection and subsequent hemophagocytosis should not be misdiagnosed as AA. AA is defined as pancytopenia with a hypocellular BM without infiltration of malignant cells or myelofibrosis. Therefore, BM biopsy is critical to assess BM cellularity and to exclude hematological malignancies or myelofibrosis. Through various comprehensive tests, AA is classified into the following entities: inherited AA, hepatitis-associated AA (HAA), IAA, and secondary AA induced by chemical exposure, drugs, radiation, and infections. Approximately 10% of childhood AA is supposedly inherited AA. HAA comprises 5–10% of childhood AA, and the remaining cases (>75%) are classified as IAA. The severity of childhood AA is usually classified into three grades: vSAA, SAA, and nSAA [19] (Table 6.2).

Table 6.1 Differential diagnosis of childhood pancytopenia

Idiopathic AA	
Secondary AA	Hepatitis associated
	Secondary (drug/chemical/radiation/infection)
Inherited AA	Fanconi anemia
	Dyskeratosis congenita
	Shwachman-Diamond syndrome
	Congenital amegakaryocytic thrombocytopenia
	Others
Myelodysplastic syndrome	Refractory cytopenia of childhood (provisional)
	Refractory cytopenia with multilineage dysplasia
	Refractory anemia with excess blast
Other diseases entity	Hemophagocytic lymphohistiocytosis
	Acute leukemia
	Bone marrow invasion of solid tumor
	Autoimmune
	Nutritious deficiency
	Myelofibrosis
	Hypersplenism

AA aplastic anemia

Table 6.2 Severity grading of childhood AA and evaluation criteria to IST

Severity grading of AA [19]		Nuetrophil	Platelet	Reticulocyte	
Moderate	At least 2 levels	$<1000/\mu L$	$<5 \times 10^4/\mu L$	$<6 \times 10^4/\mu L$	
Severe	At least 2 levels	$<500/\mu L$	$<2 \times 10^4/\mu L$	$<2 \times 10^4/\mu L$	
Very severe		$<200/\mu L$	$<2 \times 10^4/\mu L$	$<2 \times 10^4/\mu L$	
Evaluation criteria to IST [111]		Neutrophil	Platelet	Hb	Transfusion
Severe/very severe	Complete response	$\geq 1500/\mu L$	$\geq 10 \times 10^4/\mu L$	≥ 11.0 g/dL	No
	Partial response	$\geq 500/\mu L$	$\geq 3 \times 10^4/\mu L$	≥ 8.0 g/dL	No
	No response	$<500/\mu L$			
Non-severe AA	Complete response	$\geq 1500/\mu L$	$\geq 10 \times 10^4/\mu L$	≥ 11.0 g/dL	No
	Partial response	$\geq 1000/\mu L$	$\geq 3 \times 10^4/\mu L$	≥ 8.0 g/dL	No
	No response	$<1000/\mu L$			

AA aplastic anemia, *IST* immunosuppressive therapy

6.3.2   Distinguishing Inherited AA from Other Types of AA

Because the optimal management differs considerably between inherited AA and other types of AA, correct and timely diagnosis is crucial. However, no useful test for such differentiation is available, except for diagnosing Fanconi anemia. The diagnosis of inherited AA is difficult because of clinical and genetic heterogeneity. Autosomal recessive inheritance, de novo mutations, and genetic anticipation often mask familial accumulation. In addition, the BM morphology of inherited AA varies and may change over time; therefore, BM examination should be repeated to evaluate the cellularity, dysplasia, monoclonality, and leukemic changes. Because such patients are at high risk of developing MDS/AML and solid tumors, genetic diagnosis is mandatory for subsequent medical management.

6.3.3   Distinguishing AA from Myelodysplastic Syndrome

The distinction between AA and MDS is the presence of dysplasia or increased numbers of blast cells. The World Health Organization classification (2008) for MDS added the provisional entity of refractory cytopenia of childhood (RCC), defined as hypocellular marrow with mild dysplasia in one or two lineages with blast cells <5% [20]. However, a few experts agree that AA can be discriminated from refractory cytopenia with multilineage dysplasia (RCMD) and refractory anemia with excess blasts (RAEB) but not from RCC [21]. According to the reevaluation of BM morphology included in the Japanese prospective studies (AA-92 and AA-97), the response to IST, 5-year overall survival (OS), and the incidence of clonal abnormalities were comparable among AA, RCC, and RCMD groups [8]. A recent German study also reported the efficacy of IST for RCC (the response rate at 6 months, 74% in horse-ATG group [hATG] and 53% in rabbit-ATG group [rATG]) [22]. In addition, intensive genetic analyses including 88 IBFMS-associated genes and 96 myeloid malignancy-related genes in children with IAA/RCC/RCMD ($n = 168$) revealed that the somatic mutation frequency did not differ among AA, RCC, and RCMD patients [23]. *BCOR* and *PIGA* mutations were repeatedly identified. Importantly, MDS-related gene mutations such as *U2AF1* plus *SETBP1* and *TP53* mutations were found in only two patients with RCMD.

These findings suggest that AA and RCC have a common clinical and molecular-pathological nature, while the molecular pathogenesis in RCMD is heterogeneous and may progress to advanced disease.

6.4   Standard Treatment

6.4.1   Treatment Algorithm for vSAA or SAA (Fig. 6.1)

1. If a matched related donor (MRD) is available, immediate bone marrow transplant (BMT) is indicated.
2. If a MRD is not available, IST with rATG and CSA is indicated.
3. If no response is obtained at 6 months after IST, BMT from an alternative or unrelated donor (URD) is indicated.
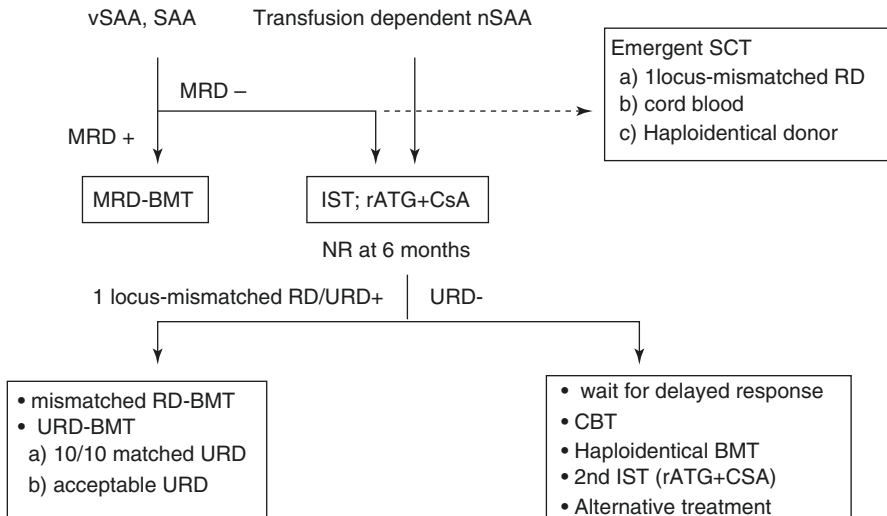
Fig. 6.1 Treatment algorism for children with SAA and vSAA. *vSAA* very severe aplastic anemia, *SAA* severe aplastic anemia, *nSAA* non-severe aplastic anemia, *MRD* matched related donor, *BMT* bone marrow transplantation, *IST* immunosuppressive therapy, *rATG* rabit anti-thymocyte-globulin, *CSA* cyclosporine, *CBT* cord blood transplantation, *NR* non renponse, *URD* unrelated donor, *SCT* stem cell transplantation

Recent reviews provide a similar treatment algorithm for childhood AA [24–27]. Figure 6.1 shows the current minimum consensus for vSAA and SAA. However, this needs to be modified based on the availability and accessibility of diagnostic technology and resources (drug and donor source). Indeed, hATG (ATGAM®) is not available in some countries including Japan. Volunteer donors and cord blood are also limited, and the coordination time for URD varies among countries.

According to the updated results comparing MRD-BMT and IST in Japanese children, failure-free survival (FFS) is significantly lower in the IST group compared with the BMT group (56% vs. 87%, respectively) [28]. Similar results were reported in a recent European study, which found that FFS was 33% for IST but 87% for up-front MRD-SCT [29].

The incidence of relapse after the first IST using hATG + CSA was 11–35% in children [30–32]. Although patients who relapse after a primary response to IST are likely to respond to a second IST, multiple courses of IST are associated with an increased incidence of Epstein-Barr virus-associated lymphoproliferative disorder (EB-LPD) after the subsequent SCT [33].

A Japanese prospective study to compare the efficacy of repeated IST with BMT from alternative donors in children with SAA who were refractory to the first IST showed that the 5-year FFS from the second-line therapy was 84% in the alternative donor BMT group but 10% in the second IST group. Therefore, alternative donor BMT is recommended for children refractory to the first IST [34].

BM is the preferred stem cell source in both MRD-SCT and URD-SCT. The use of peripheral blood stem cells (PBSC) is one of the significant risk factors for survival, acute graft-versus-host disease (aGVHD) and chronic graft-versus-host disease (cGVHD) [35–38]. In exceptional cases with a high risk of graft failure, the use of PBSC may be justified. In developing countries, several reports showed that

PBSC was associated with less graft failure and comparable survival rate compared with BM graft [39, 40].

6.4.2 Transplantation from Matched Related Donor (Tables 6.3 and 6.4)

In Japan, various conditioning regimens have been used in MRD-BMT [41]. Currently, cyclophosphamide (CY, 200 mg/kg) plus rATG (Thymoglobulin®) is the standard conditioning regimen, with successful engraftment rates with low toxicity [42]. The standard for GVHD prophylaxis comprises CSA (3 mg/kg/day, intravenously) and short-course methotrexate (sMTX) (15 mg/m² on day 1, then 10 mg/m² on days 3, 6, and 11) [43, 44]. The target trough level of CSA is 100–200 ng/mL in the whole blood. Slow weaning from CSA should be started at least 12 months after MRD-BMT. In Western countries, the higher trough level of CSA (150–250 ng/mL) is recommended for 9–12 months after transplantation [25].

A prolonged interval between diagnosis and transplantation and no ATG administered during the conditioning regimen are significant risk factors for survival [36, 45]. In addition, prior to IST as well as numerous transfusions and alloimmunization are strongly associated with graft rejection and increased risk of mixed chimerism [46]. Therefore, when MRD is available, BMT should be performed as soon as possible after diagnosis. For such high-risk patients, low-dose radiation could be justified based on the report of a low incidence of late malignancy in a Japanese study [47]. A new regimen comprising fludarabine (Flu, 120 mg/m²), CY (120 mg/kg), and rATG has been widely used in adults with excellent outcomes [48]. Higher incidence of aGVHD and cGVHD was demonstrated in CY+ radiation group compared with CY+ ATG group [49]. The superiority of radiation or alemtuzumab-containing regimens has not been demonstrated in up-front MRD-SCT [36, 38, 49].

6.4.3 Transplantation from Unrelated Donor (Tables 6.3 and 6.4)

Early studies of URD-BMT resulted in poor survival rates of 28–54% in patients with AA [50, 51]. Prior infection due to prolonged aplasia, graft rejection, and severe GVHD as well as increased toxicities caused by the myeloablative regimen, including high-dose total body irradiation (TBI), the presence of anti-HLA antibody, and organ damage by iron overload, may result in poor outcomes. From the late 1990s onward, an early indication for URD-BMT, the introduction of new HLA-typing technology and fludarabine-based non-myeloablative regimens has greatly improved the survival rate, which is currently not inferior to that of MRD-BMT in developed countries [52, 53]. However, URD grafts still have a significant impact on the incidence and severity of aGVHD and cGVHD.

Table 6.3 Outcomes of stem cell transplantation in childhood aplastic anemia (recent selected studies)

Group	Age	Number	Donor source	OS (5 years)	Graft failure (primary + late)	Acute GVHD (II-IV)	Chronic GVHD	Conditioning regimen	GVHD prophylaxis	Reference
Japan	Children	434	Related	93%				Various	Various	[90]
		298	Unrelated	88%						
		26	Cord blood	73%						
Japan	Children	30	Related	100%	3%	0%	3%	CY (200 mg/kg) + ATG, CY (200 mg/kg) + TLI, Flu (100 mg/m^2) + CY(100 mg/kg) + TLI	CSA + MTX	[41]
		31	Unrelated	94%	9%	37%	27%	CY (120-200 mg/kg) + TBI (5-10Gy) + ATG	CSA/ FK + MTX	
Korea	Children	23	Related + unrelated	90%	4%	26%	9%	Flu (120 mg/m^2) + CY (100 mg/kg) + ATG	CSA + MTX	[112]
USA	Children	15	Related	93%	7%	0%	14%	CY (200 mg/kg) + ATG/BU (only for PNH)	CSA/FK +/− MTX	[113]
		23	Unrelated	89%	4%	17%	17%	CY (200 mg/kg) + TBI (2-4Gy) + ATG/ alemtuzumab +/− CA		
UK	Children	44	Unrelated	95%	0%	2% (III-IV)	12%	Flu (150 mg/m^2) + CY (120-200 mg/kg) + alemtuzumab	CSA, CSA + MMF/ MTX, FK	[60]

(continued)

Table 6.3 (continued)

Group	Age	Number	Donor source	OS (5 years)	Graft failure (primary + late)	Acute GVHD (II-IV)	Chronic GVHD	Conditioning regimen	GVHD prophylaxis	Reference
UK/EBMT	Children	29	Upfront, unrelated	96%	3%	10%	19%	Flu (150 mg/m^2) + CY (120 mg/kg) + alemtuzumab +/− TBI (2-3Gy)	CSA +/− MMF	[82]
EBMT	Children	396	Upfront, related	91%	2%	8%	6%	Various	Various	[29]
		91	Post-IST, related, unrelated, mismatched related	83%	2%	25%	20%			
China	Children	20	Haploidentical (2–3 loci mismatch)	80%	5%	58%	17%	Flu (150 mg/m^2) + CY (120-200 mg/kg) + ATG +/− TBI (2-5Gy) +/− BU (2–6.4 mg/kg)	CSA + MTX +/− MMF, FK + MTX	[83]

ATG antithymocyte globulin, *BU* busulfan, *CA* cytarabine, *CSA* cyclosporin, *CY* cyclophosphamide, *FK* tacrolimus, *Flu* fludarabine, *GVHD* graft-versus-host disease, *IST* immunosuppressive therapy, *MMF* mycophenolate mofetil, *MTX* methotrexate, *PNH* paroxysmal nocturnal hemoglobinuria, *TBI* total body irradiation, *TLI* total lymphoid irradiation

Table 6.4 Outcomes of IST with CSA + ATG as a front-line therapy in childhood AA (selected studies)

Study group	Formulation	Patients	N	Severity	Dosage and usage		Results			
					rATG	CSA (oral)	Response at 6 months	OS	Relapse	Reference
Japan	rATG	Children	40	nSAA, SAA, vSAA	3.5 mg/kg × 5	6 mg/kg/day	48%	94%	9%	[114]
Japan/Korea/China	rATG	Children	158	SAA, vSAA	2.3–5 mg/kg × 5	6 mg/kg/day	55%	87%	6%	[68]
China	rATG	Children	46	SAA, vSAA	2.5 mg/kg × 5	5–8 mg/kg/day	65%	85%	5%	[115]
Taiwan	rATG	Children	20	SAA, vSAA	2 mg/kg × 8	5 mg/kg/day	80%	90%	5%	[116]
EWOG	rATG	Children	32	SAA, vSAA	3.75 mg/kg × 5	5 mg/kg/day	34%	92%	nd	[117]
UK	rATG	Children	43	SAA, vSAA	3.75 mg/kg × 5	5 mg/kg/day	33%	94%	31%	[60]
Poland	rATG	Children	63	SAA, vSAA	3.75 mg/kg × 5	5 mg/kg/day	49%	67%	12%	[118]
Brazil	rATG	Children	26	SAA, vSAA	5 mg/kg × 5	10 mg/kg/day	35%	74%	27%	[119]
Japan	hATG (Lymphoglobulin®)	Children	82	nSAA			55%	98%	35%	[30]
			149	SAA			61%	82%	13%	
			210	vSAA			61%	82%	12%	
Germany	hATG (Lymphoglobulin®)	Children	49	SAA			44%[a]	81%	nd	[120]
			97	vSAA			69%[a]	92%	nd	
Italy	hATG (Lymphoglobulin®)	Children	40	nSAA, SAA, vSAA			60%[b]	83%	16%	[32]
USA	hATG (ATGAM®)	Children	77	SAA, vSAA			74%	80%	33%	[31]

AA aplastic anemia, *CSA* cyclosporine, *hATG* horse antithymocyte globulin, *IST* immunosuppressive therapy, *nSAA* non-severe aplastic anemia, *OS* overall survival, *rATG* rabbit antithymocyte globulin (Thymoglobulin), *vSAA* very severe aplastic anemia

[a]Best response 1 year after start of treatment

[b]Response at 4 months

Variable doses of TBI and CY have been used in several clinical trials. Low-dose TBI (2 Gy) improved survival when added to ATG and CY (200 mg/kg total dose) in children with SAA [54]. A CY dose de-escalation study showed that 100 mg/kg or 50 mg/kg of CY combined with Flu (120 mg/m^2), ATG, and low-dose TBI (2 Gy) resulted in favorable survival (92–85%) [55, 56].

Based on these findings, Japan Childhood Aplastic Anemia Study Group has recommended a conditioning regimen with CY (3000 mg/m^2), Flu (100 mg/m^2), rATG (5 mg/kg), and low-dose TBI (3 Gy). Because AA is a benign disorder, all forms of GVHD are detrimental. In particular, cGVHD causes organ dysfunction and secondary malignancy, thus worsening quality of life in the long term. Although a direct comparison between CSA and tacrolimus has not been made, tacrolimus with sMTX has been widely used instead of CSA in Japan, and a retrospective paired-match analysis indicated the superiority of tacrolimus to CSA in URD-BMT [57]. Tacrolimus (0.02 mg/kg/day) is usually used intravenously. The level of tacrolimus is maintained in the range of 10–15 ng/mL in the whole blood. Tacrolimus should be tapered from 12 months after URD-BMT.

Donor selection is a critical issue for URD-BMT. Although a 10/10 or 8/8 allele-matched donor is desirable, such a high level of matching is not always obtained. Based on recent Japanese analysis, any single mismatch or multiple mismatching limited to HLA-C, DQ, and DRB1 may affect the incidence of aGVHD and cGVHD but not survival [58]. These findings expand the URD pool substantially, thus enabling rapid coordination for URD.

In Europe, Flu (120 mg/m^2) + CY (120 mg/kg) + rATG is currently recommended in URD-BMT as well as MRD-BMT for AA [59]. Low-dose TBI can be added in patients >14 years old or with a high risk of rejection. In addition, a UK study explored new regimens in which TBI + ATG was replaced with alemtuzumab to reduce the long-term complications. The Flu (150 mg/m^2) + CY (120 mg/kg) + alemtuzumab (FCC) regimen conferred an excellent 5-year FFS of 95% in recipients transplanted from 10/10 allele-matched URD [60]. There were no cases of graft failure, but mixed chimerism was a major concern. Another study showed an engraftment failure rate of 9%, cGVHD rate of 11%, and 5-year OS of 90% in SCT from full-matched URD and other related or mismatched URD [38]. The risk of cGVHD was significantly lower in the alemtuzumab group than in the rATG group. However, a less toxic regimen with Flu (120 mg/m^2) + CY (1200 mg/m^2) + alemtuzumab resulted in higher graft failure (9.5% for MRD-BMT and 14.5% for URD-BMT) and mixed chimerism in T cell [61].

6.4.4   Immunosuppressive Therapy

ATG is a polyclonal anti-T-cell antibody-rich serum fraction produced by immunizing mammals against human thymocytes. ATG includes several brands with different immunogens, such as hATG (Lymphoglobulin® and ATGAM®) and rATG (ATG-F® and Thymoglobulin®). Pig-ATG is also used in China. For SAA,

Table 6.5 Predictive factors to IST in childhood AA

Significance	Factor	Reference
Favorable	WBC count: $<2.0 \times 10^9$/L	[121]
	Platelet count $<25 \times 10^9$/L	[122]
	Absolute neutrophil count (ANC) $<0.2 \times 10^9$/L	[120]
	Absolute reticulocyte count $\geq 25 \times 10^9$/L and absolute lymphocyte count $\geq 1.0 \times 10^9$/L	[123]
	HLA-DR15 (+) and HLA-DR4 (−)	[124]
	Presence of PNH clone	[125, 126]
Unfavorable	Long interval from diagnosis to IST	[121, 122]
	Short telomere length	[122, 125]
	Duration of ANC = 0 longer than 2 weeks	[127]

AA aplastic anemia, *IST* immunosuppressive therapy, *PNH* paroxysmal nocturnal hemoglobinuria

combination therapy with CSA and ATG is the gold standard IST rather than CSA or ATG alone [62, 63]. The response to IST is classified as complete (CR), partial (PR), or no response (NR) (Table 6.2). Table 6.4 shows the long-term outcomes of clinical studies with standard IST.

In 2009, Lymphoglobulin® was withdrawn from the market worldwide. rATG (Thymoglobulin®) was reported to be effective as a salvage therapy for refractory or relapsed AA [64, 65]. However, the first prospective randomized study demonstrated that both response rate and OS were inferior in the rATG group (Thymoglobulin®) as compared with the hATG group (ATGAM®) (Table 6.5) [66].

The following studies using rATG (Thymoglobulin®) have shown controversial results in terms of the response rate and OS compared with hATG (Lymphoglobulin®). A European study analyzing outcomes in 105 age- and disease severity-matched pairs showed that the best response rate was comparable between rATG + CSA (60%) and hATG + CSA (67%) [67]. However, the OS at 2 years in the rATG group (68%) was inferior to that in the hATG group (86%) ($P = 0.009$). Transplant-free survival also differed significantly (52% for rATG and 76% for hATG). In the larger dataset that included 455 pediatric SAA cohorts from Asian countries, the response rate at 6 months was comparable between the hATG and rATG groups (60% vs. 55%, respectively) [68]. However, the OS in the hATG group was significantly better than that in the rATG group (2-year OS, 96% vs. 87%; 10-year OS, 92% vs. 84%, respectively). Currently, most experts conclude that hATG (ATGAM®) is preferred, but the use of rATG (Thymoglobulin®) is justified. An international prospective study to find the better rATG dose (2.5 mg/kg × 5 days vs. 3.5 mg/kg × 5 days) plus CSA (6 mg/kg) is ongoing in Japan, Korea, and China.

Several clinical and laboratory factors are useful to predict the response to IST. Pretreatment peripheral blood counts and other significant factors are shown in Table 6.6. However, these findings were based on IST using hATG + CSA. Few studies using rATG + CSA reported several predictive factors for the response; SAA rather than vSAA, the higher absolute reticulocyte count and the presence of PNH clone were shown to be favorable [69, 70]. Therefore, the predictive factors must be updated in the prospective studies using rATG + CSA.

Table 6.6 Recommended preparative regimen and GVHD prophylaxis in Japan

Donor	AA	RCC	GVHD prophylaxis
HLA-matched sibling	CY (200 mg/kg)	Flu (125 mg/m²)	CSA + sMTX
	rATG (5 mg/kg)	L-PAM (140 mg/m²)	
		rATG (5 mg/kg)	
Unrelated or HLA 1 locus mismatch related	Flu (100 mg/m²)	Flu (125 mg/m²)	FK + sMTX
	CY (3000 mg/m²)	L-PAM (140 mg/m²)	
	rATG (5 mg/kg)	rATG (5 mg/kg)	
	TBI (3 Gy)	TBI (3Gy)	
Cord blood	Flu (100 mg/m²)		FK + sMTX
	L-PAM (140 mg/m²)		
	TBI (3Gy)		

AA aplastic anemia, *CSA* cyclosporin, *CY* cyclophosphamide, *GVHD* graft-versus-host disease, *FK* tacrolimus, *Flu* fludarabine, *L-PAM* melphalan, *rATG* rabbit antithymocyte globulin, *RCC* refractory cytopenia of childhood, *sMTX* short-term methotrexate, *TBI* total body irradiation

6.4.5 Adolescent AA

Many studies have indicated that patient age >10 years old is a risk factor for survival in the setting of BMT. However, a recent report showed that the same strategy used in younger children (age <10 years old) could be applicable in this age group, with adolescent AA showing very good outcomes [71]. This European study focused on adolescents (age 12–18 years, *n* = 537) with IAA with analysis of three groups as follows: (1) MRD-SCT as first-line treatment, (2) frontline IST not followed by SCT, and (3) SCT after failure of IST. OS did not differ among the groups, but the event-free survival (EFS) was 83%, 64%, and 71%, respectively (*P* = 0.04). In the patients who underwent transplantation, an interval of 2 months or less from diagnosis to treatment, BM as a source of cells, and first-line MRD-SCT conferred a significant advantage in OS and EFS. These findings were consistent with those in younger children.

6.4.6 Hepatitis-Associated AA

HAA usually presents as SAA or vSAA, which occurs at a median of 30 days after the onset of acute hepatitis. A Japanese study assessed the outcomes of 44 HAA children who received IST with hATG and CSA [72]. The overall response at 6 months was 70% (CR 32% and PR 39%). The 10-year OS was 88%. A Chinese group reported that HAA patients had a larger proportion of CD8+ T cells, a lower ratio of CD4+/CD8+ T cells, and a smaller proportion of CD4+ CD25+ regulatory T cells, compared with non-HAA patients [73]. Therefore, HAA patients were likely to have more infection-related mortality in the first 2 years after diagnosis than non-HAA patients. In a Japanese study, two patients died of *Cytomegalovirus* (CMV) pneumonitis, which reflected the more profound immunosuppression in

HAA than in non-HAA patients [72]. In Europe, the 10-year OS after first-line IST was 69% [74]. Increasing age and delayed treatment were significant negative predictors for survival.

6.4.7   Supportive Care

1. Prophylaxis of Alloimmunization and Hemochromatosis

   Minimum transfusion is required. Platelet and hemoglobin level should be 5000–10,000/μL and 6.0–8.0 g/dL, respectively. All transfused products must be irradiated and filtered; however, repeated platelet transfusions may produce anti-HLA antibodies because MHC class I antigens are expressed on the surface of platelets. Pulmonary or intracranial hemorrhage may be fatal. In addition, the presence of anti-HLA antibodies negatively impacts engraftment after SCT [75].

   When the serum ferritin level is >1000 μg/L or the transfused volume of concentrated red cells is >40 mL/kg, iron chelation therapy is indicated.

2. Treatment of Infection

   If the patient becomes febrile, antimicrobial and antifungal therapy with G-CSF should be started immediately. Although AA patients treated with G-CSF + IST have significantly fewer infectious episodes and hospitalization days than do patients without G-CSF, treatment with G-CSF does not improve OS, EFS, response rates, or relapse rates [76]. Therefore, the routine use of G-CSF is not recommended even in vSAA patients [77].

   After the start of IST, attention to viral reactivation is necessary. rATG, CSA, and prednisolone used in IST all affect T-cell function. CMV pneumonitis and EBLPD are rare but fatal without timely diagnosis and appropriate treatment. Routine monitoring for these viruses is recommended. The preemptive use of ganciclovir should be initiated when CMV antigenemia tests positive. Rituximab is indicated for CD20-positive EBLPD.

   BMT, especially alternative donor transplantation, is associated with delayed immune reconstitution. Therefore, BK virus, JC virus, adenovirus, and human herpes virus 6 as well as CMV and EBV are frequently reactivated. Because no effective drugs are available in Japan for the treatment of BK virus, JC virus, and adenovirus infections, the dose reduction of CSA or tacrolimus is necessary so as to decrease the viral load. Since this strategy is substantially difficult in patients with active GVHD, alternative antiviral cell therapies are currently being explored [78].

3. CSA−/Tacrolimus-Related Complications

   The use of calcineurin inhibitors is often associated with hypertension, headache, renal toxicity, hyperkalemia, and encephalopathy. Precise monitoring of the blood concentrations of these drugs is therefore important.

4. Others

   Menarche should be focused on among all pubertal or immediately prepubertal females. Menstrual suppression should be initiated in actively menstruating females because of the possibility of sudden and severe hemorrhage.

6.4.8   Long-Term Complications of Transplantation

CY-based regimens for AA mostly result in normal growth and development and pregnancy [79]. However, patients with cGVHD or secondary malignancy have impaired quality of life and reduced OS. The incidence and severity of these complications are greatly affected by the donor source and conditioning regimen.

An analysis of a large European database (1176 MRD and 542 URD) demonstrated the higher incidence of late complication in URD-SCT recipients than in MRD-SCT despite the comparable OS between the two groups [80]. The cumulative incidence of late side effects was 3% or higher in MRD-SCT and did not increase over time among survivors of MRD-SCT. In contrast, the cumulative incidence in URD-SCT recipients exceeded 3% by 5 years, including gonadal dysfunction in 11%, growth disturbances in 7%, avascular necrosis in 6%, hypothyroidism in 6%, and cataracts in 5% of recipients.

In a previous large series (621 patients with idiopathic AA), the actuarial risk of developing a secondary malignancy was 14% at 20 years, including lymphoproliferative disorders [81]. However, the risk of EBLPD is currently reduced by the exact monitoring of EBV-DNA and the early use of rituximab.

In the Japanese Registry (329 MSD-BMTs), only five cases of late malignancies were found (malignant peripheral nerve sheath tumor, thyroid carcinoma, colon carcinoma, MDS, and hepatoblastoma). The cumulative incidence was 0.8% at 10 years and 2.5% at 20 years, respectively [47].

6.5   Controversial Issues

6.5.1   Up-front Unrelated Donor Transplantation

In the analysis of the European Group for Blood and Marrow Transplantation Working Party, 2-year OS in the up-front URD-SCT cohort was 96%, compared to 91% in the MRD-SCT controls and 74% in the URD-SCT post-IST failure controls [82]. The 2-year EFS in the up-front cohort was 92%, compared to 87% in MRD controls and 74% in URD-SCT post-IST failure controls. These results showed that prior IST had a significantly negative impact on the subsequent outcomes and demonstrated the superiority of frontline SCT with matched URD. However, this strategy is not feasible in Japan because the coordination time for URD is approximately 4 months.

6.5.2   Haploidentical Donor Stem Cell Transplantation (Haplo-SCT)

Haplo-SCT is associated with an increased risk of graft failure and severe GVHD. However, better conditioning regimens and new graft manipulation techniques have enabled successful transplantations. This modern form of haplo-SCT is

being increasingly used as salvage therapy even for children and adolescents with refractory SAA who lack a suitable donor [83].

Recently, the use of posttransplant CY has been shown to be an effective strategy to prevent GVHD in recipients of haplo-SCT. A small case series using a reduced intensity conditioning regimen with posttransplant CY reported promising outcomes in AA, with neutrophil engraftment of 94%, secondary graft failure of 12%, grade II–IV aGVHD of 20%, and 1-year OS of 67% [84].

Other groups have developed strategies to selectively remove alloreactive T cells in order to reduce GVHD without impairing immune reconstitution following haplo-SCT. A recent study demonstrated that CD3-/CD19-depleted peripheral stem cell grafts led to faster recovery of NK cells as compared with that in the CD34-positive selection group [85]. A Korean study enrolled 12 SAA patients who received haplo-SCT with in vitro CD3-depleted grafts [86]. Of 12 patients, 11 achieved neutrophil engraftment at a median of 10 days. Overall, three patients failed to achieve sustainable engraftment and three of the remaining nine patients developed grade II–III aGVHD.

MSCs have potent immunosuppressive effects on alloreactive T lymphocytes, which may enhance the engraftment and ameliorate the severity of GVHD. A few groups have investigated the feasibility of cotransplantation of cord blood-derived MSC in haplo-SCT, but this experimental cell therapy lacks sufficient evidence [87].

6.5.3   Cord Blood Transplantation (Table 6.4)

An early series of cord blood transplantation (CBT) using an MRD-BMT-type conditioning regimen in AA patients resulted in a high rejection rate and low survival rate; therefore, CBT has not been generally recommended [88]. However, better survival was noted in a subgroup conditioned with Flu + low-dose TBI + CY or melphalan (L-PAM). Sequential case series in adults supported the efficacy of the conditioning regimen comprising Flu (125 mg/m^2), L-PAM (80 mg/m^2), and TBI (4 Gy) [89]. Of 12 adult AA patients, 10 patients are alive, while 1 had primary graft failure, and 1 had late graft failure. Thus, the annual report of a nationwide survey in 2015 from Japanese Data Center for Hematopoietic Cell Transplantation/Japan Society for Hematopoietic Cell Transplantation reported that the OS in 26 children with AA reached 77% at 1 year and 73% at 5 years after CBT [90]. Recently a Korean group reported a successful case series ($n = 5$) using non-TBI regimen in CBT [91]. These are encouraging results; however, the optimal range of cell dose and HLA disparity that allow for sustainable engraftment are yet to be determined.

6.5.4   Strategy for Non-severe AA

Because children with nSAA have often absolute neutrophil counts >500/μL and a low risk of serious infection, the first choice in these patients is IST. A previous study for transfusion-dependent nSAA patients (mainly adults) demonstrated that

IST with CSA plus hATG was superior to CSA alone; the actuarial probability of response and FFS was 77% and 80%, respectively [92]. Therefore, combination IST has been the first-line treatment for transfusion-dependent nSAA. A Chinese retrospective study in 42 children with nSAA showed the higher response in hATG + CSA + androgen group than in CSA + androgen group (83% vs. 42%, respectively) [93]. A recent prospective analysis in 95 Japanese children using hATG plus CSA with/without androgen showed the 6-month response rate of 55% and the 10-year FFS of 44% [94].

Several studies have reported that some nSAA patients might experience spontaneous resolution, while some patients show persisting nSAA for months or years, and others progress slowly to SAA [95, 96]. However, a recent retrospective observational study showed that spontaneous remissions in nSAA in childhood were rare, and progression-free survival was only 25% at 10 years [97]. As there is no consensus on definite treatment, the timing of intervention differs among nSAA patients. The response rate to IST between transfusion-dependent group and independent group was not different statistically (49% vs. 59%, respectively) [94]. Nevertheless, IST before progression to SAA or transfusion-dependent AA may be advantageous because the duration of aplasia before IST is inversely correlated with the response in children with SAA and vSAA. A prospective study to elucidate the efficacy of early intervention in such patients is warranted.

6.5.5   Other Treatments

Many strategies have been explored as salvage therapies for patients with resistant or relapsing AA, but most studies did not show the efficacy of the study drugs which included mycophenolate mofetil [98], sirolimus [99], tacrolimus [100], and alemtuzumab [101].

6.5.5.1   Thrombopoietin-Receptor (TPO-R) Agonist

In Japan, eltrombopag is indicated for adults with chronic immune thrombocytopenic purpura. This small molecule stimulates hematopoietic stem cell as well as megakaryocytes. Indeed, multilineage bone marrow failure has been reported in patients with *TPO-R* or *TPO* mutation [102–104]. The pivotal study (NCT00922883) was conducted at the National Institutes of Health, in which eltrombopag was evaluated in 43 patients with SAA who have had an insufficient response to at least one prior IST and had a platelet count less than 30×10^9/L [105, 106]. After 12 weeks of treatment with eltrombopag, a hematologic response was observed in 17 patients (40%); the majority showed a unilineage response (13/17). A total of 14 patients who responded entered the extension phase of the trial. Of these, nine achieved a multilineage response, while five maintained the response with a median follow-up off drug of 13 months. A subsequent study (ELT 116826) also showed hematological response of 52%. Thus, the US Food and Drug Administration and the European Medicines

Agency approved the use of eltrombopag in adult patients with SAA who have had an insufficient response to IST in 2014 and 2015, respectively. However, the optimal dose and dosage in children with SAA has yet to be determined. Moreover, clonal evolution following long-term use is another serious concern. Of 43 patients, a new cytogenetic abnormality was reported in eight patients (19%), including five who had changes in chromosome 7 [105, 106]. The median time to a cytogenetic abnormality was 3 months (range, 3–13 months) after the use of eltrombopag.

6.5.5.2 CY

High-dose CY (200 mg/kg) combined with CSA therapy resulted in a higher incidence of fungal infections and death compared to conventional therapy with ATG plus CSA [107]. Moderate dose CY (120 mg/kg) plus CSA therapy also showed a response rate of 41–65% [108, 109]. High-dose CY (200 mg/kg) monotherapy showed a hematological response of 79% and EFS of 64% in a pediatric or young adult cohort (22 treatment-naïve and 6 refractory/relapsed patients) [110].

6.6 Conclusions

Treatment failure, disease recurrence, and clonal evolution are the shortcomings of IST. Long-term complications and secondary malignancy remain major concerns in URD- or haplo-BMT. Sufficient evidence is lacking to recommend CBT as an upfront therapy. In the future, genomic analyses will help to select the appropriate treatment for children with AA.

References

1. Young NS, Scheinberg P, Calado RT. Aplastic anemia. Curr Opin Hematol. 2008;15:162–8.
2. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. Blood. 2006;108:2509–19.
3. Ogawa S. Clonal hematopoiesis in acquired aplastic anemia. Blood. 2016;128:337–47.
4. Wang M, Nie N, Feng S, Shi J, Ge M, Li X, Shao Y, Huang J, Zheng Y. The polymorphisms of human leukocyte antigen loci may contribute to the susceptibility and severity of severe aplastic anemia in Chinese patients. Hum Immunol. 2014;75:867–72.
5. Yoshida N, Yagasaki H, Takahashi Y, Yamamoto T, Liang J, Wang Y, Tanaka M, Hama A, Nishio N, Kobayashi R, Hotta N, Asami K, Kikuta A, Fukushima T, Hirano N, Kojima S. Clinical impact of HLA-DR15, a minor population of paroxysmal nocturnal haemoglobinuria-type cells, and an aplastic anaemia-associated autoantibody in children with acquired aplastic anaemia. Br J Haematol. 2008;142:427–35.
6. Katagiri T, Sato-Otsubo A, Kashiwase K, Morishima S, Sato Y, Mori Y, Kato M, Sanada M, Morishima Y, Hosokawa K, Sasaki Y, Ohtake S, Ogawa S, Nakao S, Japan Marrow Donor Program. Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia. Blood. 2011;118:6601–9.

7. Kojima S, Ohara A, Tsuchida M, Kudoh T, Hanada R, Okimoto Y, Kaneko T, Takano T, Ikuta K, Tsukimoto I. Japan Childhood Aplastic Anemia Study Group Risk factors for evolution of acquired aplastic anemia into myelodysplastic syndrome and acute myeloid leukemia after immunosuppressive therapy in children. Blood. 2002;100:786–90.

8. Hama A, Takahashi Y, Muramatsu H, Ito M, Narita A, Kosaka Y, Tsuchida M, Kobayashi R, Ito E, Yabe H, Ohga S, Ohara A, Kojima S. Comparison of long-term outcomes between children with aplastic anemia and refractory cytopenia of childhood who received immuno-suppressive therapy with antithymocyte globulin and cyclosporine. Haematologica. 2015;100:1426–33.

9. Takahashi Y, Ohara A, Kobayashi R, Yabe H, Tsuchida M, Mugishima H, Morimoto A, Ohga S, Bessho F, Tsukimoto I, Nakahata T, Kojima S. Decreased incidence of clonal evolution to myelodysplastic syndrome/acute myeloid leukemia with Monosomy 7 in children with aplas-tic anemia following reduced use of G-CSF. Blood. 2007;1696a:110.

10. Socie G, Mary JY, Schrezenmeier H, Marsh J, Bacigalupo A, Locasciulli A, Fuehrer M, Bekassy A, Tichelli A, Passweg J. Granulocyte-stimulating factor and severe aplastic anemia: a survey by the European Group for Blood and Marrow Transplantation (EBMT). Blood. 2007;109:2794–6.

11. Kulasekararaj AG, Jiang J, Smith AE, Mohamedali AM, Mian S, Gandhi S, Gaken J, Czepulkowski B, Marsh JC, Mufti GJ. Somatic mutations identify a subgroup of aplastic anemia patients who progress to myelodysplastic syndrome. Blood. 2014;124:2698–704.

12. Babushok DV, Perdigones N, Perin JC, Olson TS, Ye W, Roth JJ, Lind C, Cattier C, Li Y, Hartung H, Paessler ME, Frank DM, Xie HM, Cross S, Cockroft JD, Podsakoff GM, Monos D, Biegel JA, Mason PJ, Bessler M. Emergence of clonal hematopoiesis in the majority of patients with acquired aplastic anemia. Cancer Genet. 2015;208:115–28.

13. Yoshizato T, Dumitriu B, Hosokawa K, Makishima H, Yoshida K, Townsley D, Sato-Otsubo A, Sato Y, Liu D, Suzuki H, CO W, Shiraishi Y, Clemente MJ, Kataoka K, Shiozawa Y, Okuno Y, Chiba K, Tanaka H, Nagata Y, Katagiri T, Kon A, Sanada M, Scheinberg P, Miyano S, Maciejewski JP, Nakao S, Young NS, Ogawa S. Somatic mutations and clonal hematopoiesis in aplastic anemia. N Engl J Med. 2015;373:35–47.

14. Li Y, Li X, Ge M, Shi J, Qian L, Zheng Y, Wang J. Long-term follow-up of clonal evolutions in 802 aplastic anemia patients: a single-center experience. Ann Hematol. 2011;90:529–37.

15. Timeus F, Crescenzio N, Longoni D, Doria A, Foglia L, Pagliano S, Vallero S, Decimi V, Svahn J, Palumbo G, Ruggiero A, Martire B, Pillon M, Marra N, Dufour C, Ramenghi U, Saracco P. Paroxysmal nocturnal hemoglobinuria clones in children with acquired aplastic anemia: a multicentre study. PLoS One. 2014;9:e101948.

16. MM v d H-E, Bredius RG, te Winkel ML, Tamminga R, de Kraker J, Schouten-van Meeteren AY, Bruin M, Korthof ET. Childhood paroxysmal nocturnal haemoglobinuria (PNH), a report of 11 cases in the Netherlands. Br J Haematol. 2005;128:571–7.

17. Chao YH, Wu KH, Chiou SH, Chiang SF, Huang CY, Yang HC, Chan CK, Peng CT, Wu HP, Chow KC, Lee MS. Downregulated CXCL12 expression in mesenchymal stem cells associ-ated with severe aplastic anemia in children. Ann Hematol. 2015;94:13–22.

18. Sy J, Xt X, Jiang H, Jj Z, Li F, Cao P. Low expression of basic fibroblastic growth factor in mesenchymal stem cells and bone marrow of children with aplastic anemia. Pediatr Hematol Oncol. 2014;31:11–9.

19. Camitta BM, Thomas ED, Nathan DG, Gale RP, Kopecky KJ, Rappeport JM, Santos G, Gordon-Smith EC, Storb R. A prospective study of androgens and bone marrow transplanta-tion for treatment of severe aplastic anemia. Blood. 1979;53:504–14.

20. Baumann I, Niemeyer CM, Bennett JM, Shannon K. Childhood myelodysplastic syndrome. In: Swerdlow SH, et al., editors. World Health Organization classification of tumours of hae-matopoietic and lymphoid tissues. 4th ed. Lyon: IARC Press; 2008. p. 104–7.

21. Forester CM, Sartain SE, Guo D, Harris MH, Weinberg OK, Fleming MD, London WB, Williams DA, Hofmann I. Pediatric aplastic anemia and refractory cytopenia: A retrospective analysis assessing outcomes and histomorphologic predictors. Am J Hematol. 2015;90: 320–6.

22. Yoshimi A, van den Heuvel-Eibrink MM, Baumann I, Schwarz S, Simonitsch-Klupp I, de Paepe P, Campr V, Kerndrup GB, O'Sullivan M, Devito R, Leguit R, Hernandez M, Dworzak M, de Moerloose B, Stary J, Hasle H, Smith OP, Zecca M, Catala A, Schmugge M, Locatelli F, Führer M, Fischer A, Guderle A, Nöllke P, Strahm B, Niemeyer CM. Comparison of horse and rabbit antithymocyte globulin in immunosuppressive therapy for refractory cytopenia of childhood. Haematologica. 2014;99:656–63.
23. Narita A, Okuno Y, Muramatsu H, Yoshida K, Shiraishi Y, Sekiya Y, Chiba K, Tanaka H, Wang X, Kawashima N, Nishio N, Doisaki S, Hama A, Takahashi Y, Sakaguchi H, Yoshida N, Kato K, Miyano S, Ito M, Ogawa S, Kojima S. Genetic background of idiopathic bone marrow failure syndromes in children. Blood. 2015;3610a:126.
24. Kook H, Chung NG, Kang HJ, Im HJ. Acquired aplastic anemia in Korean children: treatment guidelines from the Bone Marrow Failure Committee of the Korean Society of Pediatric Hematology Oncology. Int J Hematol. 2016;103:380–6.
25. Barone A, Lucarelli A, Onofrillo D, Verzegnassi F, Bonanomi S, Cesaro S, Fioredda F, Iori AP, Ladogana S, Locasciulli A, Longoni D, Lanciotti M, Macaluso A, Mandaglio R, Marra N, Martire B, Maruzzi M, Menna G, Notarangelo LD, Palazzi G, Pillon M, Ramenghi U, Russo G, Svahn J, Timeus F, Tucci F, Cugno C, Zecca M, Farrago P, Dufour C, Saracco P, Marrow Failure Study Group of the Pediatric Haemato-Oncology Italian Association. Diagnosis and management of acquired aplastic anemia in childhood. Guidelines from the Marrow Failure Study Group of the Pediatric Haemato-Oncology Italian Association (AIEOP). Blood Cells Mol Dis. 2015;55:40–7.
26. Miano M, Dufour C. The diagnosis and treatment of aplastic anemia: a review. Int J Hematol. 2015;101:527–35.
27. Marsh JC, Ball SE, Cavenagh J, Darbyshire P, Dokal I, Gordon-Smith EC, Keidan J, Laurie A, Martin A, Mercieca J, Killick SB, Stewart R, Yin JA, British Committee for Standards in Haematology. Guidelines for the diagnosis and management of aplastic anaemia. Br J Haematol. 2009;147:43–70.
28. Yoshida N, Kobayashi R, Yabe H, Kosaka Y, Yagasaki H, Watanabe K, Kudo K, Morimoto A, Ohga S, Muramatsu H, Takahashi Y, Kato K, Suzuki R, Ohara A, Kojima S. First-line treatment for severe aplastic anemia in children: bone marrow transplantation from a matched family donor versus immunosuppressive therapy. Haematologica. 2014;99:1784–91.
29. Dufour C, Pillon M, Sociè G, Rovò A, Carraro E, Bacigalupo A, Oneto R, Passage J, Risitano A, Tichelli A, Peffault de Latour R, Schrezenmeier H, Hocshmann B, Peters C, Kulasekararaj A, Van Biezen A, Samarasinghe S, Hussein AA, Ayas M, Aljurf M, Marsh J. Outcome of aplastic anaemia in children. A study by the severe aplastic anaemia and paediatric disease working parties of the European group blood and bone marrow transplant. Br J Haematol. 2015;169:565–73.
30. Kamio T, Ito E, Ohara A, Kosaka Y, Tsuchida M, Yagasaki H, Mugishima H, Yabe H, Morimoto A, Ohga S, Muramatsu H, Hama A, Kaneko T, Nagasawa M, Kikuta A, Osugi Y, Bessho F, Nakahata T, Tsukimoto I, Kojima S, Japan Childhood Aplastic Anemia Study Group. Relapse of aplastic anemia in children after immunosuppressive therapy: a report from the Japan Childhood Aplastic Anemia Study Group. Haematologica. 2011;96:814–9.
31. P S, Wu CO, Nunez O, Young NS. Long-term outcome of pediatric patients with severe aplastic anemia treated with antithymocyte globulin and cyclosporine. J Pediatr. 2008;153:814–9.
32. Saracco P, Quarello P, Iori AP, Zecca M, Longoni D, Svahn J, Varotto S, Del Vecchio GC, Dufour C, Ramenghi U, Bacigalupo A, Locasciulli A, Bone Marrow Failure Study Group of the AIEOP (Italian Association of Paediatric Haematology Oncology). Cyclosporin A response and dependence in children with acquired aplastic anaemia: a multicentre retrospective study with long-term observation follow-up. Br J Haematol. 2008;140:197–205.
33. Buyck HC, Ball S, Junagade P, Marsh J, Chakrabarti S. Prior immunosuppressive therapy with antithymocyte globulin increases the risk of EBV-related lymphoproliferative disorder following allo-SCT for acquired aplastic anaemia. Bone Marrow Transplant. 2009;43:813–6.

34. Kosaka Y, Yagasaki H, Sano K, Kobayashi R, Ayukawa H, Kaneko T, Yabe H, Tsuchida M, Mugishima H, Ohara A, Morimoto A, Otsuka Y, Ohga S, Bessho F, Nakahata T, Tsukimoto I, Kojima S. Japan Childhood Aplastic Anemia Study Group Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia. Blood. 2008;111:1054–9.

35. Schrezenmeier H, Passweg JR, Marsh JC, Bacigalupo A, Bredeson CN, Bullorsky E, Camitta BM, Champlin RE, Gale RP, Fuhrer M, Klein JP, Locasciulli A, Oneto R, Schattenberg AV, Socie G, Eapen M. Worse outcome and more chronic GVHD with peripheral blood progenitor cells than bone marrow in HLA-matched sibling donor transplants for young patients with severe acquired aplastic anemia. Blood. 2007;110:1397–400.

36. Bacigalupo A, Socié G, Schrezenmeier H, Tichelli A, Locasciulli A, Fuehrer M, Risitano AM, Dufour C, Passweg JR, Oneto R, Aljurf M, Flynn C, Mialou V, Hamladji RM, Marsh JC, Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (WPSAA-EBMT). Bone marrow versus peripheral blood as the stem cell source for sibling transplants in acquired aplastic anemia: survival advantage for bone marrow in all age groups. Haematologica. 2012;97:1142–8.

37. Eapen M, Le Rademacher J, Antin JH, Champlin RE, Carreras J, Fay J, Passweg JR, Tolar J, Horowitz MM, Marsh JC, Deeg HJ. Effect of stem cell source on outcomes after unrelated donor transplantation in severe aplastic anemia. Blood. 2011;118:2618–21.

38. Marsh JC, Pearce RM, Koh MB, Lim Z, Pagliuca A, Mufti GJ, Perry J, Snowden JA, Vora AJ, Wynn RT, Russell N, Gibson B, Gilleece M, Milligan D, Veys P, Samarasinghe S, McMullin M, Kirkland K, Cook G, British Society for Blood and Marrow Transplantation, Clinical Trials Committee. Retrospective study of alemtuzumab vs ATG-based conditioning without irradiation for unrelated and matched sibling donor transplants in acquired severe aplastic anemia: a study from the British Society for Blood and Marrow Transplantation. Bone Marrow Transplant. 2014;49:42–8.

39. Kumar R, Kimura F, Ahn KW, Hu ZH, Kuwatsuka Y, Klein JP, Pasquini M, Miyamura K, Kato K, Yoshimi A, Inamoto Y, Ichinohe T, Wood Jr WA, Wirk B, Seftel M, Rowlings P, Marks DI, Schultz KR, Gupta V, Dedeken L, George B, Cahn JY, Szer J, Lee JW, Ho AY, Fasth A, Hahn T, Khera N, Dalal J, Bonfim C, Aljurf M, Atsuta Y, Saber W. Comparing outcomes with bone marrow or peripheral blood stem cells as graft source for matched sibling transplants in severe aplastic anemia across different economic regions. Biol Blood Marrow Transplant. 2016;22:932–40.

40. Chen J, Lee V, Luo CJ, Chiang AK, Hongeng S, Tan PL, Tan AM, Sanpakit K, Li CF, Lee AC, Chua HC, Okamoto Y. Allogeneic stem cell transplantation for children with acquired severe aplastic anaemia: a retrospective study by the Viva-Asia Blood and Marrow Transplantation Group. Br J Haematol. 2013;162:383–91.

41. Yagasaki H, Takahashi Y, Hama A, Kudo K, Nishio N, Muramatsu H, Tanaka M, Yoshida N, Matsumoto K, Watanabe N, Kato K, Horibe K, Kojima S. Comparison of matched-sibling donor BMT and unrelated donor BMT in children and adolescent with acquired severe aplastic anemia. Bone Marrow Transplant. 2010;45:1508–13.

42. Storb R, Etzioni R, Anasetti C, Appelbaum FR, Buckner CD, Bensinger W, Bryant E, Clift R, Deeg HJ, Doney K. Cyclophosphamide combined with antithymocyte globulin in preparation for allogeneic marrow transplants in patients with aplastic anemia. Blood. 1994;84:941–9.

43. Storb R, Deeg HJ, Farewell V, Doney K, Appelbaum F, Beatty P, Bensinger W, Buckner CD, Clift R, Hansen J, et al. Marrow transplantation for severe aplastic anemia: methotrexate alone compared with a combination of methotrexate and cyclosporine for prevention of acute graft-versus-host disease. Blood. 1986;68:119–25.

44. Locatelli F, Bruno B, Zecca M, Van-Lint MT, McCann S, Arcese W, Dallorso S, Di Bartolomeo P, Fagioli F, Locasciulli A, Lawler M, Bacigalupo A. Cyclosporin A and short-term methotrexate versus cyclosporin A as graft versus host disease prophylaxis in patients with severe aplastic anemia given allogeneic bone marrow transplantation from an HLA-identical sibling: results of a GITMO/EBMT randomized trial. Blood. 2000;96:1690–7.

45. Bacigalupo A, Socie' G, Lanino E, Prete A, Locatelli F, Locasciulli A, Cesaro S, Shimoni A, Marsh J, Brune M, Van Lint MT, Oneto R, Passweg J, Severe Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation. Fludarabine, cyclophosphamide, antithymocyte globulin, with or without low dose total body irradiation, for alternative donor transplants, in acquired severe aplastic anemia: a retrospective study from the EBMT-SAA Working Party. Haematologica. 2010;95:976–82.

46. Kobayashi R, Yabe H, Hara J, Morimoto A, Tsuchida M, Mugishima H, Ohara A, Tsukimoto I, Kato K, Kigasawa H, Tabuchi K, Nakahata T, Ohga S, Kojima S, Japan Childhood Aplastic Anemia Study Group. Preceding immunosuppressive therapy with antithymocyte globulin and ciclosporin increases the incidence of graft rejection in children with aplastic anaemia who underwent allogeneic bone marrow transplantation from HLA-identical siblings. Br J Haematol. 2006;135:693–6.

47. Kikuchi A, Yabe H, Kato K, Koh K, Inagaki J, Sasahara Y, Suzuki R, Yoshida N, Kudo K, Kobayashi R, Tabuchi K, Kawa K, Kojima S. Long-term outcome of childhood aplastic anemia patients who underwent allogeneic hematopoietic SCT from an HLA-matched sibling donor in Japan. Bone Marrow Transplant. 2013;48:657–60.

48. Maury S, Bacigalupo A, Anderlini P, Aljurf M, Marsh J, Socié G, Oneto R, Passweg JR, Severe Aplastic Anemia Working Party, European Group for Blood and Marrow Transplantation (EBMT-SAAWP). Improved outcome of patients older than 30 years receiving HLA-identical sibling hematopoietic stem cell transplantation for severe acquired aplastic anemia using fludarabine-based conditioning: a comparison with conventional conditioning regimen. Haematologica. 2009;94:1312–5.

49. Ades L, Mary JY, Robin M, Ferry C, Porcher R, Esperou H, Ribaud P, Devergie A, Traineau R, Gluckman E, Socié G. Long-term outcome after bone marrow transplantation for severe aplastic anemia. Blood. 2004;103(7):2490.

50. Hows J, Szydlo R, Anasetti C, Camitta B, Gajewski J, Gluckman E. Unrelated donor marrow transplants for severe acquired aplastic anemia. Bone Marrow Transplant. 1992;10(Suppl 1): 102–6.

51. Margolis D, Camitta B, Pietryga D, Keever-Taylor C, Baxter-Lowe LA, Pierce K, Kupst MJ, French 3rd J, Truitt R, Lawton C, Murray K, Garbrecht F, Flomenberg N, Casper J. Unrelated donor bone marrow transplantation to treat severe aplastic anaemia in children and young adults. Br J Haematol. 1996;94:65–72.

52. Locasciulli A, Oneto R, Bacigalupo A, Socié G, Korthof E, Bekassy A, Schrezenmeier H, Passweg J, Führer M, Severe Aplastic Anemia Working Party of the European Blood and Marrow Transplant Group. Outcome of patients with acquired aplastic anemia given first line bone marrow transplantation or immunosuppressive treatment in the last decade: a report from the European Group for Blood and Marrow Transplantation (EBMT). Haematologica. 2007;92:11–8.

53. Viollier R, Socié G, Tichelli A, Bacigalupo A, Korthof ET, Marsh J, Cornish J, Ljungman P, Oneto R, Békássy AN, Fuehrer M, Maury S, Schrezenmeier H, van Lint MT, Wojcik D, Locasciulli A, Passweg JR. Recent improvement in outcome of unrelated donor transplantation for aplastic anemia. Bone Marrow Transplant. 2008;41:45–50.

54. Deeg HJ, O'Donnell M, Tolar J, Agarwal R, Harris RE, Feig SA, Territo MC, Collins RH, McSweeney PA, Copelan EA, Khan SP, Woolfrey A, Storer B. Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. Blood. 2006;108:1485–91.

55. Tolar J. Fludarabine-based conditioning for marrow transplantation from unrelated donors in severe aplastic anemia: early results of a cyclophosphamide dose deescalation study show life-threatening adverse events at predefined cyclophosphamide dose levels. Biol Blood Marrow Transplant. 2012;18:1007–11.

56. Anderlini P, Wu J, Gersten I, Ewell M, Tolar J, Antin JH, Adams R, Arai S, Eames G, Horwitz ME, McCarty J, Nakamura R, Pulsipher MA, Rowley S, Leifer E, Carter SL, DiFronzo NL, Horowitz MM, Confer D, Deeg HJ, Eapen M. Cyclophosphamide conditioning in patients with severe aplastic anaemia given unrelated marrow transplantation: a phase 1-2 dose deescalation study. Lancet Haematol. 2015;2:e367–75.

57. Yagasaki H, Kojima S, Yabe H, Kato K, Kigasawa H, Sakamaki H, Tsuchida M, Kato S, Kawase T, Muramatsu H, Morishima Y, Kodera Y. Tacrolimus/Methotrexate versus cyclosporine/methotrexate as graft-versus-host disease prophylaxis in patients with severe aplastic anemia who received bone marrow transplantation from unrelated donors: results of matched pair analysis. Biol Blood Marrow Transplant. 2009;15:1603–8.

58. Yagasaki H, Kojima S, Yabe H, Kato K, Kigasawa H, Sakamaki H, Tsuchida M, Kato S, Kawase T, Morishima Y, Kodera Y, Japan Marrow Donor Program. Acceptable HLA-mismatching in unrelated donor bone marrow transplantation for patients with acquired severe aplastic anemia. Blood. 2011;118:3186–90.

59. Apperley J, Carreras E, Gluckman E, Masszi T, editors. The 2012 revised edition of the EBMT-ESH handbook on haematopoietic stem cell transplantation. Paris: European School of Haematology.

60. Samarasinghe S, Steward C, Hiwarkar P, Saif MA, Hough R, Webb D, Norton A, Lawson S, Qureshi A, Connor P, Carey P, Skinner R, Vora A, Pelidis M, Gibson B, Stewart G, Keogh S, Goulden N, Bonney D, Stubbs M, Amrolia P, Rao K, Meyer S, Wynn R, Veys P. Excellent outcome of matched unrelated donor transplantation in paediatric aplastic anaemia following failure with immunosuppressive therapy: a United Kingdom multicentre retrospective experience. Br J Haematol. 2012;157:339–46.

61. Marsh JC, Gupta V, Lim Z, Ho AY, Ireland RM, Hayden J, Potter V, Koh MB, Islam MS, Russell N, Marks DI, Mufti GJ, Pagliuca A. Alemtuzumab with fludarabine and cyclophosphamide reduces chronic graft-versus-host disease after allogeneic stem cell transplantation for acquired aplastic anemia. Blood. 2011;118:2351–7.

62. Frickhofen N, Kaltwasser JP, Schrezenmeier H, Raghavachar A, Vogt HG, Herrmann F, Freund M, Meusers P, Salama A, Heimpel H. Treatment of aplastic anemia with antilymphocyte globulin and methylprednisolone with or without cyclosporine. The German Aplastic Anemia Study Group. N Engl J Med. 1991;324:1297–304.

63. Frickhofen N, Heimpel H, Kaltwasser JP, Schrezenmeier H, German Aplastic Anemia Study Group. Antithymocyte globulin with or without cyclosporin A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. Blood. 2003;101:1236–42.

64. Di Bona E, Rodeghiero F, Bruno B, Gabbas A, Foa P, Locasciulli A, Rosanelli C, Camba L, Saracco P, Lippi A, Iori AP, Porta F, De Rossi G, Comotti B, Iacopino P, Dufour C, Bacigalupo A. Rabbit antithymocyte globulin (r-ATG) plus cyclosporine and granulocyte colony stimulating factor is an effective treatment for aplastic anaemia patients unresponsive to a first course of intensive immunosuppressive therapy. Gruppo Italiano Trapianto di Midollo Osseo (GITMO). Br J Haematol. 1999;107:330–4.

65. Scheinberg P, Nunez O, Young NS. Retreatment with rabbit anti-thymocyte globulin and ciclosporin for patients with relapsed or refractory severe aplastic anaemia. Br J Haematol. 2006;133:622–7.

66. Scheinberg P, Nunez O, Weinstein B, Scheinberg P, Biancotto A, CO W, Young NS. Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. N Engl J Med. 2011;365:430–8.

67. Marsh JC, Bacigalupo A, Schrezenmeier H, Tichelli A, Risitano AM, Passweg JR, Killick SB, Warren AJ, Foukaneli T, Aljurf M, Al-Zahrani HA, Höchsmann B, Schafhausen P, Roth A, Franzke A, Brummendorf TH, Dufour C, Oneto R, Sedgwick P, Barrois A, Kordasti S, Elebute MO, Mufti GJ, Socie G, European Blood and Marrow Transplant Group Severe Aplastic Anaemia Working Party. Prospective study of rabbit antithymocyte globulin and cyclosporine for aplastic anemia from the EBMT Severe Aplastic Anaemia Working Party. Blood. 2012;119:5391–6.

68. Jeong DC, Chung NG, Cho B, Zou Y, Ruan M, Takahashi Y, Muramatsu H, Ohara A, Kosaka Y, Yang W, Kim HK, Zhu X, Kojima S. Long-term outcome after immunosuppressive therapy with horse or rabbit antithymocyte globulin and cyclosporine for severe aplastic anemia in children. Haematologica. 2014;99:664–71.

69. Zhang L, Jing L, Zhou K, Wang H, Peng G, Li Y, Li Y, Li J, Ye L, Shi L, Fan H, Zhao X, Wang J, Zhang F. Rabbit antithymocyte globulin as first-line therapy for severe aplastic anemia. Exp Hematol. 2015;43:286–94.

70. Afable 2nd MG, Shaik M, Sugimoto Y, Elson P, Clemente M, Makishima H, Sekeres MA, Lichtin A, Advani A, Kalaycio M, Tiu RV, O'Keefe CL, Maciejewski JP. Efficacy of rabbit anti-thymocyte globulin in severe aplastic anemia. Haematologica. 2011;96:1269–75.
71. Dufour C, Pillon M, Passweg J, Socié G, Bacigalupo A, Franceschetto G, Carraro E, Oneto R, Risitano AM, Peffault de Latour R, Tichelli A, Rovo A, Peters C, Hoechsmann B, Samarasinghe S, Kulasekararaj AG, Schrezenmeier H, Aljurf M, Marsh J. Outcome of aplastic anemia in adolescence: a survey of the Severe Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation. Haematologica. 2014;99:1574–81.
72. Osugi Y, Yagasaki H, Sako M, Kosaka Y, Taga T, Ito T, Yamamoto M, Ohara A, Sato T, Mimaya J, Tsukimoto I, Kojima S, Japan Childhood Aplastic Anemia Study Group. Antithymocyte globulin and cyclosporine for treatment of 44 children with hepatitis associated aplastic anemia. Haematologica. 2007;92:1687–90.
73. Wang H, Tu M, Fu R, Wu Y, Liu H, Xing L, Shao Z. The clinical and immune characteristics of patients with hepatitis-associated aplastic anemia in China. PLoS One. 2014;9:e98142.
74. Locasciulli A, Bacigalupo A, Bruno B, Montante B, Marsh J, Tichelli A, Socié G, Passweg J, Severe Aplastic Anemia Working Party of the European Blood and Marrow Transplant Group (SAA-WP, EBMT). Hepatitis-associated aplastic anaemia: epidemiology and treatment results obtained in Europe A report of The EBMT aplastic anaemia working party. Br J Haematol. 2010;149:890–5.
75. Zhu H, He J, Cai J, Yuan X, Jiang H, Luo C, Wang J, Luo C, Pan Z, Terasaki PI, Ding L, Chen J. Pre-existing anti-HLA antibodies negatively impact survival of pediatric aplastic anemia patients undergoing HSCT. Clin Transplant. 2014;28:1225–33.
76. Tichelli A, Schrezenmeier H, Socié G, Marsh J, Bacigalupo A, Dührsen U, Franzke A, Hallek M, Thiel E, Wilhelm M, Höchsmann B, Barrois A, Champion K, Passweg JR. A randomized controlled study in patients with newly diagnosed severe aplastic anemia receiving antithymocyte globulin (ATG), cyclosporine, with or without G-CSF: a study of the SAA Working Party of the European Group for Blood and Marrow Transplantation. Blood. 2011;117:4434–41.
77. Marsh JC. Hematopoietic growth factors in the pathogenesis and for the treatment of aplastic anemia. Semin Hematol. 2000;37:81–90.
78. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, Leung K, Carrum G, Gee AP, Vera JF, Krance RA, Brenner MK, Rooney CM, Heslop HE, Leen AM. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci Transl Med. 2014;6:242ra83.
79. Sanders JE, Woolfrey AE, Carpenter PA, Storer BE, Hoffmeister PA, Deeg HJ, Flowers ME, Storb RF. Late effects among pediatric patients followed for nearly 4 decades after transplantation for severe aplastic anemia. Blood. 2011;118:1421–8.
80. Buchbinder D, Nugent DJ, Brazauskas R, Wang Z, Aljurf MD, Cairo MS, Chow R, Duncan C, Eldjerou LK, Gupta V, Hale GA, Halter J, Hayes-Lattin BM, Hsu JW, Jacobsohn DA, Kamble RT, Kasow KA, Lazarus HM, Mehta P, Myers KC, Parsons SK, Passweg JR, Pidala J, Reddy V, Sales-Bonfim CM, Savani BN, Seber A, Sorror ML, Steinberg A, Wood WA, Wall DA, Winiarski JH, LC Y, Majhail NS. Late effects in hematopoietic cell transplant recipients with acquired severe aplastic anemia: a report from the late effects working committee of the center for international blood and marrow transplant research. Biol Blood Marrow Transplant. 2012;18:1776–84.
81. Deeg HJ, Leisenring W, Storb R, Nims J, Flowers ME, Witherspoon RP, Sanders J, Sullivan KM. Long-term outcome after marrow transplantation for severe aplastic anemia. Blood. 1998;91:3637–45.
82. Dufour C, Veys P, Carraro E, Bhatnagar N, Pillon M, Wynn R, Gibson B, Vora AJ, Steward CG, Ewins AM, Hough RE, de la Fuente J, Velangi M, Amrolia PJ, Skinner R, Bacigalupo A, Risitano AM, Socie G, Peffault de Latour R, Passweg J, Rovo A, Tichelli A, Schrezenmeier H, Hochsmann B, Bader P, van Biezen A, Aljurf MD, Kulasekararaj A, Marsh JC, Samarasinghe S. Similar outcome of upfront-unrelated and matched sibling stem cell transplantation in idiopathic paediatric aplastic anaemia. A study on behalf of the UK Paediatric BMT Working Party, Paediatric Diseases Working Party and Severe Aplastic Anaemia Working Party of EBMT. Br J Haematol. 2015;171:585–94.

83. Zhu H, Luo RM, Luan Z, Lee V, Zhu YP, Luo CJ, Tang XF, Si YJ, Chen J. Unmanipulated haploidentical haematopoietic stem cell transplantation for children with severe aplastic anaemia. Br J Haematol. 2016;174:799–805.

84. Esteves I, Bonfim C, Pasquini R, Funke V, Pereira NF, Rocha V, Novis Y, Arrais C, Colturato V, de Souza MP, Torres M, Fernandes JF, Kerbauy FR, Ribeiro AA, Santos FP, Hamerschlak N. Haploidentical BMT and post-transplant Cy for severe aplastic anemia: a multicenter retrospective study. Bone Marrow Transplant. 2015;50:685–9.

85. Handgretinger R. Negative depletion of CD3(+) and TcRαβ(+) T cells. Curr Opin Hematol. 2012;19:434–9.

86. Im HJ, Koh KN, Choi ES, Jang S, Kwon SW, Park CJ, Chi HS, Seo JJ. Excellent outcome of haploidentical hematopoietic stem cell transplantation in children and adolescents with acquired severe aplastic anemia. Biol Blood Marrow Transplant. 2013;19:754–9.

87. Wu Y, Cao Y, Li X, Xu L, Wang Z, Liu P, Yan P, Liu Z, Wang J, Jiang S, Wu X, Gao C, Da W, Han Z. Cotransplantation of haploidentical hematopoietic and umbilical cord mesenchymal stem cells for severe aplastic anemia: successful engraftment and mild GVHD. Stem Cell Res. 2014;12:132–8.

88. Yoshimi A, Kojima S, Taniguchi S, Hara J, Matsui T, Takahashi Y, Azuma H, Kato K, Nagamura-Inoue T, Kai S, Kato S, Japan Cord Blood Bank Network. Unrelated cord blood transplantation for severe aplastic anemia. Biol Blood Marrow Transplant. 2008;14: 1057–63.

89. Yamamoto H, Kato D, Uchida N, Ishiwata K, Araoka H, Takagi S, Nakano N, Tsuji M, Asano-Mori Y, Matsuno N, Masuoka K, Izutsu K, Wake A, Yoneyama A, Makino S, Taniguchi S. Successful sustained engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with severe aplastic anemia. Blood. 2011;117:3240–2.

90. Hematopoietic Cell Transplantation in Japan. Annual report of nationwide survey 2015. The Japanese Data Center for Hematopoietic Cell Transplantation/The Japan Society for Hematopoietic Cell Transplantation. 2016.

91. Jaing TH, Huang IA, Chen SH, Yang CP, Liang DC, Hung IJ. Cord blood transplantation in children with relapsed or refractory severe aplastic anemia. J Pediatr Hematol Oncol. 2011;33:18–21.

92. Marsh J, Schrezenmeier H, Marin P, Ilhan O, Ljungman P, McCann S, Socie G, Tichelli A, Passweg J, Hows J, Raghavachar A, Locasciulli A, Bacigalupo A. Prospective randomized multicenter study comparing cyclosporin alone versus the combination of antithymocyte globulin and cyclosporin for treatment of patients with nonsevere aplastic anemia: a report from the European Blood and Marrow Transplant (EBMT) Severe Aplastic Anaemia Working Party. Blood. 1999;93:2191–5.

93. Jiang S, Wang Y, Shi W, Shao Y, Qiao X, Lin J, Kuang H, Xie X. The benefit of ATG in immunosuppressive therapy of children with moderate aplastic anemia. Pediatr Hematol Oncol. 2009;26:313–20.

94. Nishikawa E, Yagasaki H, Hama A, Yabe H, Ohara A, Kosaka Y, Kudo K, Kobayashi R, Ohga S, Morimoto A, Watanabe K, Yoshida N, Muramatsu H, Takahashi Y, Kojima S. Long-term outcomes of 95 children with moderate aplastic anemia treated with horse antithymocyte globulin and cyclosporine. Pediatr Blood Cancer. 2016 Nov 3; doi:10.1002/pbc.26305. [Epub ahead of print].

95. Howard SC, Naidu PE, XJ H, Jeng MR, Rodriguez-Galindo C, Rieman MD, Wang WC. Natural history of moderate aplastic anemia in children. Pediatr Blood Cancer. 2004;43: 545–51.

96. Wang S, Chen Y, Zou Y, Zheng Y, Zhu X. The progression risk factors of children with transfusion-independent non-severe aplastic anemia. Int J Hematol. 2013;97:210–5.

97. Nishio N, Yagasaki H, Takahashi Y, Muramatsu H, Hama A, Yoshida N, Kudo K, Kojima S. Natural history of transfusion-independent non-severe aplastic anemia in children. Int J Hematol. 2009;89:409–13.

98. Scheinberg P, Nunez O, Wu C, Young NS. Treatment of severe aplastic anaemia with combined immunosuppression: anti-thymocyte globulin, ciclosporin and mycophenolate mofetil. Br J Haematol. 2006;133:606–11.

99. Scheinberg P, CO W, Nunez O, Scheinberg P, Boss C, Sloand EM, Young NS. Treatment of severe aplastic anemia with a combination of horse antithymocyte globulin and cyclosporine, with or without sirolimus: a prospective randomized study. Haematologica. 2009;94:348–54.

100. Alsultan A, Goldenberg NA, Kaiser N, Graham DK, Hays T. Tacrolimus as an alternative to cyclosporine in the maintenance phase of immunosuppressive therapy for severe aplastic anemia in children. Pediatr Blood Cancer. 2009;52:626–30.

101. Scheinberg P, Nunez O, Weinstein B, Scheinberg P, CO W, Young NS. Activity of alemtuzumab monotherapy in treatment-naive, relapsed, and refractory severe acquired aplastic anemia. Blood. 2012;119:345–54.

102. Dasouki MJ, Rafi SK, Olm-Shipman AJ, Wilson NR, Abhyankar S, Ganter B, Furness LM, Fang J, Calado RT, Saadi I. Exome sequencing reveals a thrombopoietin ligand mutation in a Micronesian family with autosomal recessive aplastic anemia. Blood. 2013;122:3440–9.

103. Ihara K, Ishii E, Eguchi M, Takada H, Suminoe A, Good RA, Hara T. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. Proc Natl Acad Sci U S A. 1999;96(6):3132.

104. Muraoka K, Ishii E, Tsuji K, Yamamoto S, Yamaguchi H, Hara T, Koga H, Nakahata T, Miyazaki S. Defective response to thrombopoietin and impaired expression of c-mpl mRNA of bone marrow cells in congenital amegakaryocytic thrombocytopenia. Br J Haematol. 1997;96:287–92.

105. Olnes MJ, Scheinberg P, Calvo KR, Desmond R, Tang Y, Dumitriu B, Parikh AR, Soto S, Biancotto A, Feng X, Lozier J, CO W, Young NS, Dunbar CE. Eltrombopag and improved hematopoiesis in refractory aplastic anemia. N Engl J Med. 2012;367:11–9.

106. Desmond R, Townsley DM, Dumitriu B, Olnes MJ, Scheinberg P, Bevans M, Parikh AR, Broder K, Calvo KR, CO W, Young NS, Dunbar CE. Eltrombopag restores trilineage hematopoiesis in refractory severe aplastic anemia that can be sustained on discontinuation of drug. Blood. 2014;123:1818–25.

107. Tisdale JF, Dunn DE, Geller N, Plante M, Nunez O, Dunbar CE, Barrett AJ, Walsh TJ, Rosenfeld SJ, Young NS. High-dose cyclophosphamide in severe aplastic anaemia: a randomised trial. Lancet. 2000;356:1554–9.

108. Scheinberg P, Townsley D, Dumitriu B, Scheinberg P, Weinstein B, Daphtary M, Rios O, CO W, Young N. Moderate-dose cyclophosphamide for severe aplastic anemia has significant toxicity and does not prevent relapse and clonal evolution. Blood. 2014;124:2820–3.

109. Zhang F, Zhang L, Jing L, Zhou K, Wang H, Peng G, Li Y, Li Y, Li J, Ye L, Shi L, Fan H, Zhao X, Chu Y, Hao Y, Wang J. High-dose cyclophosphamide compared with antithymocyte globulin for treatment of acquired severe aplastic anemia. Exp Hematol. 2013;41:328–34.

110. Gamper CJ, Takemoto CM, Chen AR, Symons HJ, Loeb DM, Casella JF, Dezern AE, King KE, McGonigle AM, Jones RJ, Brodsky RA. High-dose cyclophosphamide is effective therapy for pediatric severe aplastic anemia. J Pediatr Hematol Oncol. 2016;38:627–35.

111. Kojima S, Hibi S, Kosaka Y, Yamamoto M, Tsuchida M, Mugishima H, Sugita K, Yabe H, Ohara A, Tsukimoto I. Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony-stimulating factor in children with acquired aplastic anemia. Blood. 2000;96:2049–54.

112. Chung NG, Lee JW, Jang PS, Jeong DC, Cho B, Kim HK. Reduced dose cyclophosphamide, fludarabine and antithymocyte globulin for sibling and unrelated transplant of children with severe and very severe aplastic anemia. Pediatr Transplant. 2013;17:387–93.

113. Kennedy-Nasser AA, Leung KS, Mahajan A, Weiss HL, Arce JA, Gottschalk S, Carrum G, Khan SP, Heslop HE, Brenner MK, Bollard CM, Krance RA. Comparable outcomes of matched-related and alternative donor stem cell transplantation for pediatric severe aplastic anemia. Biol Blood Marrow Transplant. 2006;12:1277–84.

114. Takahashi Y, Muramatsu H, Sakata N, Hyakuna N, Hamamoto K, Kobayashi R, Ito E, Yagasaki H, Ohara A, Kikuchi A, Morimoto A, Yabe H, Kudo K, Watanabe K, Ohga S, Kojima S, Japan Childhood Aplastic Anemia Study Group. Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia. Blood. 2013;121:862–3.

115. Chen C, Xue HM, HG X, Li Y, Huang K, Zhou DH, Guo HX, Fang JP, Huang SL. Rabbit-antithymocyte globulin combined with cyclosporin A as a first-line therapy: improved, effective, and safe for children with acquired severe aplastic anemia. J Cancer Res Clin Oncol. 2012;138:1105–11.

116. Huang IA, Jaing TH, Yang CP, Hung IJ, Tsay PK, Luo CC, Sun CF. Single-Center Experience: immunosuppressive therapy as frontline treatment for 33 children with acquired severe aplastic anemia. Pediatr Hematol Oncol. 2009;26:487–95.

117. Yoshimi A, Niemeyer CM, Führer MM, Strahm B. Comparison of the efficacy of rabbit and horse antithymocyte globulin for the treatment of severe aplastic anemia in children. Blood. 2013;121:860–1.

118. Pawelec K, Salamonowicz M, Panasiuk A, Demkow U, Kowalczyk J, Balwierz W, Zaleska-Czepko E, Chybicka A, Szmyd K, Szczepanski T, Bubala H, Wysocki M, Kurylak A, Wachowiak J, Szpecht D, Młynarski W, Bulas M, Krawczuk-Rybak M, Leszczynska E, Urasinski T, Peregud-Pogorzelski J, Balcerska A, Kaczorowska-Hac B, Matysiak M. First-line immunosuppressive treatment in children with aplastic anemia: rabbit antithymocyte globulin. Adv Exp Med Biol. 2015;836:55–62.

119. Garanito MP, Carneiro JD, Odone Filho V, Scheinberg P. Outcome of children with severe acquired aplastic anemia treated with rabbit antithymocyte globulin and cyclosporine A. J Pediatr (Rio J). 2014;90:523–7.

120. Führer M, Rampf U, Baumann I, Faldum A, Niemeyer C, Janka-Schaub G, Friedrich W, Ebell W, Borkhardt A, Bender-Goetze C. Immunosuppressive therapy for aplastic anemia in children: a more severe disease predicts better survival. Blood. 2005;106:2102–4.

121. Yoshida N, Yagasaki H, Hama A, Takahashi Y, Kosaka Y, Kobayashi R, Yabe H, Kaneko T, Tsuchida M, Ohara A, Nakahata T, Kojima S. Predicting response to immunosuppressive therapy in childhood aplastic anemia. Haematologica. 2011;96:771–4.

122. Sakaguchi H, Nishio N, Hama A, Kawashima N, Wang X, Narita A, Doisaki S, Xu Y, Muramatsu H, Yoshida N, Takahashi Y, Kudo K, Moritake H, Nakamura K, Kobayashi R, Ito E, Yabe H, Ohga S, Ohara A, Kojima S, Japan Childhood Aplastic Anemia Study Group. Peripheral blood lymphocyte telomere length as a predictor of response to immunosuppressive therapy in childhood aplastic anemia. Haematologica. 2014;99:1312–6.

123. Scheinberg P, CO W, Nunez O, Young NS. Predicting response to immunosuppressive therapy and survival in severe aplastic anaemia. Br J Haematol. 2009;144:206–16.

124. Song EY, Kang HJ, Shin HY, Ahn HS, Kim I, Yoon SS, Park S, Kim BK, Park MH. Association of human leukocyte antigen class II alleles with response to immunosuppressive therapy in Korean aplastic anemia patients. Hum Immunol. 2010;71:88–92.

125. Narita A, Muramatsu H, Sekiya Y, Okuno Y, Sakaguchi H, Nishio N, Yoshida N, Wang X, Xu Y, Kawashima N, Doisaki S, Hama A, Takahashi Y, Kudo K, Moritake H, Kobayashi M, Kobayashi R, Ito E, Yabe H, Ohga S, Ohara A, Kojima S, Japan Childhood Aplastic Anemia Study Group. Paroxysmal nocturnal hemoglobinuria and telomere length predicts response to immunosuppressive therapy in pediatric aplastic anemia. Haematologica. 2015;100:1546–52.

126. Tutelman PR, Aubert G, Milner RA, Dalal BI, Schultz KR, Deyell RJ. Paroxysmal nocturnal haemoglobinuria phenotype cells and leucocyte subset telomere length in childhood acquired aplastic anaemia. Br J Haematol. 2014;164:717–21.

127. Yagasaki H, Shichino H, Ohara A, Kobayashi R, Yabe H, Ohga S, Hamamoto K, Ohtsuka Y, Shimada H, Inoue M, Muramatsu H, Takahashi Y, Kojima S. Immunosuppressive therapy with horse anti-thymocyte globulin and cyclosporine as treatment for fulminant aplastic anemia in children. Ann Hematol. 2014;93:747–52.

Chapter 7
Inherited Bone Marrow Failure Syndrome, TAM

Etsuro Ito, Kiminori Terui, and Tsutomu Toki

Abstract Inherited bone marrow failure syndromes (IBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and increased risk of malignant disease. IBMFS may affect all blood cell lineages, causing clinical symptoms similar to aplastic anemia, or they may be restricted to one or two blood cell lineages with symptoms specific to the affected cell lineage. Early and accurate diagnosis of the disease is important, as there are implications for management and long-term follow-up. However, diagnosis is often difficult due to the wide varieties of clinical presentation. Recent advances in our understanding of IBMFS have largely come from the identification of the causative genes and investigations of their pathways. In this chapter, advances in the pathobiology and clinical management of two representative diseases, Diamond-Blackfan anemia and Fanconi's anemia, will be described.

Trisomy 21, the genetic hallmark of Down syndrome (DS), is the most frequent human chromosomal abnormality. In neonates with DS, about 5–10% develop transient abnormal myelopoiesis (TAM). Almost all cases of TAM have mutations in *GATA1*. In most cases, it resolves spontaneously within 3 months. However, early death occurs in about 20% of the cases. Furthermore, approximately 20% of TAM patients develop myeloid leukemia of DS (ML-DS) within 4 years of life. Human tumors have been shown to progress by the accumulation of genetic abnormalities. The malignant progression from TAM to ML-DS offers a unique model to study the stepwise development of cancer pathogenesis. Recent studies have provided fascinating insights into the pathogenesis of TAM, details that may provide insight not only into DS leukemia but also contribute to our understanding of the pathogenesis of other leukemias.

E. Ito, M.D., Ph.D. (✉) • K. Terui, M.D., Ph.D. • T. Toki, Ph.D.
Department of Pediatrics, Hirosaki University Graduate School of Medicine,
Hirosaki, Japan
e-mail: eturou@hirosaki-u.ac.jp

7.1 Inherited Bone Marrow Failure Syndrome

7.1.1 Diamond-Blackfan Anemia

7.1.1.1 Introduction

Diamond-Blackfan anemia (DBA) is a congenital pure red cell aplasia characterized by normochromic macrocytic anemia and reticulocytopenia in the peripheral blood and the loss of erythroblasts in the bone marrow. It generally presents in the first year of life. Approximately 40% of DBA patients have variable malformations [1–3]. In addition, the patients have a predisposition for malignancies [4]. DBA was first reported by Dr. Josephs in 1936 and refined as a distinct clinical entity by Drs. Diamond and Blackfan in 1938 [5, 6].

DBA has been associated with heterozygous mutations or large deletions in ribosomal protein (RP) genes in more than 50% of patients [7–17], suggesting that DBA is caused by ribosome dysfunction [18]. Corticosteroids and transfusion are the mainstays of treatment. Approximately 80% of patients respond to an initial course of steroids; however, only 60–70% of patients ultimately become transfusion-independent [3]. Bone marrow transplantation is the only curative treatment, but it requires an HLA-matched sibling and is primarily reserved for patients with severe complications [19].

7.1.1.2 Epidemiology

Ten to 20% of DBA families have more than one affected individual, and the majority of these cases appear to be dominantly inherited. The remaining cases are sporadic or familial with other inheritance patterns. The incidence is estimated to be 5–10 per million live births without ethnic predilection, with both sexes equally affected [2, 19–22]. In the Registration of the Japanese Society of Pediatric Hematology and Oncology, 175 patients with DBA, including patients diagnosed with idiopathic pure red cell anemia, were reported from 1988 to 2011 [23].

7.1.1.3 Pathogenesis

Following the observation that a DBA patient had an X;19 chromosomal translocation, a major DBA locus was mapped to chromosomal location 19q13, and the breakpoint was shown to occur in the *RPS19* gene, which encodes one of 80 ribosomal proteins [9]. Subsequent large-scale studies established that *RPS19* is mutated in approximately 25% of DBA patients [24]. To date, 15 RP genes including *RPS7*, *RPS10*, *RPS15A*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPS27*, *RPS28*, *RPS29*, *RPL5*, *RPL11*, *RPL26*, *RPL27*, and *RPL35A* have been reported to be responsible for DBA [7–17, 136]. A sizable fraction of DBA patients have a large deletion of RP genes that are not detected by sequencing [25, 26]. Although 10–20% of patients have inherited DBA, most patients are sporadic and have de novo mutations. DBA has

been associated with heterozygous mutations or large deletions in RP genes in up to 60% of patients, except for rare germline *GATA1* mutation reported in three X-linked DBA families [137].

The ribosome is an intracellular organelle and is the site of protein synthesis and translation of messenger RNA into continuous chains of amino acids. The mammalian ribosome (80S) consists of a large subunit (60S) and a small subunit (40S), and each subunit is composed of ribosomal RNAs and RPs. RPs of the small and large subunits are called RPS and RPL, respectively. RPS19, RPS24, RPS10, and RPS26 play important roles in maturation of the 18S rRNA and assembly of the 40S ribosomal subunit [8, 27–30]. RPL35A, RPL5, and RPL11 play important roles in the maturation of the 28S and 5.8S rRNAs and assembly of the 60S ribosomal subunit [10, 12]. Therefore, deficiency in RPs leads to a relative lack of the 40S or 60S ribosomal subunits and a decline in translation initiation. The fact that all of the causative genes for DBA are ribosomal proteins, except for *GATA1*, suggests that insufficiency in ribosomal function may be the underlying cause of red cell aplasia in patients with DBA. Although the mechanism whereby mutations in the ribosomal protein genes cause specific defects in red cell maturation is not fully understood, many lines of evidence indicate that p53 activation caused by ribosomal dysfunction may be central to DBA pathogenesis (Fig. 7.1) [31].
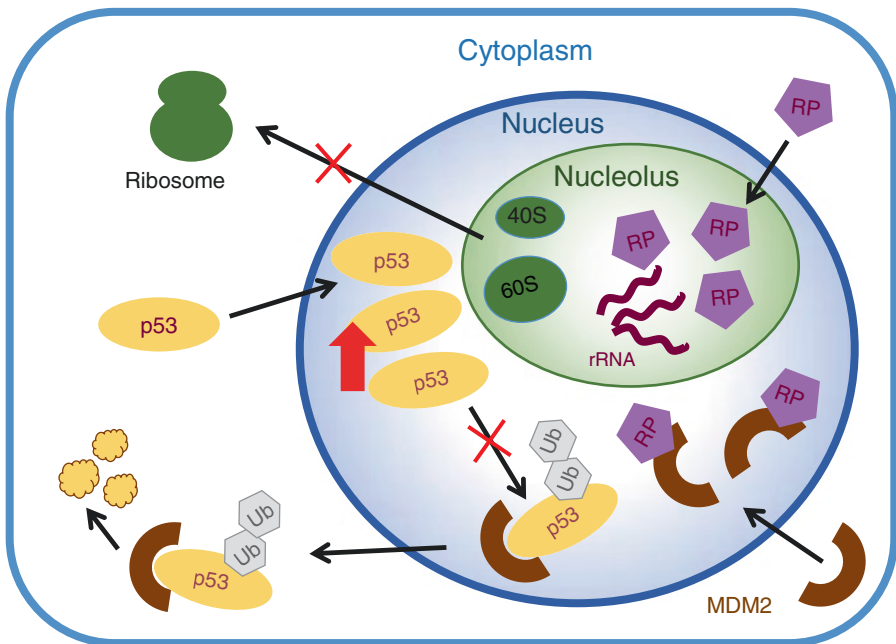
Fig. 7.1 Model of p53 stabilization in response to impaired ribosome biogenesis in DBA. Normal erythroblasts produce large numbers of ribosomes for protein synthesis. Levels of p53 remain low via a feedback loop whereby MDM2, a transcriptional p53 target, ubiquitinates p53 to promote its degradation by proteasomes. However, haploinsufficiency for specific RPs causes accumulation of other RPs, which bind to MDM2, thereby inhibiting its ability to promote p53 degradation. Consequently, p53 accumulates and triggers cell cycle arrest and apoptosis

7.1.1.4 Clinical Management

Clinical Presentation

Anemia

DBA is a congenital pure red cell aplasia characterized by normochromic macrocytic anemia and reticulocytopenia in the peripheral blood and the loss of erythroblast from bone marrow. Approximately 90% of affected individuals present in infancy or early childhood, although a "nonclassical" mild phenotype may not be diagnosed until later in life [2, 3].

Congenital Anomalies

Clinical manifestations of DBA are variable. In about 40% of patients, DBA is associated with physical anomalies and growth retardation, but in some patients, no congenital anomalies are found [3]. Table 7.1 summarizes the range of congenital abnormalities found in DBA patients.

Cancer Predisposition

Twenty-nine cases were reported among more than 700 patients in the literature [3]. A recent prospective study showed that among 608 patients, 15 solid tumors, two acute myeloid leukemias (AML), and two cases of myelodysplastic syndrome (MDS) were diagnosed at a median age of 41 years in patients who had not received a bone marrow transplant. The rate of development of solid tumors or leukemia was 5.4-fold higher in DBA patients than expected in a demographically matched comparison with the general population. Furthermore, specific tumors had significantly elevated incidence ratios for MDS, AML, adenocarcinoma of the colon, osteogenic sarcoma, and female genital cancer [138].

Table 7.1 Congenital abnormalities observed in Diamond-Blackfan anemia (DBA)

Head, face, palate	Hypertelorism, cleft palate, high-arched palate, microcephaly, micrognathia, low-set ears, low hairline, epicanthus, ptosis, etc
Upper limb	Triphalangeal, duplex or bifid, hypoplastic thumb; flat thenar eminence; syndactyly; absent radial artery
Renal, urogenital	Absent kidney, horseshoe kidney, hypospadias
Cardiopulmonary	Ventricular septal defect, atrial septal defect, coarctation of the aorta, complex cardiac anomalies
Other	
Neck	Short neck, webbed neck
Eyes	Congenital glaucoma, strabismus, congenital cataract
Neuromotor	Learning difficulties
Short stature	

Diagnostic Procedures

Diagnostic Criteria

The diagnostic criteria for "classical" DBA include macrocytic (or normocytic) anemia with no other significant cytopenia presenting prior to the first birthday, reticulocytopenia, and normal marrow cellularity with a paucity of erythroid precursors, and it is accompanied by congenital anomalies in some cases. However, diagnosis of DBA is often difficult due to the incompleteness of the phenotypes and the wide variability in clinical expression. Even mutations in individual RP genes lead to widely variable phenotypic expression; family members with the same mutation in an RP gene can present with clinical differences. For example, *RPS19* mutations are found in some first-degree relatives presenting only with isolated high erythrocyte adenosine deaminase activity and/or macrocytosis [24]. Therefore, it is very difficult to make a diagnosis on the basis of clinical phenotype alone. Molecular diagnosis enables the detection of carriers and the avoidance of hematopoietic stem cell transplantation from sibling donors with these mutations. However, about 40% have no known pathogenic mutations. The diagnostic and supporting criteria for diagnosis of DBA, including mild type, are described in Table 7.2. We have modified the criteria from the international clinical consensus conference [3] based on our results for the novel DBA biomarker reduced glutathione (GSH) [32].

Table 7.2 Diagnostic criteria for patients with Diamond-Blackfan anemia (DBA)

Diagnostic criteria:
• Age less than 1 year
• Macrocytic anemia with no other significant cytopenias
• Reticulocytopenia
• Normal marrow cellularity with paucity of erythroid precursors
Supporting criteria:
Major:
• Gene mutation described in "classical" DBA
• Positive family history
Minor:
• Elevated erythrocyte adenosine deaminase (eADA) activity and erythrocyte-reduced glutathione (eGSH)[a]
• Congenital anomalies described in "classical" DBA
• Elevated HbF
• No evidence of other inherited bone marrow failure syndromes
"Classical" DBA fulfills all four diagnostic criteria
"Nonclassical" DBA fulfills one of the following:
1. Three of the diagnostic criteria and one major or two minor supporting criteria
2. Two of the diagnostic criteria and three minor supporting criteria
3. Two major supporting criteria

[a]eADA activities and eGSH should be measured simultaneously, and the data statistically analyzed using linear analysis according to SNV methods to predict DBA [32]

Differential Diagnosis

In children, transient erythrocytopenia in childhood (TEC) should be a major consideration [3]. TEC usually presents in children older than 1 year of age following viral infection. Most cases resolve spontaneously within 1–2 months. Patients with TEC present with normocytic anemia, and, in contrast to those with DBA, their HbF levels and erythrocyte adenosine deaminase (eADA) activities are normal. Congenital bone marrow failure syndromes accompanied by physical anomalies include dyskeratosis congenita, Shwachman-Diamond syndrome, congenital amegakaryocytic thrombocytopenia, and Pearson syndrome. Although all of these diseases are extremely rare, it is possible to discriminate them from specific clinical findings. Recently, because the causative genes for all of these diseases have been identified, the molecular etiology may be clarified in the near future, and genetic diagnosis may be possible in many patients.

Treatment

Transfusion Therapy

Patients who do not respond to steroids may require chronic transfusion therapy every ~4–8 weeks. Although maintaining hemoglobin levels above 8 g/dL is recommended, a disadvantage of chronic transfusion therapy is hemosiderosis by iron overload. To avoid liver dysfunction, diabetes, and myocardiopathy due to iron deposition, chelation therapy with deferasirox or deferoxamine is recommended. However, methods for oral iron chelation therapy with deferasirox in neonates have not been established.

Drug Therapy

Approximately 80% of patients respond to steroids. The recommended starting dose is 2 mg/kg/day of prednisone at initial treatment. In over 20% of DBA patients, steroids eventually are stopped completely [3]. It should be mentioned that important steroid side effects include growth retardation, osteoporosis, obesity, hypertension, diabetes mellitus, cataract, and glaucoma. The therapy is not generally recommended in babies under 6 months of age. Alternative treatments, including cyclosporine, metoclopramide, EPO and a combination of prednisolone and cyclosporine, have been mentioned, but evaluations have not been provided.

Hematopoietic Stem Cell Transplantation

Chronic transfusion dependence with refractoriness to steroids is considered an acceptable indication for hematopoietic stem cell transplantation (HSCT). The outcomes of HSCT for DBA in Japan are better than those reported in other countries. To date, 19 patients have received transplants of allogeneic hematopoietic stem cells

(HSCs). Thirteen patients underwent BMT (six cases with HLA-matched siblings and seven cases with unrelated donors), and all have disease-free survival [33]. Five patients underwent cord blood transplantation (CBT), and two of these with transplants from siblings have survived. However, in three patients treated with unrelated donor cord blood, two were not successfully engrafted, and the third died of lymphoproliferative disease, despite confirmed engraftment. Consequently, bone marrow should be selected as a source of HSCs for transplantation at present. Better therapeutic outcomes have been observed with a conditioning therapy combining busulfan (oral administration, 16 mg/kg or 560 mg/m^2) and cyclophosphamide (120–200 mg/kg). Although the reported number of patients is few, a conditioning therapy using a half dose of busulfan leads to good outcomes when using unrelated HSCs transplantation. When busulfan was omitted from pretreatment, the number of cases with engraftment failure was modestly higher. There is not sufficient evidence supporting the value of bone marrow nondestructive pretreatment therapy for HSCT in DBA [34].

7.1.1.5 Future Challenge

Recent studies have provided fascinating insights into the pathogenesis of DBA. However, several important questions remain to be answered. For example, it is not clear how haploinsufficiency of ribosomal proteins leads to a phenotype of defective erythropoiesis and why mutations in some ribosomal protein genes such as *RPL5* have more profound impact on fetal development than mutations in other RP genes. Although corticosteroids remain the mainstay of treatment of DBA more than half a century after the original report of their efficacy, their mechanism of action is still unknown. DBA will necessarily lead us toward a further understanding of bone marrow failure syndrome and potentially to novel ways of treatment.

Recently, using DBA models in zebra fish and mice, it has been reported that administration of L-leucine, an essential amino acid, provided relief from anemia [35, 36]. Clinical trials to evaluate the therapeutic effects of L-leucine are underway in the United States. About half of DBA patients have not been identified causative mutations. Comprehensive genetic analysis using next-generation sequencing in the remaining DBA patients would be valuable in the identification of other causative gene mutations.

7.1.2 Fanconi's Anemia

7.1.2.1 Introduction

Fanconi's anemia (FA) is characterized by progressive bone marrow failure, congenital abnormalities, and a pronounced susceptibility for malignancy, including myelodysplastic syndrome (MDS), leukemia, and solid tumors such

as squamous cell carcinoma. FA was first reported by Dr. Fanconi in 1927 [37]. Lymphocytes from FA patients exhibit chromosomal instability [38], a characteristic that was increased by mitomycin C treatment [39]. Mutations in 19 different genes (FA genes) have been identified, the products of which cooperate with other gene products in a common pathway regulating DNA repair [40]. Stem cell transplantation is the only curative treatment for FA. In recent years, HSCT from alternative donors has become more popular and has been successfully employed [41, 42].

7.1.2.2   Epidemiology

About 5–10 cases are diagnosed with FA, and the incidence in Japan is estimated to be 5 in 1,000,000 newborns. The carrier frequency for FA in Japan may be 1 in 200–300 people [23].

7.1.2.3   Pathogenesis

FA is a multigenic disorder with 19 genes currently identified (*A*, *B*, *C*, *D1*, *D2*, *E*, *F*, *G*, *I*, *J*, *L*, *M*, *N*, *O*, *P*, *Q*, *R*, *S*, *and T*) [40]. Among them, *FANCT* was the first FA-causative gene discovered by a Japanese group [43]. *FANCD1*, *FANCJ*, and *FANCN* were identified as *BRCA2*, *BRIP1*, and *PALB2*, respectively. These three FA genes are associated with breast and ovarian cancer in heterozygotes. With the exception of the X-linked *FANCB* gene, the remaining 18 FA genes are autosomal recessive. The most common gene in FA is *FANCA* (60–70%), and more than 80% of FA patients have mutations in *FANCA* or *FANCG* in Japan. These FA proteins cooperate with other gene products, in a common pathway (FA-BRCA pathway) that regulates DNA repair (Fig. 7.2). The FA pathway plays an important role in the proliferation of hematopoietic stem cells and tumor suppression. The FANCD1 and FANCN subtypes initiate a malignant tumor in early childhood, and their prognosis is very poor. In contrast, clonal expansion of hematopoietic cells with reversion mosaicism can restore normal hematopoiesis [44].

Recent studies in mice have suggested that the FA proteins might counteract aldehyde-induced genotoxicity in hematopoietic stem cells [139, 140]. Acetaldehyde dehydrogenase 2 (ALDH2) primarily catalyzes the conversion of acetaldehyde to acetic acid; it also converts a range of endogenous toxic aldehydes. ALDH2 deficiency resulting from a Glu504Lys substitution (rs671, hereinafter referred to as the A allele) is highly prevalent in East Asian populations, including Japanese. The A allele (Lys504) acts as a dominant negative, since the variant form can suppress the activity of the Glu504 form (G allele) in GA heterozygotes by the formation of heterotetramers [45]. Individuals with the A variant experience flushing when drinking alcohol and have an elevated risk of esophageal cancer with habitual drinking. Compared to individuals with GG homozygotes, enzymatic activity is reduced by
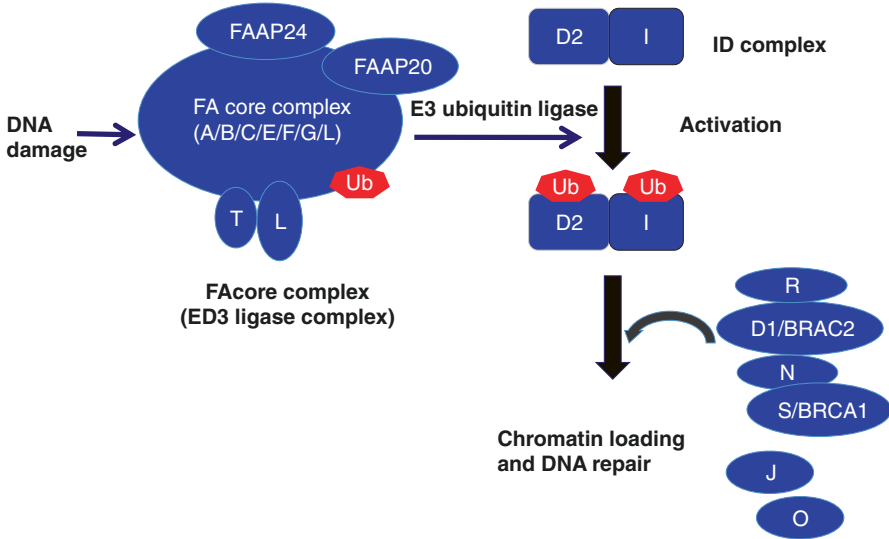
Fig. 7.2 Schematic of the Fanconi's anemia DNA repair pathway. After DNA damage, the FA core complex is activated, which then functions as an E3 ubiquitin ligase and monoubiquitinates FANCD2 and FANCI. The monoubiquitinated FANCD2/FANCI complex (ID complex) is then targeted to chromatin where it forms a complex with additional FA proteins and other DNA repair proteins

60–80% or nearly abolished (~4%) in GA or AA individuals, respectively [46]. Recently, Hira et al. showed that the presence of the A allele strongly accelerated progression of BMF in Japanese FA patients. Although *ALDH2* status did not influence the number of developmental abnormalities, malformations at some specific anatomic locations were observed more frequently in ALDH2-deficient patients [47]. These results indicate that the level of ALDH2 activity impacts the pathogenesis of FA.

7.1.2.4 Clinical Management

Clinical Presentation

Pancytopenia

Pancytopenia was the most common presentation, particularly when the red cell mean cell volume (MCV) and fetal hemoglobin (HbF) were elevated for age. Bone marrow biopsy examination most often showed hypocellularity for age, due to decreased numbers of hematopoietic precursors with normal morphology. The median age at diagnosis is 6.5 years, ranging from birth to adult. The diagnostic age in the reported cases was similar in both sexes [48].

Congenital Anomalies

Clinical manifestations of FA are variable. In about 60% of patients, FA is associated with physical anomalies and growth retardation, but in some patients, no congenital anomalies are found [48]. The most common characteristics at birth are skin hyperpigmentation, *café au lait* spots, short stature, abnormal thumbs, and radii [49].

Cancer Predisposition

Patients with FA are at a particularly high risk of developing MDS/AML and specific solid tumors at unusually young ages, including head, neck, esophageal, and gynecological squamous cell carcinoma. In the literature, about 15–20% of FA patients had hematologic neoplasms, and 5–10% had nonhematologic neoplasms [50, 51]. Of these neoplasms in Japanese FA patients, 33% were hematologic and 10.4% were nonhematologic [49]. Solid tumors were observed both after and before HSCT, and they developed between 1 and 39 years of age.

Diagnostic Procedures

Diagnostic Evaluation

FA is an autosomal recessive disorder associated with a high frequency of bone marrow failure, leukemia, and solid tumors. FA is a complex disease that can affect many systems of the body. Initially, patients are recognized when they have the combination of aplastic anemia and birth defects. However, current diagnostic criteria are more extensive and rely on demonstration of chromosomal aberrations in cells cultured with DNA cross-linking agents. The suspected diagnosis is usually confirmed by demonstration of chromosomal aberrations in blood lymphocytes cultured with a DNA cross-linking agent such as diepoxybutane (DEB) or mitomycin C (MMC) [48]. Following DNA damage, the complex of upstream FA gene products (A, B, C, E, F, G, I, L, M) leads to ubiquitination of the product of *FANCD2*, detected with a Western blot with a D2-specific antibody [52].

However, some FA patients have hematopoietic somatic mosaicism. These cases have corrected one mutated allele in a bone marrow stem cell, leading to an acquired heterozygosity in the blood cells. If diagnostic test results of blood are not conclusive and there is a high probability of FA, skin or bone marrow fibroblast cultures are required to demonstrate sensitivity to DNA cross-linking agents. Finally, FA mutation analysis determines the initial complementation group. Target next-generation sequencing can be performed to determine the relevant mutations.

Differential Diagnosis

Chromosome instability syndrome is a group of inherited conditions associated with chromosomal instability and breakage, and the following chromosome instability syndromes are known: xeroderma pigmentosum, ataxia-telangiectasia, Bloom syndrome, and Nijmegen syndrome.

Treatment

Transfusion Therapy

FA patients can be transfused to maintain minimal trough hemoglobin of 6 g/dL and minimal trough platelet of 5000/μL.

Drug Therapy

Patients who choose not to pursue transplant for severe cytopenias may benefit from treatment with androgens. Androgens can improve cytopenias in all three lineages, erythroid, myeloid, and platelets, but the effects are typically most pronounced for the erythroid lineage [48]. The effects of androgens were recognized in half of FA patients, but its effect seems to have lasted only temporarily [53]. Androgen may exacerbate the risk of liver tumors, and prior exposure to androgens is the one of the risk factors affecting survival after unrelated transplant [54]. Metenolone acetate is used in Japan. A clinical trial of danazol, which has less virilizing side effects, is currently under way. The use of steroid hormones should be avoided.

Hematopoietic Stem Cell Transplantation

Currently, the only cure for the hematological abnormalities of FA remains HSCT. The optimal timing of transplantation is challenging because the best outcomes are achieved prior to the development of complications such as infections from chronic severe neutropenia, high transfusion burden to treat anemia/thrombocytopenia, and the development of MDS or AML. Patients with FA are exquisitely sensitive to genotoxic agents such as cyclophosphamide, busulfan, and ionizing radiation. FA patients are also susceptible to the damaging inflammatory side effects of graft-versus-host disease. The use of high-dose cyclophosphamide (CY) and radiation in preparative regimens for FA patients often resulted in excessive organ toxicity and death. Therefore, efforts have focused on reducing the doses required for transplant preparative regimens, choosing nongenotoxic regimens to prevent graft-versus-host disease and using alternative conditioning regimens [48]. The use of low-dose CY (20–40 mg/kg) combined with 4–6 Gy of thoracoabdominal irradiation (TAI) or total body irradiation (TBI) reduced procedure toxicity and improved the outcome for FA patients transplanted from HLA-matched sibling donors [55]. Non-radiation regimens have been increasingly used for FA patients to reduce the secondary malignancies associated with radiation [56, 57].

A significant proportion of FA patients undergoing HSCT can now be dramatically cured, even in the absence of an HLA-identical sibling, especially if the conditioning regimen includes fludarabine [42, 58]. In Japanese FA patients transplanted using a fludarabine regimen, the 3-year estimate of overall survival (OS) for eight patients was 100% when the donor was an HLA-matched sibling [59], and 26 patients out of 27 patients transplanted from an alternative donor are alive [42]. It is essential to test all potential sibling donors for FA regardless of clinical findings since the phenotypic variation even within a given family is broad.

HSCT does not correct the nonhematological manifestations of Fanconi's anemia. Solid tumor risk, particularly head and neck squamous cell carcinoma, continues to increase after transplant, particularly in FA patients experiencing severe graft-versus-host disease [60]. A retrospective study comparing solid tumor risks in transplanted versus non-transplanted FA patients reported a 4.4-fold higher age-specific hazard rate of squamous cell carcinoma in patients treated by transplantation. The tumors appeared at an earlier age in the transplanted cohort [61]. Data on solid tumor risks in patients transplanted with the newer regimens are as yet limited.

7.1.2.5   Future Challenges

Patients with FA usually are diagnosed in childhood, and their registrations have been managed in the Japanese Society of Pediatric Hematology/Oncology in Japan. Although the adult FA population is small, it is important to understand transplanted/non-transplanted adult FA patients, who have or have not developed bone marrow failure, hematological malignancy, and solid tumors. Fludarabine-based preconditioning regimen can be used satisfactorily in alternative HSCT for FA. However, long-term observation of secondary cancers and other late effects will be required to determine the therapeutic utility of this approach. A recent report showed endogenously produced aldehydes impact the severity of FA, suggesting the possibility of a novel therapeutic approach [47]. For example, Alda-1 can stimulate the enzymatic activity of both the normal and variant ALDH2 [62]. ALDH2 agonists such as Alda-1 might be a novel protective drug against BMF in FA patients.

7.2   TAM

7.2.1   Introduction

Trisomy 21, the genetic hallmark of Down syndrome (DS), is the most frequent human chromosomal abnormality. In Japan, the incidence in the general population is 1 in 600–800 live births. Neonates with DS are at a high risk of developing a hematologic disorder referred to as transient abnormal myelopoiesis (TAM), which is characterized by the rapid growth of abnormal blast cells expressing megakaryocytic markers [63]. It has been estimated that 5–10% of infants with DS exhibit the disorder, and in most cases, it resolves spontaneously within 3 months. However, approximately 20% of severe cases are subject to fatal complications, and 20–30% of patients who escape from early death develop AMKL referred to as myeloid leukemia of DS (ML-DS) within the first 4 years of life [63–66].

Human tumors have been shown to progress by the accumulation of genetic abnormalities [67]. The malignant progression from TAM to AMLK offers a unique model to study the stepwise developments of cancer pathogenesis. The blasts in TAM are indistinguishable from true leukemic cells in both morphology and
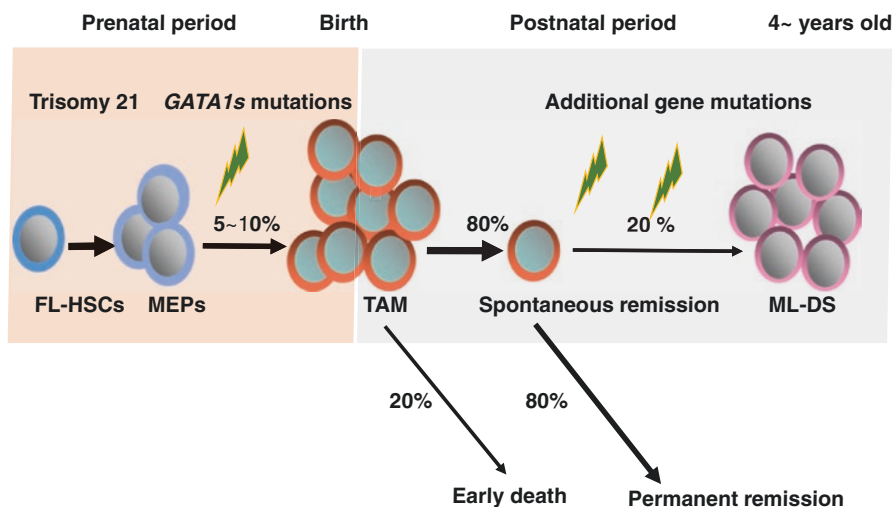
Fig. 7.3 Natural history and pathogenesis of TAM and ML-DS. Trisomy 21, the genetic hallmark of Down syndrome (DS), is the most frequent human chromosomal abnormality. Five to 10% of neonates with DS develop transient abnormal myelopoiesis (TAM), which is characterized by rapid growth of abnormal megakaryoblastic cells. Almost all cases of TAM have mutations in *GATA1*. In most cases, it resolves spontaneously within 3 months. However, early death occurs in about 20% of the cases. Furthermore, about 20% of patients develop myeloid leukemia of DS (ML-DS) within 4 years of life after acquisition of additional mutations in the genes encoding multiple cohesion components, CTCF, and epigenetic regulations such as EZH2, as well as genes encoding common signaling pathways, such as JAK family kinases and Ras pathway genes. FL-HSCs: Hematopoietic stem cells in fetal livers, MEPs: Megakaryocyte-erythroid progenitors

expressed surface markers. Blast cells in most patients with TAM as well as those with ML-DS have mutations in the gene encoding the transcription factor GATA1 [68–74], which is essential for normal development of erythroid and megakaryocytic cells [75–77]. Recent reports showed that TAM is caused by a single *GATA1* mutation and constitutive trisomy 21, and subsequent AMKL evolves from a preexisting TAM clone through the acquisition of additional mutations in genes coding for cohesin components, CCCTC-binding factor (CTCF), epigenetic regulators, as well as tyrosine kinases/Ras pathway genes (Fig. 7.3) [78]. In this chapter, advances in the pathobiology and clinical management of TAM will be described.

7.2.2   Epidemiology

The actual incidence of TAM has been difficult to define. Estimates of the frequency of TAM vary from 3.8 to 30% of neonates with DS, depending on the diagnostic and eligibility criteria used in each study and the sensitivity of the methodologies used for screening [66, 79–81]. Zipursky et al. screened a small series of DS neonates by

microscopic examination of blood films and found that 10% had blasts in the periph-eral blood [66]. It is possible that some cases with relatively few blasts would have been missed. Pine et al. screened Guthrie cards from 590 DS infants for *GATA1* mutations by single-strand conformation polymorphisms (SSCP) and direct sequencing of PCR products and showed that 3.8% of the infants harbored *GATA1* mutations [80]. A recent prospective study of 200 DS neonates showed an 8.5% frequency of *GATA1* mutations, using a combination of standard Sanger sequencing with direct high-performance liquid chromatography (Ss/HPLC) [81]. All of these patients had more that 10% blasts in the peripheral blood. Furthermore, low abun-dant minor *GATA1*-mutant clones were detected in 20.4% of DS neonates without detectable *GATA1* mutant clones with Ss/HPLC, by more sensitive targeted next-generation sequencing that can detect as few as 0.3% TAM blasts. They referred to these patients as "silent TAM." This study indicates approximately 30% of DS neonates have *GATA1* mutations [81].

7.2.3   Pathogenesis

7.2.3.1   *GATA1* Mutations in TAM and ML-DS

Cell differentiation is controlled in part by cell lineage-restricted transcription fac-tors. The transcription factor GATA1 is expressed within the hematopoietic hierar-chy in erythroid, megakaryocytic, eosinophilic, and mast cell lineages [82–85], as well as in Sertoli cells of the testis [86, 87]. Gene targeting experiments have revealed that GATA1 is required for the terminal differentiation of definitive erythroid and megakaryocytic cells [88–90].

GATA1 mRNAs are abundantly expressed in blast cells from TAM patients, as well as ML-DS patients [91]. Almost all patients with TAM and ML-DS have *GATA1* mutations [68–74]. The mutations lead to a loss of expression of the full-length GATA1 protein and the expression of a truncated GATA1 protein (GATA1s) that lacks the N-terminal transactivation domain. GATA1s, which is translated from the second methionine at codon 84, retains intact zinc finger regions, binds appro-priately to the GATA consensus sequence, and interacts normally with its essential cofactor FOG-1.

Several cell culture-based rescue experiments have reported that the N-terminal activation domain appears to be dispensable during erythroid and megakaryocytic cell differentiation [92–95]. Furthermore, distinct regions in the N-terminus of GATA1, which regulates the proliferation of immature megakaryocytic progenitors, are required for terminal megakaryocyte differentiation and controlling the growth of immature precursors [96, 97]. GATA1, but not GATA1s, interacts physically with the transcription factor E2F, which can trigger reentry into the cell cycle. That observation suggests that the failure of GATA1s to repress E2F activity in cellular transformation of fetal progenitors may be caused by this failure in direct or indirect interaction [98]. Supporting this hypothesis, Toki et al. recently discovered novel

GATA1 mutants in TAM that lack just the E2F interaction domain in the N-terminus of GATA1 [99].

On the other hand, mice harboring a heterozygous *GATA1* knockdown allele frequently develop erythroblastic leukemia [100]. These observations indicate that the expression level of GATA1 is crucial for the proper development of erythroid and megakaryocytic cells and that compromised GATA1 expression is a causal factor in leukemia [101]. Recently, Kanezaki et al. showed that the spectrum of transcripts derived from the mutant genes affects GATA1s protein expression. Mutations resulting in low GATA1s levels are significantly associated with a risk of progression to ML-DS [102]. However, a subsequent report contended that the *GATA1* mutational spectrum did not differ between TAM and AMKL and that the type of *GATA1* sequence mutation is not a reliable tool and is not prognostic of which patients with TAM are likely to develop ML-DS [103]. To resolve this problem, a prospective study with a large series of TAM patients is necessary.

7.2.3.2 The Target Cells of TAM and ML-DS

TAM is a disorder found mainly in patients with DS during the newborn period and is sometimes associated with liver fibrosis, which usually takes a lethal course. Based on pathological observation of TAM cases with fatal liver fibrosis, Miyauchi et al. have proposed a hypothesis that TAM blasts originate from fetal liver hematopoietic progenitors [104]. Analyses of *GATA1* mutations provide direct evidence for this hypothesis. The presence of identical *GATA1* mutations in the blasts of both TAM and ML-DS in an identical twin suggested that *GATA1* mutations occur early during prenatal hematopoiesis [105]. *GATA1* mutations were detected in hematopoietic tissues from DS fetuses that had no pathological evidence of leukemia [106] and in Guthrie newborn screening cards at birth from DS infants who later developed ML-DS [107].

In addition, mice expressing GATA1s presented with hyperproliferation of yolk sac and fetal liver progenitors [108]. Consistent with this finding, GATA1-deficient mice rescued with transgenic expression of GATA1s exhibited hyper-megakaryopoiesis during a limited embryonic and postnatal period, resembling the phenotype in human TAM cases [109]. These data from mouse experiments also indicated the possibility that fetal megakaryocytic progenitors are the target cells of TAM and ML-DS.

7.2.3.3 The Essential Roles of Trisomy 21 in Leukemogenesis of DS

For the following reasons, trisomy 21 is thought to be the first genetic event in the development of ML-DS. First, TAM occurs almost exclusively in patients with DS [66]. Second, when TAM occurs in patients with mosaic trisomy 21, the TAM clones involve only the cells with trisomy 21 [110]. Third, an inherited mutation in

humans leading to production of only GATA1s is associated with impaired erythro-poiesis and granulopoiesis, but does not promote leukemia in the absence of trisomy 21 [111]. Fourth, trisomy 21 itself is associated with enhanced expansion of human fetal erythroid and megakaryocytic precursors, independent of *GATA1* mutations [112, 113].

The mechanisms by which trisomy 21 predisposes individuals for the development of acute leukemia are thought to involve increased expression of a gene, or genes, on chromosome 21 that stimulate abnormal proliferation of hematopoietic stem cells in infancy [114]. A recent genotype-phenotype study in 30 DS individuals with rare segmental trisomy 21 led to the identification of a critical region of 8.35 Mb that is likely to contribute to increased risk of developing TAM and AMKL [115]. This region includes the *RUNX1*, *DYRK1A*, *ERG*, and *ETS* genes.

ERG and *ETS2*

Both ERG and ETS2 transcription factors belong to the ETS family. ERG is expressed in TAM and AMKL in DS, and its forced expression in erythroleukemia cells causes a phenotypic shift toward the megakaryocytic lineage. ERG binds the hematopoietic enhancer of *SCL/TAL1* that regulates its expression in hematopoietic stem cells [116]. SCL1/TAL1 overexpression is known to force progenitor cells toward the megakaryocytic lineage. Ectopic expression of *ERG* in fetal hematopoietic progenitors promotes megakaryopoiesis, and *ERG* alone acts as a potent oncogene *in vivo* leading to rapid onset of leukemia in mice. Furthermore, ERG also strongly cooperates with GATA1s to immortalize megakaryocytic progenitors [117]. Overexpression of *ETS2* and *ERG* immortalizes GATA1 knock-down and GATA1s knockin, but not wild-type fetal liver progenitors. Immortalization is accompanied by activation of the JAK-STAT pathway, commonly seen in megakaryocytic malignancies [118]. These results indicate that there is a specific synergy between loss of full-length GATA1 and overexpression of ETS family members in the control of self-renewal of megakaryocyte progenitors.

RUNX1

Both GATA1 and GATA1s are associated with the RUNX1 transcription factor [119], which cooperates with GATA1 during megakaryocyte differentiation [120]. Analysis using a transchromosomic system, in which mouse embryonic stem cells (ESCs) carried an extra human chromosome 21, showed that trisomy 21 causes hyperproduction of multipotential immature hematopoietic precursors, accompanied by increased expression of GATA2, c-Kit, and TIE-2. A panel of partial trisomy 21 ESCs, which was mapped by 3.5 kbp-resolution tiling arrays, helped to identify two different, nonoverlapping regions related to abnormal hematopoiesis. A human-specific siRNA silencing experiment revealed that an extra copy of

RUNX1, but not *ERG* or *ETS2*, raised the levels of TIE-2/c-Kit [121]. Interestingly, the expression levels of c-KIT and SCF/KIT signaling are significantly increased in primary TAM blasts [122].

A recent study with induced pluripotent stem cell (iPSC) lines demonstrated synergistic interaction of trisomy 21 and *GATA1* mutations in hematopoietic abnormalities. Banno et al. established systematic TAM models using human iPSCs and genome/chromosome-editing technologies. They found that trisomy 21 promoted expansion of early hematopoiesis. Furthermore, GATA1s is upregulated by trisomy 21, leading to aberrant megakaryopoiesis [123]. Assays for expression of chromosome 21 genes in trisomic and disomic iPSC-derived CD34$^+$CD38$^-$ cells identified a group of genes with relatively higher expression levels in trisomic iPSCs. These genes included *RUNX1*, *ETS2*, and *ERG*, which are clustered within a 4 Mb region on chromosome 21. Notable, trisomy of the 4 Mb region is critical to the perturbation of megakaryocyte development via GATA1s upregulation. Knockout of either *RUNX1*, *ETS2*, or *ERG* genes in a single chromosome revealed that the loss of one copy of the *RUNX1* gene canceled the accelerating effect on early hematopoiesis, and the increased gene dosage of *RUNX1*, *ETS2*, and *ERG* perturbed megakaryocyte differentiation synergistically via GATA1s upregulation.

DIRK1A

Malinge et al. recently used a mouse model of three oncogenic events to show that trisomy of 33 gene orthologs of human chromosome 21, a *GATA1* mutation, and an *MPL* mutation were sufficient to induce ML-DS in vivo. Furthermore, functional screening of the trisomic genes identified *DYRK1A*, which encodes dual-specificity tyrosine-phosphorylation-regulated kinase 1A, as a potent megakaryoblastic tumor-promoting gene that contributes to leukemogenesis through dysregulation of nuclear factor of activated T cells (NFAT) activation [124].

7.2.3.4 Additional Genetic Events During the Progression from TAM to AMKL

It has been proposed that other genetic alterations must be acquired in addition to the *GATA1* mutation for progression from TAM to ML-DS. Available evidence indicates that acute leukemia could arise from cooperation between one class of mutations that interferes with differentiation, such as loss-of-function mutations in hematopoietic transcription factors, and a second class of mutations that confers a proliferative advantage to cells, such as activating mutations in the hematopoietic tyrosine kinases [125]. Indeed, mutations in *JAK1*, *JAK2*, *JAK3*, *FLT3*, and *TP53* were reported in some ML-DS patients [65, 126–130]. However, the possible presence of additional mutations in ML-DS remains unknown in most cases.

Recently, Yoshida et al. reported genomic profiling of 41 TAM, 49 ML-DS, and 19 non-DS-AMKL samples, including whole-genome and/or exome-sequencing of 15 TAM and 14 ML-DS samples. Non-silent mutations in TAM blasts are primarily limited to the *GATA1* gene. In contrast, ML-DS blasts carry a higher burden of mutations, with frequent mutations of cohesin components (53%), *CTCF* (20%), *EZH2*, *KANSL1*, and other epigenetic regulators (45%), as well as common signaling pathways, such as the *JAK* family kinases, *MLP*, *SH2B3* (*LNK*), and multiple *RAS* pathway genes (47%) (Fig. 7.2) [78].

Cohesin is a multiprotein complex consisting of four core components, including the SMC1, SMC3, RAD21, and STAG proteins. In concert with functionally associated proteins such as NIPBL, cohesin is engaged in sister chromatids cohesion, post-replicative DNA repair, and transcriptional regulation. CTCF is a zinc finger protein implicated in long-range regulation of gene expression in collaboration with cohesin. Importantly, all mutations and deletions in different cohesin components were mutually exclusive, suggesting that cohesin function was the common target of these mutations.

Genes commonly observed with chromatin modification were frequently mutated in ML-DS. Especially, *EZH2*, which encodes a catalytic subunit of the polycomb repressive complex 2 (PRC2), was another recurrent mutational target in ML-DS. The frequency of the mutations was 33%, which is much higher than that in other hematological malignancies. In erythroid cells, PRC2 is involved in epigenetic silencing of a subset of GATA1-repressed genes, such as *KIT* and *GATA2*.

Collectively, these findings support a multistep leukemogenesis model whereby TAM is caused by a single *GATA1* mutation and constitutive trisomy 21, and subsequent AMKL evolve from a preexisting TAM clone through the acquisition of additional mutations (Fig. 7.3).

7.2.4 Clinical Management

7.2.4.1 Clinical Presentation

TAM presents in a wide variety of ways, from asymptomatic alterations in the blood counts to fulminant hepatic fibrosis. Recent prospective and retrospective studies revealed a median age at diagnosis of TAM is 3–7 days [131–133]. About 10–25% of TAM patients are asymptomatic at presentation. In symptomatic infants, hepatomegaly, splenomegaly, pleural/pericardial effusions, ascites, jaundice, and bleeding diatheses (bruising, petechiae, or bleeding) are most common findings, whereas hepatic fibrosis, hydrops fetalis, renal dysfunction/failure, and rash are less common. Patients may develop liver fibrosis due to blast cell infiltration that can cause fulminant liver failure and early death. Smrcek et al. reported that 11 of 79 (14%) patients with DS had fetal hydrops, and three of these patients (27%) presented with hepatosplenomegaly and TAM in the second and third trimesters [134].

7.2.4.2 Diagnostic Procedures

Generally accepted diagnostic criteria for TAM have not been established. Each study group uses its own criteria [79, 131]. For example, according to the Japan Pediatric Leukemia/Lymphoma Study Group (JPLSG), diagnosis of TAM is made if infants with trisomy 21 or mosaic trisomy 21 are up to 90 days at presentation with blasts in the peripheral blood or if infants with normal karyotype are up to 90 days at presentation with TAM-like blasts in the peripheral blood, which have trisomy 21 and *GATA1* mutations.

Myeloid blasts in the peripheral blood with megakaryoblastic features are diagnostic hallmarks of TAM. The blasts express variable combinations of CD33, CD41, CD42b, CD117, CD7, CD34, CD45, and CD56. However, a recent study showed that about 98% of DS neonates have circulating blasts [81], suggesting that it is very difficult to diagnose TAM morphologically. The most reliable method to identify TAM blasts is the detection of a *GATA1* mutation. We recommend direct sequencing analysis by using complementary DNA (cDNA) prepared from total RNA extracted from peripheral blood cells, and then confirmation of the results using genomic DNA. Sequence analysis of cDNA for the *GATA1* mutation is more sensitive than that of genomic DNA because only TAM blasts express *GATA1* in the peripheral blood. Recently, Roberts showed that targeted next-generation sequencing (NGS) can detect *GATA1* mutation with a sensitivity of 0.3% and that the estimated frequency of TAM is around 30% in DS neonates [81].

7.2.4.3 Treatment

Most neonates with TAM do not need treatment. The TAM neonates without severe disease can be safely monitored without treatment since their outcome is favorable [79]. The Pediatric Oncology Group conducted the first prospective study of TAM. Three children with life-threatening disease received low-dose cytosine arabinoside (LDCA) (0.4–1.5 mg/kg every 12 h for 5 or 7 days). The disease resolved in all of the patients [135]. In the BFM trial, chemotherapy with LDCA has a beneficial effect on the outcome of those children with risk factors for early death. LDCA (0.5–1.5 mg/kg for 3–12 days) improved the 5-year event-free survival from 28 to 52%. The main aim of treating high risk TAM patients is to immediately improve life-threatening features of TAM. Treatment of TAM did not alter the risk of developing subsequent ML-DS [131].

7.2.4.4 Prognostic Factors

Prospective and retrospective studies from the United States, Germany, and Japan confirmed that TAM is not a benign disease, as early death was reported in 15–20% of patients [131–133]. Risk factors for early death include high white blood cell (WBC) count, preterm delivery, bleeding diatheses, failure of spontaneous remission,

increased bilirubin, and liver enzymes. Recent prospective study (TAM-10) of the Japan Pediatric Leukemia/Lymphoma Study Group (JPLSG) confirmed that a high WBC count and effusion are risk factors for early death (unpublished data).

7.2.5   Future Challenges

Recent studies have provided fascinating insights into the pathogenesis of TAM and AMKL in DS. Most important is the discovery of acquired mutations in *GATA1* in TAM and additional genetic events during TAM to AMKL progression. Furthermore, several candidate genes on chromosome 21 that may cooperate with GATA1s have been identified. However, there are still many important questions to be answered. For example, why do *GATA1s* mutations occur to such a high degree exclusively in children with DS? What is the true incidence of TAM? What are the epigenetic events that occur subsequent to *GATA1* mutations that cause malignant transformation from TAM to ML-DS?

Population-based study with more sensitive methods for *GATA1* mutation screening will clarify the true incidence of TAM in DS and risk factors that predict malignant transformation from TAM to AMKL. Studies of ML-DS have now begun to lead us toward a fuller understanding of acute myeloid leukemia in children and adults and potentially to novel ways of prevention and treatment of this devastating disorder.

References

1. Ito E, et al. Molecular pathogenesis in Diamond-Blackfan anemia. Int J Hematol. 2010;92:413–8.
2. Lipton JM, et al. Improving clinical care and elucidating the pathophysiology of Diamond Blackfan anemia: an update from the Diamond Blackfan Anemia Registry. Pediatr Blood Cancer. 2006;46:558–64.
3. Vlachos A, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol. 2008;142:859–76.
4. Vlachos A, et al. Incidence of neoplasia in Diamond Blackfan anemia: a report from the Diamond Blackfan Anemia Registry. Blood. 2012;119:3815–9.
5. Diamond LK, Blackfan KD. Hypoplastic anemia. Am J Dis Child. 1938;56:464–7.
6. Josephs HW, et al. Anaemia of infancy and early childhood. Medicine. 1936;15:307.
7. Cmejla R, et al. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Hum Mutat. 2007;28:1178–82.
8. Doherty L, et al. Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. Am J Hum Genet. 2010;86:222–8.
9. Draptchinskaia N, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nat Genet. 1999;21:169–75.
10. Farrar JE, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. Blood. 2008;112:1582–92.

11. Gazda HT, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. Am J Hum Genet. 2006;79:1110–8.
12. Gazda HT, et al. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. Am J Hum Genet. 2008;83:769–80.
13. Gazda HT, et al. Frameshift mutation in p53 regulator RPL26 is associated with multiple physical abnormalities and a specific pre-ribosomal RNA processing defect in Diamond-Blackfan anemia. Hum Mutat. 2012;33:1037–44.
14. Gerrard G, et al. Target enrichment and high-throughput sequencing of 80 ribosomal protein genes to identify mutations associated with Diamond-Blackfan anaemia. Br J Haematol. 2013;162:530–6.
15. Konno Y, et al. Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia. Haematologica. 2010;95:1293–9.
16. Mirabello L, et al. Whole-exome sequencing and functional studies identify RPS29 as a novel gene mutated in multi-case Diamond-Blackfan anemia families. Blood. 2014;124:24–32.
17. Wang R, et al. Loss of function mutations in RPL27 and RPS27 identified by whole-exome sequencing in Diamond-Blackfan anemia. Br J Haematol. 2015;168(6):854–64.
18. Narla A, Ebert BJ. Ribosomopathies: human disorders of ribosome dysfunction. Blood. 2010;115:3196–205.
19. Ohga S, et al. Diamond-Blackfan anemia in Japan: clinical outcomes of prednisolone therapy and hematopoietic stem cell transplantation. Int J Hematol. 2004;79:22–30.
20. Ball SE, et al. Diamond-Blackfan anaemia in the U.K.: analysis of 80 cases from a 20-year birth cohort. Br J Haematol. 1996;94:645–53.
21. Ramenghi U, et al. Diamond-Blackfan anaemia in the Italian population. Br J Haematol. 1999;104:841–8.
22. Willig TN, et al. Identification of new prognosis factors from the clinical and epidemiologic analysis of a registry of 229 Diamond-Blackfan anemia patients. DBA group of Societe d'Hematologie et d'Immunologie Pediatrique (SHIP), Gesellschaft fur Padiatrische Onkologie und Hamatologie (GPOH), and the European Society for Pediatric Hematology and Immunology (ESPHI). Pediatr Res. 1999;46:553–61.
23. Ohara A. Japan National Registry of aplastic anemia in children 1998–2005: clinical features and prognosis. Jpn J Pediatr Hematol. 2008;22:53–62.
24. Willig TN, et al. Mutations in ribosomal protein S19 gene and Diamond Blackfan anemia: wide variations in phenotypic expression. Blood. 1999;94:4294–306.
25. Farrar JE, et al. Ribosomal protein gene deletions in Diamond-Blackfan anemia. Blood. 2011;118(26):6943–51.
26. Kuramitsu M, et al. Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia. Blood. 2012;119:2376–84.
27. Choesmel V, et al. Impaired ribosome biogenesis in Diamond-Blackfan anemia. Blood. 2007;109:1275–83.
28. Choesmel V, et al. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in a ribosome biogenesis disorder. Hum Mol Genet. 2008;17:1253–63.
29. Flygare J, et al. Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. Blood. 2007;109:980–6.
30. Leger-Silvestre I, et al. Specific role for yeast homologs of the Diamond Blackfan Anemia-associated Rps19 protein in ribosome synthesis. J Biol Chem. 2005;280:38177–85.
31. Fumagalli S, et al. Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rpL11-translation-dependent mechanism of p53 induction. Nat Cell Biol. 2009;11:501–8.
32. Utsugisawa T, et al. Erythrocyte glutathione is a novel biomarker of Diamond-Blackfan anemia. Blood Cell Mol Dis. 2016;59:31–6.
33. Mugishima H, et al. Hematopoietic stem cell transplantation for Diamond-Blackfan anemia: a report from the Aplastic Anemia Committee of the Japanese Society of Pediatric Hematology. Pediatr Transplant. 2007;11:601–7.

34. Yabe H, et al. Allogeneic stem cell transplantation for Diamond-Blackfan anemia in Japan; a report from the Inborn Errors Working Group of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Bone Marrow Transplant. 2013;48(suppl):s152.

35. Jaako P, et al. Dietary L-leucine improves the anemia in a mouse model for Diamond-Blackfan anemia. Blood. 2012;120:2225–8.

36. Payne EM. L-Leucine improves the anemia and developmental defects associated with Diamond-Blackfan anemia and del (5q) MDS by activating the mTOR pathway. Blood. 2012;120:2214–24.

37. Fanconi G. Familial constitutional panmyelopathy, Fanconi's anemia. 1. Clinical aspects. Semin Hematol. 1967;4:233–40.

38. Schroeder TM, et al. Spontane chromosomenaberrationen bei familiarer panmyelopathie. Humangenetik. 1964;1:194–6.

39. Sasaki MS, Tonomura A. A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res. 1973;33:1829–36.

40. Michl J, et al. Interplay between Fanconi anemia and homologous recombination pathways in genome integrity. EMBO J. 2016;35(9):909–23.

41. de la Fuente J, et al. Non-TBI stem cell transplantation protocol for Fanconi anaemia using HLA-compatible sibling and unrelated donors. Bone Marrow Transplant. 2003;32:653–6.

42. Yabe H, et al. Allogeneic haematopoietic cell transplantation from alternative donors with a conditioning regimen of low dose irradiation, fludarabine and cyclophosphamide in Fanconi anemia. Br J Haematol. 2006;134:208–12.

43. Hira A, et al. Mutations in the gene encoding the E2 conjugating enzyme UBE2T cause Fanconi anemia. Am J Hum Genet. 2015;96(6):1001–7.

44. Soulier J, et al. Related articles, links abstract detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCS pathway. Blood. 2005;105:1329–36.

45. Crabb DW, et al. Genotypes for aldehyde dehydrogenase deficiency and alcohol sensitivity. The inactive ALDH2(2) allele is dominant. J Clin Invest. 1989;83(1):314–6.

46. Gross ER, et al. A personalized medicine approach for Asian-Americans with the aldehyde dehydrogenase 2*2 variant. Annu Rev Pharmacol Toxicol. 2015;55:107–27.

47. Hira A, et al. Variant ALDH2 is associated with accelerated progression of bone marrow failure in Japanese Fanconi anemia patients. Blood. 2013;122:3206–9.

48. Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. Blood Rev. 2010;24:101–22.

49. Yabe M. Diagnosis and treatment for Fanconi anemia. J Jpn Pediatr Soc. 2012;116:1205–12.

50. Alter BP. Cancer in Fanconi anemia, 1927–2001. Cancer. 2003;97:425–40.

51. Kulter DI, et al. 20-year perspective on the International Fanconi Anemia Registry. Blood. 2003;101:1249–56.

52. Shimamura A, et al. A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood. 2002;100(13):4649–54.

53. Shahidi N, Diamond L. Testosterone-induced remission in aplastic anemia of both acquired and congenital types. Further observations in 24 cases. N Engl J Med. 1961;264:953–67.

54. Guardiola P, et al. Outcome of 69 allogeneic stem cell transplantations for Fanconi anemia using HLA-matched unrelated donors. Blood. 2000;95:422–9.

55. Socie G, et al. Transplantation for Fanconi's anaemia: long-term follow-up of fifty patients transplanted from a sibling donor after low-dose cyclophosphamide and thoraco-abdominal irradiation for conditioning. Br J Haematol. 1998;193:249–55.

56. de Medeios CR, et al. Bone marrow transplantation for patients with Fanconi anemia : reduced doses of cyclophosphamide without irradiation as conditioning. Bone Marrow Transplant. 1999;24:849–52.

57. Socie G, et al. Increased incidence of solid malignant tumors after bone marrow transplantation for severe aplastic anemia. Blood. 1991;78:277–9.

58. Locatelli F, et al. The outcome of children with Fanconi anemia given hematopoietic stem cell transplantation and the influence of fludarabine in the conditioning regimen: a report from the Italian pediatric group. Haematologica. 2007;92:1381–8.
59. Yabe M, et al. Matched sibling donor stem cell transplantation for Fanconi anemia patients with T-cell somatic mosaicism. Pediatr Transplant. 2012;16:340–5.
60. Deeg HJ, et al. Malignancies after marrow transplantation for aplastic anemia after Fanconi anemia: a joint Seattle and Paris analysis of results in 700 patients. Blood. 1996;87:386–92.
61. Rosenberg PS, et al. Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood. 2005;105:67–73.
62. Chen CH, et al. Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. Science. 2008;321(5895):1493–5.
63. Hasle H, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003;17:277–82.
64. Hitzler JK. Acute megakaryoblastic leukemia in Down syndrome. Pediatr Blood Cancer. 2007;49:1066–9.
65. Malinge S, et al. Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in Down syndrome. Blood. 2009;113(12):2619–28.
66. Zipursky A, et al. Leukemia in Down syndrome. A review. Pediatr Hematol/Oncol. 1992;9:139–49.
67. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61:759–67.
68. Greene ME, et al. Mutations in GATA1 in both transient myeloproliferative disorder and acute megakaryoblastic leukemia of Down syndrome. Blood Cell Mol Dis. 2003;31:351–6.
69. Groet J, et al. Acquired mutations in GATA1 in neonates with Down's syndrome with transient myeloid disorder. Lancet. 2003;361:1617–20.
70. Hitzler JK, et al. GATA1 mutations in transient leukemia and acute megakaryoblastic leukemia of Down syndrome. Blood. 2007;101:4301–4.
71. Mundschau G, et al. Mutagenesis of GATA1 is an initiating event in Down syndrome leukemogenesis. Blood. 2003;101:4298–300.
72. Rainis L, et al. Mutations in exon 2 of GATA1 are early events in megakaryocytic malignancies associated with trisomy 21. Blood. 2003;102:981–6.
73. Wechsler J, et al. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. Nat Genet. 2002;32:148–52.
74. Xu G, et al. Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. Blood. 2003;102:2960–8.
75. Ferreira R, et al. GATA1 function, a paradigm for transcription factors in hematopoiesis. Mol Cell Biol. 2005;25:1215–27.
76. Gutierrez L, et al. Ablation of Gata1 in adult mice results in aplastic crisis, revealing its essential role in steady-state and stress erythropoiesis. Blood. 2008;111:4375–85.
77. Morceau F, et al. GATA-1: friends, brothers, and coworkers. Ann N Y Acad Sci. 2004;1030:537–54.
78. Yoshida K, et al. The landscape of somatic mutations in Down syndrome-related myeloid disorders. Nat Genet. 2013;45(11):1293–9.
79. Gamis AS, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. Blood. 2011;118:6752–9.
80. Pine SR, et al. Incidence and clinical implications of *GATA1* mutations in newborns with Down syndrome. Blood. 2007;110(21):28–2131.
81. Roberts I, et al. GATA1-mutant clones are frequent and often unsuspected in babies with Down syndrome: identification of a population at risk of leukemia. Blood. 2013;122:3908–17.
82. Martin DI, et al. Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages. Nature. 1990;344:444–7.

83. Romeo PH, et al. Megakaryocytic and erythrocytic lineages share specific transcription factors. Nature. 1990;344:447–9.
84. Tsai SF, et al. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature. 1989;339:446–51.
85. Zon LI, et al. Expression of mRNA for the GATA-binding proteins in human eosinophils and basophils: potential role in gene transcription. Blood. 1993;81:3234–41.
86. Ito E, et al. Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis. Nature. 1993;362:466–8.
87. Yomogida K, et al. Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. Development. 1994;120:1759–66.
88. Pevny L, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature. 1991;349:257–60.
89. Shivdasani RA, et al. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J. 1997;16:3965–73.
90. Takahashi S, et al. Role of GATA-1 in proliferation and differentiation of definitive erythroid and megakaryocytic cells in vivo. Blood. 1998;92:434–42.
91. Ito E, et al. Expression of erythroid-specific genes in acute megakaryoblastic leukaemia and transient myeloproliferative disorder in Down's syndrome. Br J Haematol. 1995;90:607–14.
92. Blobel GA, et al. Rescue of GATA-1-deficient embryonic stem cells by heterologous GATA-binding proteins. Mol Cell Biol. 1995;15:626–33.
93. Shimizu R, et al. In vivo requirements for GATA-1 functional domains during primitive and definitive erythropoiesis. EMBO J. 2001;20:5250–60.
94. Visvader JE, et al. The C-terminal zinc finger of GATA-1 or GATA-2 is sufficient to induce megakaryocytic differentiation of an early myeloid cell line. Mol Cell Biol. 1995;15:634–41.
95. Weiss MJ, et al. Erythroid-cell-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell line. Mol Cell Biol. 1997;17:1642–51.
96. Kuhl C, et al. GATA1-mediated megakaryocyte differentiation and growth control can be uncoupled and mapped to different domains in GATA1. Mol Cell Biol. 2005;25:8592–606.
97. Muntean AG, Crispino JD. Differential requirements for the activation domain and FOG-interaction surface of GATA-1 in megakaryocyte gene expression and development. Blood. 2005;106:1223–31.
98. Klusmann JH, et al. Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis. Genes Dev. 2010;24:1659–72.
99. Toki T, et al. Naturally occurring oncogenic GATA1 mutants with internal deletions in transient abnormal myelopoiesis in Down syndrome. Blood. 2013;121:3181–4.
100. Shimizu R, et al. Leukemogenesis caused by incapacitated GATA-1 function. Mol Cell Biol. 2004;24:10814–25.
101. Shimizu R, et al. GATA1-related leukaemias. Nat Rev Cancer. 2008;8:279–87.
102. Kanezaki R, et al. Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia. Blood. 2010;116:4631–8.
103. Alford KA, et al. Analysis of GATA1 mutations in Down syndrome transient myeloproliferative disorder and myeloid leukemia. Blood. 2011;118:2222–38.
104. Miyauchi J, et al. Unusual diffuse liver fibrosis accompanying transient myeloproliferative disorder in Down's syndrome: a report of four autopsy cases and proposal of a hypothesis. Blood. 1992;80:1521–7.
105. Shimada A, et al. Fetal origin of the *GATA-1* mutation in identical twins with transient myeloproliferative disorder and acute megakaryoblastic leukemia accompanying Down's syndrome. Blood. 2004;103:366.
106. Taub JW, et al. Prenatal origin of GATA1 mutations may be an initiating step in the development of megakaryocytic leukemia in Down syndrome. Blood. 2004;104:1588–9.

107. Ahmed M, et al. Natural history of GATA1 mutations in Down syndrome. Blood. 2004;103:2480–9.

108. Li Z, et al. Developmental stage-selective effect of somatically mutated leukemogenic transcription factor GATA1. Nat Genet. 2005;37:613–9.

109. Shimizu R, et al. Induction of hyperproliferative fetal megakaryopoiesis by an N-terminally truncated GATA1 mutant. Genes Cells. 2009;14:1119–31.

110. Zubizarreta P, et al. Transient myeloproliferative disorder associated with trisomy 21, a wide range syndrome: report of two cases with trisomy 21 mosaicism. Med Pediatr Oncol. 1995;25:60–4.

111. Hollanda LM, et al. An inherited mutation leading to production of only the short isoform of GATA-1 is associated with impaired erythropoiesis. Nat Genet. 2006;38:807–12.

112. Chou ST, et al. Trisomy 21 enhances human fetal erythro-megakaryocytic development. Blood. 2008;112:4503–6.

113. Tunstall-Pedoe O, et al. Abnormalities in the myeloid progenitor compartment in Down syndrome fetal liver precede acquisition of GATA1 mutations. Blood. 2008;112:4507–11.

114. Look AT. A leukemogenic twist for GATA1. Nat Genet. 2002;32:83–4.

115. Korbel JO, et al. The genetic architecture of Down syndrome phenotypes revealed by high-resolution analysis of human segmental trisomies. Proc Natl Acad Sci U S A. 2009;106:12031–6.

116. Rainis L, et al. The proto-oncogene ERG in megakaryoblastic leukemias. Cancer Res. 2005;65:7596–602.

117. Salek-Ardakani S, et al. ERG is a megakaryocytic oncogene. Cancer Res. 2009;69:4665–7463.

118. Stankiewicz MJ, Crispino JD. ETS2 and ERG promote megakaryopoiesis and synergize with alterations in GATA-1 to immortalize hematopoietic progenitor cells. Blood. 2009;113:3337–47.

119. Xu G, et al. Physical association of the patient-specific GATA1 mutants with RUNX1 in acute megakaryoblastic leukemia accompanying Down syndrome. Leukemia. 2006;20:1002–8.

120. Elagib KE, et al. RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood. 2003;101:4333–41.

121. De Vita S, et al. Trisomic dose of several chromosome 21 genes perturbs haematopoietic stem and progenitor cell differentiation in Down's syndrome. Oncogene. 2010;29:6102–14.

122. Toki T, et al. The key role of stem cell factor/KIT signaling in the proliferation of blast cells from Down syndrome-related leukemia. Leukemia. 2009;23:95–103.

123. Banno K, et al. Systematic cellular disease models reveal synergistic interaction of trisomy 21 and GATA1 mutations in hematopoietic abnormalities. Cell Rep. 2016;15:1228–4121.

124. Malinge S, et al. Increased dosage of the chromosome 21 ortholog Dyrk1a promotes megakaryoblastic leukemia in a murine model of Down syndrome. J Clin Investig. 2012;122:948–62.

125. Deguchi K, Gilliland DG. Cooperativity between mutations in tyrosine kinases and in hematopoietic transcription factors in AML. Leukemia. 2002;16:740–4.

126. Blink M, et al. Frequency and prognostic implications of JAK 1-3 aberrations in down syndrome acute lymphoblastic and myeloid leukemia. Leukemia. 2011;25:1365–8.

127. Kiyoi H, et al. JAK3 mutations occur in acute megakaryoblastic leukemia both in Down syndrome children and non-Down syndrome adults. Leukemia. 2007;21:574–6.

128. Malinge S, et al. Activating mutations in human acute megakaryoblastic leukemia. Blood. 2008;112:4220–6.

129. Sato T, et al. Functional analysis of JAK3 mutations in transient myeloproliferative disorder and acute megakaryoblastic leukemia accompanying Down syndrome. Br J Haematol. 2008;141:681–8.

130. Walters DK, et al. Activating alleles of JAK3 in acute megakaryoblastic leukemia. Cancer Cell. 2002;10:65–75.

131. Klusmann JH, et al. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. Blood. 2008;111(6):2991–8.

132. Massey GV, et al. A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's Oncology Group (COG) Study POG-9481. Blood. 2006;107(12):4606–13.
133. Muramatsu H, et al. Risk factors for early death in neonates with Down syndrome and transient leukaemia. Br J Haematol. 2008;142(4):610–5.
134. Smercek JM, et al. Fetal hydrops and hepatosplenomegaly in the second half of pregnancy: a sign of myeloproliferative disorder in fetuses with trisomy 21. Ultrasound Obstet Gynecol. 2001;17:403–9.
135. Al-Kasim F, et al. Incidence and treatment potentially lethal diseases in transient leukemia of Down syndrome: Pediatric Oncology Group Study. J Pediatr Hematol Oncol. 2002;24:9–13.
136. Ikeda F, et al. Exome sequencing identified as a novel causative gene for Diamond-Blackfan anemia. Haematologica. 2017;102(3):e93–e96.
137. Sankaran VG, et al. Exome sequencing identifies GATA1 mutations resulting Diamond-Blackfan anemia. J Clin Invest. 2012;122:2439–43.
138. Vlachos A, et al. Incidence of neoplasia in Diamond Blackfan anemia: a report from the Diamond Blackfan Anemia Registry. Blood. 2012;119:3815–19.
139. Langevin F, et al. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. Nature. 2011;475:53–8.
140. Garaycoechea J, et al. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature. 2012;489:571–5.

Part IV
Platelet and Coagulation Disorders

Chapter 8
Immune and Inherited Thrombocytopenia in Children

Masue Imaizumi

Abstract Immune thrombocytopenia (ITP), the most common isolated thrombocytopenia in children, is regarded collectively as various thrombocytopenic diseases commonly involved in immunological mechanisms. ITP children can be treated with multiple therapeutic modalities including novel biological agents. However, without available means of confirmative diagnosis yet, ITP more or less requires exclusion diagnosis. As recent investigation of inherited thrombocytopenic disorders has revealed that genetic thrombocytopenia is frequently overlooked, inherited thrombocytopenias (ITs) might become increasingly important as diseases of differential diagnosis. Misdiagnosis of ITP not only prevents IT patients from correct management but also exposes them to inappropriate and potentially harmful treatments. Therefore, correct diagnosis based on combined clinical examinations and gene analyses may become more necessary for appropriate management and treatment choice of children with thrombocytopenia.

8.1 Introduction

Immune thrombocytopenia (ITP), the most common isolated thrombocytopenia in children, is characterized by increased platelet destruction in the spleen and reticuloendothelial system and by impaired platelet production in the bone marrow [1, 2]. Formally, ITP stands for idiopathic thrombocytopenic purpura, which literally means that it has unknown cause. It therefore requires diagnosis based on exclusion of any definite disease. However, an accumulation of findings that include pre-existing infection or vaccination, effectiveness of splenectomy, and presence of antiplatelet antibodies and/or platelet antigen-responsive T cells has

M. Imaizumi
Department of Hematology and Oncology, Miyagi Children's Hospital,
4-3-17 Ochiai, Aoba-ku, Sendai 989-3126, Japan
e-mail: imaizumi@miyagi-children.or.jp

shed light on pathological mechanism(s) that are common both in ITP of unknown cause and thrombocytopenia associated with immunological diseases. Therefore, ITP is regarded not as a single disease but collectively as various thrombocytopenic diseases that are commonly involved in immunological mechanisms [1, 3].

As the categories of diseases covered by primary and secondary ITP have become more numerous, inherited thrombocytopenic disorders might become increasingly important as diseases of differential diagnosis [4]. Especially in cases of inherited thrombocytopenias (ITs) with inconspicuous characteristic features or findings other than thrombocytopenia, patients might be misdiagnosed as ITP patients who would show chronic course with inadequate therapeutic response to standard treatments for ITP. Moreover, recent advances in genetic analyses have increased knowledge of ITs, suggesting that genetic thrombocytopenia is frequently overlooked [5].

This article presents a review of recent findings related to clinical, diagnostic, and pathological aspects of childhood ITP as well as on those of inherited disorders with thrombocytopenia that should be examined as differential diagnoses of ITP.

8.2   Clinical Picture of ITP

ITP is representative of hemorrhagic disorders in children. With the help of the previous epidemiological studies [6, 7], the annual incidence of children with ITP is estimated at approximately 1000 children in Japan. However, the number of children with thrombocytopenia in the registry of the Japanese Society of Pediatric Hematology was 439 on an average annual basis from 2006 to 2009 [8], suggesting that ITP in children might be seen not only by pediatric hematologists but also by general pediatricians.

Figure 8.1 presents results of a population-based retrospective study conducted using the Japanese public health system disease registry [9]. Results show that the incidence of patients with ITP is associated closely with age and sex. Approximately 80% of children with ITP developed the disease before school age [10], although postadolescent patients tend to become more numerous gradually along with age. Three peaks of incidence are apparent across all age groups. The first is in children under 4 years of age, with boys predominant, the second in middle age with female predominance, and the third in elderly people.

Table 8.1 shows that major bleeding manifestations of children with ITP were purpura (92.6%) and nasal bleeding (29.7%), the frequency of which was significantly higher than that in adults with ITP. It is particularly interesting that intracranial hemorrhage, a severe complication, is extremely rare in children (0.1%), but less in adults (0.7%), suggesting an involvement of age-related physiological changes [10]. The clinical features at diagnosis, such as >10 years of age, $>2 \times 10^4/$ μL of platelet counts, and the absence of precedent infection or vaccination, are correlated significantly with the risk of transition to chronic ITP [11].
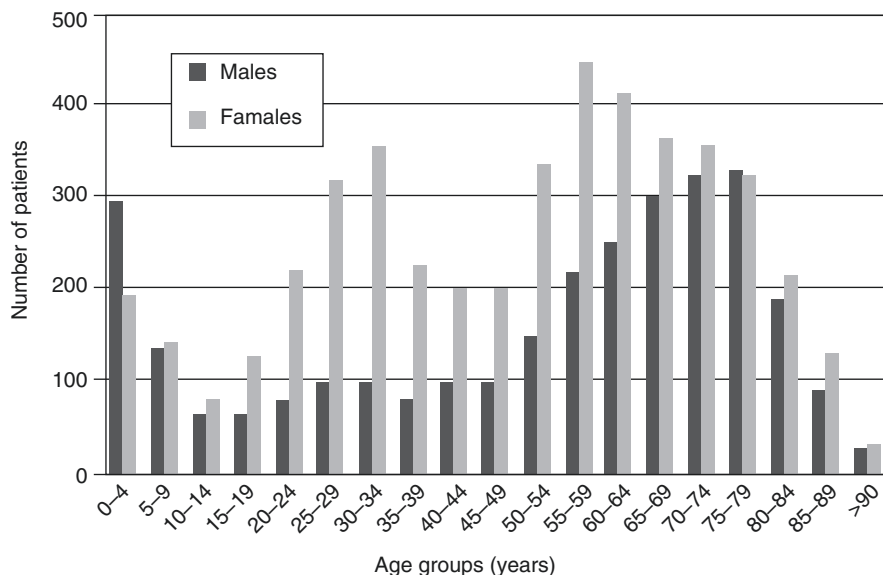
Fig. 8.1 Population-based epidemiological study of 7774 patients with ITP in a Japanese population [9]. A population of 7774 patients with ITP were analyzed retrospectively using the database registry of the Ministry of Health, Labour, and Welfare of Japan from 2004 to 2007

Table 8.1 Comparative study of hemorrhagic manifestations between pediatric and adult patients with ITP [9]

Hemorrhagic manifestations	Pediatric patients (%)	Adult patients (%)	p Value
Purpura	860 (92.6)	4300 (62.8)	$p < 0.001$
Gingival bleeding	175 (18.8)	1365 (19.9)	ns
Nasal bleeding	276 (29.7)	687 (10.0)	$p < 0.001$
Hematuria	54 (5.8)	453 (6.6)	ns
Melena	43 (4.6)	259 (3.8)	ns
Hypermenorrhea	11 (1.2)	264 (3.9)	$p < 0.001$
Intracranial hemorrhage	1 (0.1)	45 (0.7)	$p < 0.05$
Other bleedings	54 (5.8)	214 (3.1)	$p < 0.001$
Total	929	6845	

8.3   Immune-Mediated Mechanism(s) Causing Thrombocytopenia

Although clinical findings, such as frequent preceding infectious events and therapeutic effect of splenectomy, suggest the involvement of immunological mechanisms in the onset of ITP, it is also known that ITP children often lack direct evidence of immune-mediated mechanisms [1]. Recently, "a cryptic epitope model" in cellular and molecular pathogenesis has been presented for adult ITP, where

autoreactive CD4+ T cells that can recognize the cryptic peptides derived from platelets might promote peripheral B-cell production of antiplatelet GPIIb/IIa antibodies [12, 13].

Compared to ITP in adult, childhood ITP has clinical features that are often characterized by remission after a short duration and precedent events such as infection or vaccination, suggesting the background factors of physiologically and developmentally immature immunological regulation systems [14].

Not only peripheral platelets but also megakaryocytes in bone marrow can be targets of immunological responses attributable to the expression of GPIIb/IIIa molecules on the megakaryocytes, leading to impaired platelet production or aberrant megakaryopoiesis [15, 16].

8.4 International Standardization of Terminology, Definitions, and Outcome Criteria

The Japanese Society of Pediatric Hematology proposed conventional guidelines of diagnosis, treatment, and management for childhood ITP [17]. The guideline, in which ITP stands for idiopathic thrombocytopenic purpura of unknown causes, had been used for many years and prevailed in Japan. Recently, international standardization of terminology, definitions, and outcome criteria has been proposed and acknowledged around the country [3]. This standardization proposes ITP as "immune thrombocytopenia," which is not a single disease but comprising various diseases with thrombocytopenia commonly caused by immunological mechanisms.

Firstly, as shown in Table 8.2, the international standardization classifies ITP into two categories, primary and secondary ITP, in which the primary ITP corresponds to conventional ITP of unknown causes. The secondary ITP includes disorders showing thrombocytopenia, which are strongly associated with immunological pathogenesis, such as underlying immune-mediated diseases or drug-induced reactions. Consequently, several diseases that have been exclusive from conventional ITP can become inclusive of the secondary ITP, leading to increased importance of some inherited disorders with thrombocytopenia as diseases in differential diagnosis of ITP.

Secondly, the international standardization has introduced the concept of "phases of the disease (newly diagnosed, persistent, and chronic ITP)" in substitution of types of the disease (acute and chronic ITP) to avoid an uncertain type of disease at onset, for which confirmation by definition requires evaluation for a certain period. Moreover, it advises 12 months of observation until diagnosis of the chronic ITP to avoid excessive treatment for numerous patients who can have remission of thrombocytopenia within 12 months after onset. In addition, severe ITP is defined as the state with the presence of bleeding symptoms sufficient to

Table 8.2 Standardization of terminology, definitions, and outcome criteria in ITP [3]

A. Definitions of disease
Abbreviation of ITP: *i*mmune *t*hrombocyto*p*enia
Primary ITP
• Isolated thrombocytopenia (peripheral blood platelet count <10 × 10⁴/μL) in the absence of other causes or disorders that may be associated with thrombocytopenia
• The diagnosis of primary ITP remains one of exclusion; no robust clinical or laboratory parameters are currently available to establish its diagnosis with accuracy
• The main clinical problem of primary ITP is an increased risk of bleeding, although bleeding symptoms may not always be present
Secondary ITP
• All forms of immune-mediated thrombocytopenia except primary ITP
• Secondary forms of thrombocytopenia that are due to an underlying disease or to drug exposure. The name of the associated disease should follow the designation, for example, secondary ITP (SLE associated) or (HIV associated)
B. Phase of the disease
Newly diagnosed ITP: within 3 months from diagnosis
Persistent ITP: between 3 and 12 months from diagnosis, includes patients not reaching spontaneous remission or not maintaining complete response off therapy
Chronic ITP: lasting for more than 12 months
Severe ITP: presence of bleeding symptoms at presentation sufficient to mandate treatment or occurrence of new bleeding symptoms requiring additional therapeutic intervention with a different platelet-enhancing agent or an increased dose
C. Criteria for assessing response to ITP treatment
CR(complete response): platelet count ≥10 × 10⁴/μL and absence of bleeding
R(response): platelet count ≥3 × 10⁴/μL and at least twofold increase the baseline platelet count and absence of bleeding
NR(no response): platelet count <3 × 10⁴/μL or less than twofold increase of baseline count or bleeding
Loss of CR or R: platelet count below 10 × 10⁴/μL or bleeding (from CR) or below 3 × 10⁴/μL or less than twofold increase of baseline platelet count or bleeding (from R)
D. Refractory ITP (all need to be met)
• Failure to achieve at least R or loss of R after splenectomy
• Need of treatment(s) to minimize the risk of clinically significant bleeding. Need of on demand or adjunctive therapy alone does not qualify the patient as refractory
• *Primary ITP confirmed by excluding other supervened causes of thrombocytopenia*

mandate treatment or occurrence of new bleeding symptoms requiring additional therapeutic intervention.

Thirdly, the international standardization has proposed outcome criteria that define the conditions to evaluate therapeutic effects: CR, complete response; R, response; NR, no response; and refractory state of ITP. Although these criteria are apparently rather broad and simple, the consensus criteria for therapeutic evaluation and disease states are indispensable for comparative and cooperative investigations among different study groups.

8.5   Treatments [17, 18]

8.5.1   Conventional Treatments

Steroid and intravenous immunoglobulin (IVIG) are two major conventional drugs in the first-line therapy for children with ITP. Children with newly diagnosed ITP presenting with bleeding symptom and $<2 \times 10^4/\mu L$ of platelet counts are treated with steroids (2 mg/kg) or IVIG (1–2 g/kg), although a wait-and-see approach might be used for patients with minimal bleeding. Approximately, 70–80% of children with newly diagnosed ITP are likely to have complete response within 6–12 months after diagnosis. For children with chronic, but not severe ITP, close observation without treatment is a possible consideration in cases of minimal bleeding, even at $<2 \times 10^4/\mu L$ of platelet count. However, patients with severe ITP in chronic phase or refractory ITP need treatment including second-line therapy.

Conventional second-line treatment for severe or refractory ITP includes splenectomy and immunosuppressive agents such as cyclophosphamide, cyclosporin A, and dapsone (diaphenylsulfone). Although splenectomy is a reliable second-line therapy, its application to children has become less frequent since the introduction of new agents such as rituximab and thrombopoietin receptor (TPO-R) agonists in clinical areas. A review of these two therapeutic agents is presented below.

8.5.2   Rituximab

Rituximab is a chimeric monoclonal antibody that targets CD20 antigen on the surface of B cells. It is applied initially to B-cell lymphoma and, then, expanded to autoimmune diseases. In contrast to the wide use of rituximab in adult patients, few data are available for the long-term efficacy of rituximab for childhood ITP [19, 20]. Recently, a retrospective study in Japan of the long-term effects of rituximab for 22 children with refractory ITP [21] reported that the initial CR rate as high as 41% (9/22) decreased gradually to a 14% (3/22) relapse-free CR rate at 5 years after the first rituximab therapy. Consequently, although sustained effects of rituximab on children with refractory ITP might occur only with low probability, repeated rituximab administration might be a promising therapy because patients who have received multiple courses of rituximab after relapse responded each time without adverse effects and obtained remission.

8.5.3   TPO-R Agonists

Eltrombopag and romiplostim, TPO-R agonists in the second generation, are low-molecular compounds that stimulate signaling through TPO-R without inducing a neutralizing antibody against TPO. TPO-R agonists were effective and safe in

approximately 80% of adult patients with refractory ITP [22, 23]. These agents have two specific properties: a loss of effect after withdrawal and an increased risk of myelofibrosis. Although TPO-R agonists are effective also for children with refractory ITP, a retrospective study of children receiving TPO-R agonists for up to 53 months revealed that one of 24 patients developed myelofibrosis in grade 2 [24]. The guideline of the American Society of Hematology continues to advise a cautious attitude related to TPO-R agonists for children with ITP [25].

8.6 Inherited Thrombocytopenia

Recent advances of genetic investigation of inherited thrombocytopenias (ITs) have greatly enhanced the understanding of ITs at the molecular and genetic levels, leading to identification of new responsible genes and a better characterization of ITs [4]. To date, ITs constitute at least 22 disorders caused by mutations of 25 genes with different degrees of complexity in phenotypes and variation in prognosis (Table 8.3) [5]. Conventionally, ITs are classified using characteristic small-, normal-, or large-sized platelets, respectively. The platelet size is denoted either by the mean platelet diameter (upper limit of normal range, 4 μm) or by the mean platelet volume (MPV) (normal range, 7.2–11.1 fL). Recently, with greater emphasis on symptoms, Pecci has categorized ITs into two groups according to symptoms and organs involved by ITs: syndromic and non-syndromic forms [5].

Despite increasing knowledge of ITs, its diagnosis remains difficult, and these disorders are probably still underdiagnosed. In fact, several patients with ITs might have had a prior wrong diagnosis of ITP [5, 63]. Therefore, physicians should consider ITs during differential diagnosis of ITP in children, especially those who exhibit chronic or refractory ITP with small- or normal-sized platelets, positive familial history, or skeletal malformation. Not only clinical examinations, including familial history, but also gene analyses are invariably important to make a correct diagnosis of ITs.

For this article, among ITs with identified entities and gene mutations, we specifically review mainly ITs with small- or normal-sized platelets. ITs with small platelets are represented by diseases with mutation in *WAS* gene, whereas ITs with normal-sized platelets consist of various syndromic forms of diseases that entail complications such as skeletal anomaly or predisposition to malignant diseases.

8.6.1 *ITs with Small-Sized Platelets*

Wiskott–Aldrich syndrome (WAS), an X-linked primary immunodeficiency caused by mutations of the *WAS* gene, is clinically characterized as having small-sized thrombocytopenia, increased susceptibility to infection, and refractory eczema

Table 8.3 Main clinical features of inherited thrombocytopenia

Disease (abbreviation, OMIM entry)	Inheritance	Gene (locus)	Platelet size	Bleeding[a]	Main clinical feature	Reference
Syndromic forms						
MYH9-related disease (MYH9-RD, na)	AD	MYH9 (22q12)	Large	A to Mi	Sensorineural deafness, nephropathy, cataract, and/or elevated liver enzymes. Giant platelets. Döhle-like inclusions in granulocytes. Also non-syndromic[b]	[26–28]
Wiskott–Aldrich syndrome (WAS, 301000)	XL	WAS (Xp11)	Small	S	Severe immunodeficiency leading to early death. Eczema	[29, 30]
					Increased risk of malignancies and autoimmunity	
					Reduced platelet sizw	
X-linked thrombocytopenia (XLT, 313900)	XL	WAS (Xp11)	Small	A to Mo	Mild immunodeficiency. Mild transient eczema	[29, 31]
					Increased risk of malignancies and autoimmunity	
					Also non-syndromic[b]	
Paris–Trousseau thrombocytopenia (TCPT, 188025)	AD	Deletions in 11q23	Normal	Mo to S	Physical growth delay, mental retardation, facial and skull dysmorphisms, malformations of the cardiovascular system, CNS, gastrointestinal apparatus, kidney, and/or urinary tract; other malformations	[32, 33]
Jacobsen syndrome (JBS, 147791)						
Thrombocytopenia-absent radius syndrome (TAR, 274000)	AR	RBM8A (1q21)	Normal	S	Bilateral radial aplasia +/− other upper and lower limb bone abnormalities. Kidney, cardiac, and/or CNS malformations. Reduced/absent megakaryocytes in BM	[33–35]
					Platelet count tends to raise over time	

Disease	Inheritance	Gene (location)	Platelet size	Severity	Clinical features	Refs
Thrombocytopenia associated with sitosterolaemia (STSL, 210250)	AR	ABCG5, ABCG8 (2p21)	Large	A to Mi	Tendon and tuberous xanthomas. Premature atherosclerosis. Hemolytic anemia with stomatocytosis / Large platelets. Also non-syndromic[b]	[34, 36, 37]
Radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT, 605432)	AD	HOXA11 (7p15) / EVI1 (3q26)	Normal	S	Bilateral radio-ulnar synostosis +/− other malformations / Reduced/absent megakaryocytes in BM. Possible evolution to bone marrow aplasia	[38–40]
GATA1-related disease: X-linked thrombocytopenia with thalassemia (XLTT, 314050), X-linked thrombocytopenia with dyserythropoietic anemia (XLTDA, 300367)	XL	GATA1 (Xp11)		Mi to S	Hemolytic anemia with laboratory abnormalities resembling beta-thalassemia, splenomegaly, or dyserythropoietic anemia / Congenital erythropoietic porphyria	[41]
FLNA-related thrombocytopenia (FLNA-RT, na)	XL	FLNA (Xq28)	Large	Mi to Mo	Periventricular nodular heterotopia (OMIM 300049) / Large platelets. Also non-syndromic[b]	[42]
Non-syndromic forms						
Bernard–Soulier syndrome (BSS, 231200/153670)		GP1BA (17p13)	Large			[43–46]
Biallelic	AR	GPIBB (22q11)		S	Giant platelets	
Monoallelic	AD	GP9 (3q21)		A to Mi	Large platelets	
Congenital amegakaryocytic thrombocytopenia (CAMT, 604498)	AR	MPL (1p34.2)	Normal	S	Reduced/absent megakaryocytes in BM. Evolution to fatal bonemarrow aplasia in infancy in all patients	[47]
Familial platelet disorder with propensity to acute myelogenous leukemia (FPD-AML, 601399)	AD	RUNX1 (21q22)	Normal	A to Mo	Over 40% of patients acquire acute myelogenous leukemia or myelodysplastic syndromes. Increased risk of T acute lymphoblastic leukemia	[48]

(continued)

Table 8.3 (continued)

Disease (abbreviation, OMIM entry)	Inheritance	Gene (locus)	Platelet size	Bleeding[a]	Main clinical feature	Reference
ANKRD26-related thrombocytopenia (ANKRD26-RT or THC2, 188000)	AD	ANKRD26 (10p12)	Normal	A to Mo	About 8% of patients acquire myeloid malignancies. Some patients have increased hemoglobin levels and/or leukocytosis	[49]
Gray platelet syndrome (GPS, 139090)	AR	NBEAL2 (3p21)	Large	A to Mi	Platelet count decreases over time. Development of progressive myelofibrosis and splenomegaly. Elevated serum vitamin B12 levels. Pale platelets due to severe alpha-granule deficiency	[50, 51]
ACTN1-related thrombocytopenia (ACTN1-RT, 615193)	AD	ACTN1 (14q24)	Large	A to Mi	Large platelets	[52, 53]
Platelet-type von Willebrand disease (PTvWD, 177820)	AD	GP1BA (17p13)		A to Mi	Platelet count can decrease under stress	[54]
ITGA2B/ITGB3-related thrombocytopenia (ITGA2B/ITGB3-RT, 187800)	AD	ITGA2B (17q21) ITGB3 (17q21)	Large	Mi to Mo	Large platelets	[55]
ETV6-related thrombocytopenia (ETV6-RT, na)	AD	ETV6 (12p13)	Normal	A to Mo	Increased risk of myeloid and lymphoid malignancies	[56, 57]
TUBB1-related thrombocytopenia (TUBB1-RT, 613112)	AD	TUBB1 (20q13)	Large	A to Mi	Large platelets	[58]
CYCS-related thrombocytopenia (CYCS-RT or THC4, 612004)	AD	CYCS (7p15)	Normal/small	A	Normal/reduced platelet size	[59]
GFI1b-related thrombocytopenia (GFI1b-RT, 187900)	AD	GFI1B (9q34)	Large	Mo to S	Some pale platelets reflecting a variable alpha-granule deficiency	[60, 61]
PRKACG-related thrombocytopenia (PRKACG-RT, na)	AR	PRKACG (9q21)	Large	S	Large platelets	[62]

The content was quoted from the review of Pecci A [5] with additional modifications

AD autosomal-dominant, *AR* autosomal recessive, *XL* X-linked, *BM* bone marrow, *CNS* central nervous system, *na* not available

[a]Bleeding, severity of bleeding tendency in the majority of patients reported for each form: *A* absent, *Mi* mild, *Mo* moderate, *S* severe

[b,a]'Also non-syndromic' indicates syndromic forms for which some patients having only thrombocytopenia (without the associated defects) have been reported

[29, 30]. Also, X-linked thrombocytopenia (XLT), a mild variant of WAS, is derived from mutations that do not abolish WAS expression completely and which often present with mild thrombocytopenia [31]. These two are important diseases for differential diagnosis of boys with chronic ITP at an early age of onset. Hematological examinations show small thrombocytopenia decreases (MPV, 6.0–6.2 fL) and no increase of megakaryocytes in bone marrow. Compared to ITP, the risk of intracranial hemorrhage is increased in WAS and XLT because of impaired platelet aggregation.

8.6.2   ITs with Progression to Pancytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT) is an autosomal recessive disease characterized by onset in the newborn period or early infancy, diminished megakaryocytes in bone marrow, increased TPO levels, and progression to pancytopenia [47]. This disease results from mutations of *MPL* gene encoding TPO-R, the signaling of which promotes megakaryocytic maturation and platelet production [64].

8.6.3   ITs with Skeletal Anomalies

Several disorders with skeletal malformation and distinct etiology are included in this category. Thrombocytopenia with absent radii (TAR) with autosomal recessive inheritance is characterized by defect of the radius, thrombocytopenia in early infancy showing alleviation with growth, and decreased megakaryocytes in BM [33, 35]. Actually, TAR results from mutations of *RBM8A* gene encoding a component of RNA-processing complexes [34].

Radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT) is characterized by congenital fusion of the radius and ulna, with progression to pancytopenia [38]. Heterozygous mutations of homeotic gene, *HOXA11*, have been reported as a cause of RUSAT in two families with autosomal-dominant inheritance [39]. More recently, Niihori et al. identified de novo heterozygous missense mutations in *EVI1* gene as a novel entity of RUSAT [40]. Missense mutations were clustered within the eighth zinc finger motif of the C-terminal zinc finger domain of EVI1, suggesting that EVI1 has important functions in hematopoiesis and stem cell self-renewal, and in the development of forelimbs in humans. Patients with *EVI1* mutations were treated with stem cell transplantation at 4–18 months of age because of its more rapid progression of bone marrow failure as compared to that of RUSAT with *HOXA11* mutation.

8.6.4   ITs with Predisposition to Malignant Diseases

This category includes several diseases with distinct etiology. Familial platelet disorder with propensity to acute myeloid leukemia (FPD-AML) is an autosomal-dominant disease caused by mutations of *RUNX1* gene encoding the hematopoietic transcription factor [48, 65]. FPD-AML shows a mild to moderate thrombocytopenia, but those patients have an approximately 35% of lifetime risk to develop AML or MDS. The process to malignant development in FPD-AML might be involved by mutations in additional genes such as *CDC25C*, a cell cycle regulator [66].

Autosomal-dominant thrombocytopenia, thrombocytopenia 2 (THC2), is an autosomal-dominant disease caused by mutations of *ANKRD26* gene [49]. It shows moderate thrombocytopenia and platelet dysfunction. A small percentage (<5%) of patients with THC2 also present a lifetime risk of developing myeloid malignancies such as AML, MDS, and CMML.

More recently, germline mutations in *ETV6* were found to be responsible for a third form of autosomal-dominant thrombocytopenia that is predisposed to hematological malignancies and skin and colon cancers with an uncertain degree of risk [56, 57].

8.7   Conclusion

With unavailable means of confirmative diagnosis yet, childhood ITP more or less requires exclusion diagnosis. Inherited thrombocytopenia is a disease that should be considered because misdiagnosis of ITP not only prevents IT patients from correct management of their diseases, but it also exposes them to inappropriate and potentially harmful treatments. The combination of clinical examinations and gene analyses is expected to be important for the medical treatment of children with ITP.

References

1. Nugent DJ. Immune thrombocytopenic purpura of childhood. Hematol Am Soc Hematol Educ Program. 2006;2006:97–103.
2. Cines DB, Bussel JB, Liebman HA, et al. The ITP syndrome: pathogenic and clinical diversity. Blood. 2009;113:6511–21.
3. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009;113:2386–93.
4. Drachman JG. Inherited thrombocytopenia: when a low platelet count does not mean ITP. Blood. 2004;103:390–8.
5. Pecci A. Diagnosis and treatment of inherited thrombocytopenias. Clin Genet. 2016;89: 141–53.
6. Kuhne T, Imbach P, Bolton-Maggs PHB, et al. Newly diagnosed idiopathic thrombocytopenic purpura in childhood: an observational study. Lancet. 2001;358:2122–5.

7. Zeller B, Rajantie J, Hedlund-Treutiger I, et al. Childhood idiopathic thrombocytopenic purpura in the Nordic countries: epidemiology and predictors of chronic disease. Acta Paediatr. 2005;94:178–84.
8. Horibe K. 2009 Annual report of disease registry by Japanese Society of Pediatric Hematology (in Japanese). Jpn J Pediatr Hematol. 2010;24:182–9.
9. Kurata Y, Fujimura K, Kuwana M, et al. Epidemiology of primary immune thrombocytopenia in children and adults in Japan: a population-based study and literature review. Int J Hematol. 2011;93:329–35.
10. Shirahata A, Fujisawa K, Ishii E, et al. A nationwide survey of newly diagnosed childhood idiopathic thrombocytopenic purpura in Japan. J Pediatr Hematol Oncol. 2009;31:27–32.
11. Kubota M, Adachi S, Usami I, et al. Characterization of chronic idiopathic thrombocytopenic purpura in Japanese children: a retrospective multi-center study. Int J Hematol. 2010;91:252–7.
12. Kuwana M, Kaburaki J, Kitasato H, et al. Immunodominant epitopes on glycoprotein IIb-IIIa recognized by autoreactive T cells in patients with immune thrombocytopenic purpura. Blood. 2001;98:130–9.
13. Kuwana M, Okazaki Y, Ikeda Y. Detection of circulating B cells producing anti-GPIb autoantibodies in patients with immune thrombocytopenia. PLoS One. 2014;9:e86943.
14. Liu B, Zhao H, Poon MC, et al. Abnormality of CD4(+)CD25(+) regulatory T cells in idiopathic thrombocytopenic purpura. Eur J Haematol. 2007;78:139–43.
15. Houwerzijl EJ, Blom NR, van der Want JJ, et al. Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. Blood. 2004;103:500–6.
16. McMillan R, Wang L, Tomer A, et al. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. Blood. 2004;103:1364–9.
17. Shirahata A, Ishii E, Eguchi H, et al. Consensus guideline for diagnosis and treatment of childhood idiopathic thrombocytopenic purpura. Int J Hematol. 2006;83:29–38.
18. Neunert C, Lim W, Crowther M, et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood. 2010;117:4190–207.
19. Arnold DM, Dentali F, Crowther MA, et al. Systematic review: efficacy and safety of rituximab for adults with idiopathic thrombocytopenic purpura. Ann Intern Med. 2007;146:25–33.
20. Patel VL, Mahévas M, Lee SY, et al. Outcome 5 years after response to rituximab therapy in children and adults with immune thrombocytopenia. Blood. 2012;119:5989–95.
21. Matsubara K, Takahashi Y, Hayakawa A, et al. Long-term follow-up of children with refractory immune thrombocytopenia treated with rituximab. Int J Hematol. 2014;99:429–36.
22. Cheng G, Saleh MN, Marcher C, et al. Eltrombopag for management of chronic immune thrombocytopenia (RAISE): a 6-month, randomised, phase 3 study. Lancet. 2010;377: 393–402.
23. Bussel JB, Buchanan GR, Nugent DL, et al. A randomized, double-blind study of romiplostim to determine its safety and efficacy in children with immune thrombocytopenia. Blood. 2011;118:28–36.
24. Ramaswamy K, Hsieh L, Leven E, et al. Thrombopoietic agents for the treatment of persistent and chronic immune thrombocytopenia in children. J Pediatr. 2014;165:600–5.
25. Provan D, Stasi R, Newland AC, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. Blood. 2010;115:168–86.
26. Pecci A, Klersy C, Gresele P, et al. MYH9-related disease: a novel prognostic model to predict the clinical evolution of the disease based on genotype-phenotype correlations. Hum Mutat. 2014;35:236–47.
27. Balduini CL, Pecci A, Savoia A. Recent advances in the understanding and management of MYH9-related inherited thrombocytopenias. Br J Haematol. 2011;154:161–74.
28. Pecci A, Biino G, Fierro T, et al. Alteration of liver enzymes is a feature of the MYH9-related disease syndrome. PLoS One. 2012;7:e35986.
29. Albert MH, Notarangelo LD, Ochs HD. Clinical spectrum, pathophysiology and treatment of theWiskott-Aldrich syndrome. Curr Opin Hematol. 2011;18:42–8.

30. Massaad MJ, Ramesh N, Geha RS. Wiskott-Aldrich syndrome: a comprehensive review. Ann N Y Acad Sci. 2013;1285:26–43.
31. Albert MH, Bittner TC, Nonoyama S, et al. X-linked thrombocytopenia(XLT) due to WAS mutations: clinical characteristics, long-term outcome and treatment options. Blood. 2010;115:3231–8.
32. Mattina T, Perrotta CS, Grossfeld P. Jacobsen syndrome. Orphanet J Rare Dis. 2009;4:9.
33. Greenhalgh KL, Howell RT, Bottani A, et al. Thrombocytopenia-absent radius syndrome: a clinical genetic study. J Med Genet. 2002;39:876–81.
34. Albers CA, Paul DS, Schulze H, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. Nat Genet. 2012;44:435–9.
35. Toriello HV. Thrombocytopenia-absent radius syndrome. Semin Thromb Hemost. 2011;37:707–12.
36. Merkens LS, Myrie SB, Steiner RD, et al., editors. GeneReviews™ [Internet]. 2013. http://www.ncbi.nlm.nih.gov/books/NBK 131810.
37. Rees DC, Iolascon A, Carella M, et al. Stomatocytic haemolysis and macrothrombocytopenia (Mediterranean stomatocytosis/macrothrombocytopenia) is the haematological presentation of phytosterolaemia. Br J Haematol. 2005;130:297–309.
38. Thompson AA, Woodruff K, Feig SA, et al. Congenital thrombocytopenia and radio-ulnar synostosis: a new familial syndrome. Br J Haematol. 2001;113:866–70.
39. Thompson AA, Nguyen LT. Amegakaryocytic thrombocytopenia and radio-ulnar synostosis are associated with HOXA11 mutation. Nat Genet. 2000;26:397–8.
40. Niihori T, Ouchi-Uchiyama M, Sasahara Y, et al. Mutations in MECOM, encoding oncoprotein EVI1, cause radioulnar synostosis with amegakaryocytic thrombocytopenia. Am J Hum Genet. 2015;97:848–54.
41. Millikan PD, Balamohan SM, Raskind WH, et al. Inherited thrombocytopenia due to GATA-1 mutations. Semin Thromb Hemost. 2011;37:682–9.
42. Nurden P, Debili N, Coupry I, et al. Thrombocytopenia resulting from mutations in filamin A can be expressed as an isolated syndrome. Blood. 2011;118:5928–37.
43. Berndt MC, Andrews RK. Bernard-Soulier syndrome. Haematologica. 2011;96:355–9.
44. Noris P, Perrotta S, Bottega R, et al. Clinical and laboratory features of 103 patients from 42 Italian families with inherited thrombocytopenia derived from the monoallelic Ala156Val mutation of GPIbα (Bolzano mutation). Haematologica. 2012;97:82–8.
45. Kunishima S, Imai T, Hamaguchi M, et al. Novel heterozygous missense mutation in the second leucine rich repeat of GPIbalpha affects GPIb/IX/V expression and results in macrothrombocytopenia in a patient initially misdiagnosed with idiopathic thrombocytopenic purpura. Eur J Haematol. 2006;76:348–55.
46. Savoia A, Kunishima S, De Rocco D, et al. Spectrum of the mutations in Bernard-Soulier syndrome. Hum Mutat. 2014;35:1033–45.
47. Ballmaier M, Germeshausen M. Congenital amegakaryocytic thrombocytopenia: clinical presentation, diagnosis, and treatment. Semin Thromb Hemost. 2011;37:673–81.
48. Owen CJ, Toze CL, Legare RD, et al. Five new pedigrees with inherited RUNX1 mutations causing familial platelet disorder with propensity to myeloid malignancy. Blood. 2008;112:4639–45.
49. Noris P, Perrotta S, Seri M, et al. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families. Blood. 2011;117:6673–80.
50. Gunay-Aygun M, Zivony-Elboum Y, Gumruk F, et al. Gray platelet syndrome: natural history of a large patient cohort and locus assignment to chromosome 3p. Blood. 2010;116:4990–5001.
51. Kahr WH, Hinckley J, Li L, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. Nat Genet. 2011;43:738–40.
52. Bottega R, Marconi C, Faleschini M, et al. ACTN1-related thrombocytopenia: identification of novel families for phenotypic characterization. Blood. 2015;125:869–72.

53. Kunishima S, Okuno Y, Yoshida K, et al. ACTN1 mutations cause congenital macrothrombo-cytopenia. Am J Hum Genet. 2013;92:431–8.
54. Othman M, Emsley J. Platelet-type von Willebrand disease: toward an improved understanding of the "sticky situation". Semin Thromb Hemost. 2014;40:146–50.
55. Nurden AT, Pillois X, Fiore M, et al. Glanzmann thrombasthenia-like syndromes associated with macrothrombocytopenias and mutations in the genes encoding the αIIbβ3 integrin. Semin Thromb Hemost. 2011;37:698–706.
56. Zhang MY, Churpek JE, Keel SB, et al. Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. Nat Genet. 2015;47:180–5.
57. Noetzli L, Lo RW, Lee-Sherick AB, et al. Germline mutations in ETV6 are associated with thrombocytopenia, red cell macrocytosis and predisposition to lymphoblastic leukemia. Nat Genet. 2015;47:535–8.
58. Kunishima S, Kobayashi R, Itoh TJ, et al. Mutation of the beta1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly. Blood. 2009;113: 458–61.
59. Morison IM, Cramer Borde EM, Cheesman EJ, et al. A mutation of human cytochrome c enhances the intrinsic apoptotic pathway but causes only thrombocytopenia. Nat Genet. 2008;40:387–9.
60. Stevenson WS, Morel-Kopp MC, Chen Q, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11:2039–47.
61. Monteferrario D, Bolar NA, Marneth AE, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. N Engl J Med. 2014;370:245–53.
62. Manchev VT, Hilpert M, Berrou E, et al. A new form of macrothrombocytopenia induced by a germ-line mutation in the PRKACG gene. Blood. 2014;124:2554–63.
63. Ouchi-Uchiyama M, Sasahara Y, Kikuchi A, et al. Analyses of genetic and clinical parameters for screening patients with inherited thrombocytopenia with small or normal-sized platelets. Pediatr Blood Cancer. 2015;62:2082–8.
64. Ballmaier M, Germeshausen M. Advances in the understanding of congenital amegakaryocytic thrombocytopenia. Br J Haematol. 2009;146:3–16.
65. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet. 1999;23:166–75.
66. Yoshimi A, Toya T, Kawazu M, et al. Recurrent CDC25C mutations drive malignant transformation in FPD/AML. Nat Commun. 2014;5:4770.

Chapter 9
Pathogenesis and Treatment of Hemophilia

Keiji Nogami and Midori Shima

Abstract Hemophilia A and B are congenital inherited bleeding disorders, occurred by genetic abnormalities of blood coagulation factor (F)VIII and FIX molecules, respectively. The clinical abnormality is associated with bleeding episodes affecting especially joints and muscles, and repeated hemorrhage results in chronic arthropathy finally associated with loss of joint movement. The current hemostatic treatment is the replacement therapy of plasma-derived or recombinant (r)FVIII or FIX concentrates with on demand or prophylaxis. Development of this therapy had improved the quality of life of hemophiliacs more dramatically than before. The primary and secondary prophylaxis is recently becoming widespread for the prevention of arthropathy. There remain some issues such as frequent intravenous injection, however. Extended half-life rFVIII and rFIX concentrates have been recently developed, and some products are available. However, in 20–30% of severe hemophilia A and 3–5% of hemophilia B which had multi-transfused, anti-FVIII (FIX) alloantibodies (inhibitors) appeared, resulting in difficulty of hemostatic management. Immune tolerance induction therapy to eradicate inhibitors has been actively conducted. The bypassing agent therapy is treated for hemophilia patients with inhibitor. Subcutaneous injection therapy such as FVIII-mimetic bispecific antibody or gene therapy is currently ongoing in the clinical trials for the future prospective therapy.

K. Nogami, M.D., Ph.D. (✉) • M. Shima
Department of Pediatrics, Nara Medical University,
840 Shijo-cho, Kashihara, Nara 634-8522, Japan
e-mail: roc-noga@naramed-u.ac.jp

9.1 Hemophilia A

9.1.1 Introduction

Hemophilia A is an inherited bleeding disorder caused by deficiency or dysfunction of the coagulation protein, factor (F)VIII. FVIII plays an essential role in the intrinsic pathway during blood coagulation, and the genetic defect causes a decreased and delayed generation of thrombin leading to disordered clot formation and a bleeding diathesis. The clinical abnormality is associated with bleeding episodes affecting joints, muscles, and soft tissue. Repeated hemorrhage results in chronic arthropathy finally associated with loss of joint movement. Both hemophilia A and hemophilia B (discussed below) are inherited as X-linked recessive traits; i.e., male individuals are affected and female individuals are asymptomatic or mildly affected carriers.

9.1.2 FVIII Protein and FVIII Gene (F8) (Fig. 9.1)

The FVIII protein was first isolated and purified over 30 years ago [1], and the human FVIII gene (*F8*) was initially cloned in 1984 [2, 3]. *F8* is located in the most distal band (Xq28) of the long arm of the X chromosome. The gene contains 25 introns and 26 exons, 24 of which vary in length from 69 to 262 base pairs (bp), while the two larger exons 14 and 26 contain 3106 and 1958 bp, respectively. Most of exon 26 consists of a 3′ untranslated sequence, however, and exon 14, therefore, bears by far the largest exonic coding sequence, principally that of the B domain. Spliced FVIII mRNA is approximately 9 kb in length and predicts a precursor protein of 2351 amino acids.

Removal of a 19 peptide secretory leader sequence of FVIII results in the mature sequence of 2332 amino acids. The FVIII molecule consists of three homologous A domains, a B domain, and two homologous C domains structurally in the order A1-A2-B-A3-C1-C2. Prior to secretion, FVIII is processed into a series of metal ion-linked heterodimers produced by cleavage at the B-A3 junction together with a number of additional cleavages within the B domain. FVIII is highly sensitive to proteolytic processing after secretion, and only a small fraction of circulating FVIII retains a single-chain form. Most consist of heavy chains of variable length (including sequences of the A1, A2, and B domains) linked ion covalently to light chains composed of the A3, C1, and C2 domains [4]. Limited proteolysis at Arg372 and Arg1689 by thrombin or FXa generates cofactor activity (FVIIIa). Expression of recombinant (r)FVIII lacking the entire length of the B domain has confirmed, however, that this domain is dispensable for the activation and procoagulant function of the protein. The cofactor function of FVIIIa accelerates the activation of FX by activated FIX (FIXa) on a suitable phospholipid surface, thus amplifying the clotting stimulus by several orders of magnitude. Specific proteolytic cleavages between the FVIII domains both activate and inactivate the cofactor [5]. The active form
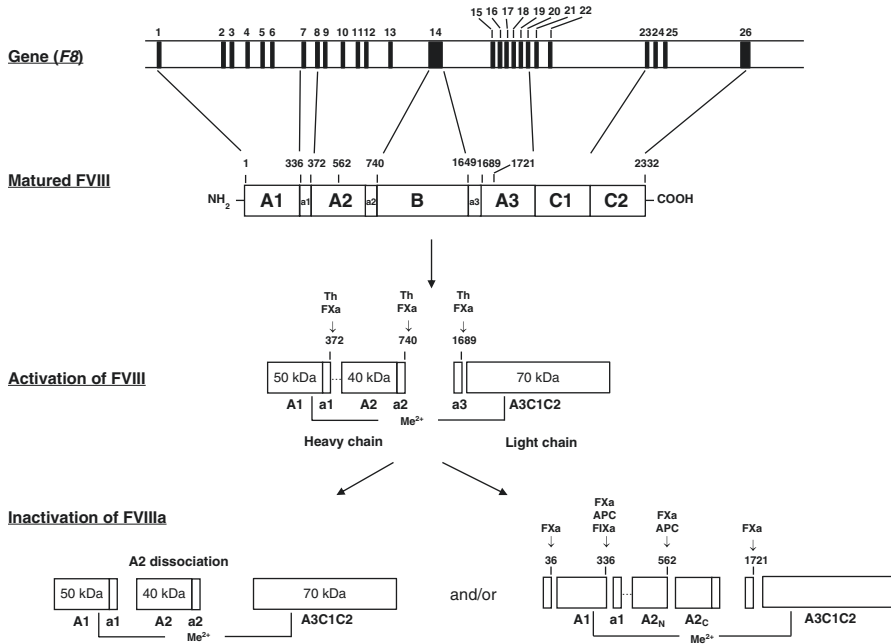
Fig. 9.1 Linear representation of *F8*, the structure of FVIII, and its cleavage site. The 26 exons (*above*) and domain organization (*below*) of FVIII based on amino acid homology. Activation of FVIII leads to release of the B and a3 domains. In the activated FVIII heterotrimer, the A1 and A3 domains retain the metal ion-mediated interaction, and the stable A1/A3-C1-C2 dimer is weakly associated with the A2 subunit through electrostatic interactions. Either spontaneous dissociation of A2 or proteolysis of FVIIIa results in loss of its function. *Th* thrombin

(FVIIIa) cation-dependently bonds to the A3-C1-C2 light chain together with the heavy chain held by electrostatic association between the A1 and A2 domains. FVIIIa is highly unstable owing to spontaneous dissociation of the A2 subunit and proteolytic inactivation of FVIIIa through cleavage at Arg336 by activated protein C (APC) and FXa. An additional cleavage site for APC at Arg562 in the A2 subunit results in complete inactivation of FVIIIa [5].

For convenience, *F8* defects associated with hemophilia A may be divided into several categories: (1) gross gene rearrangements, (2) deletions or insertions of genetic sequence of a size varying from one base pair up to the entire gene, and (3) single DNA base substitutions resulting in either amino acid replacement ("missense"), premature peptide chain termination ("nonsense" or stop mutations), or mRNA splicing defects. More than 2500 unique molecular defects in *F8* have been described and are included in the worldwide Factor VIII Variant Database (http://www.factorviii-db.org). Molecular characterization of patients with hemophilia A is dependent initially upon the analysis of intron 22 and intron 1 in *F8* [6, 7]. The single, clinically most important defect is a gene rearrangement (an inversion) involving intron 22 (Fig. 9.2), which is evident in approximately 50% of all severe

a Normal

b Intron 22 inversion
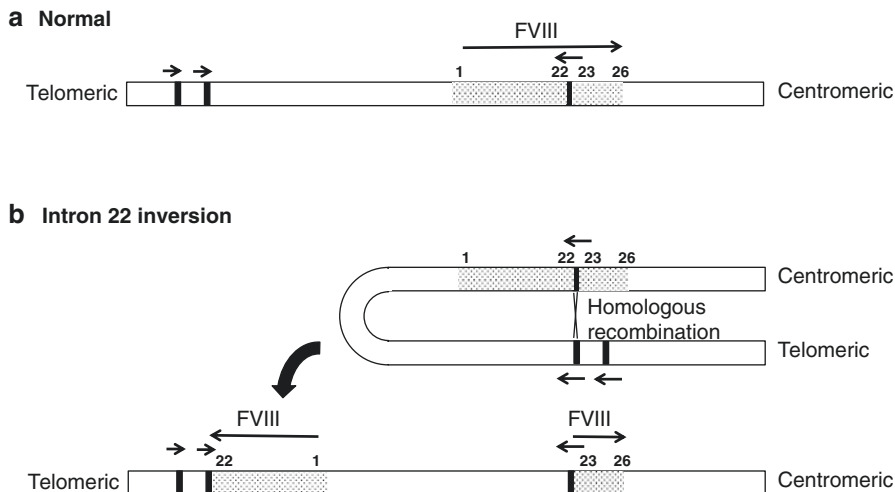
Fig. 9.2 Simplified representation of the intron 22 inversion. Recombination between homologous sequences in intron 22 and ~400 kb telomeric to the gene leads to separation of exons 1–22 from exons 23–26

disease cases [6]. If genetic variations in intron 1 or intron 22 are not detected, full *F8* mutation screening is generally performed by direct sequencing, covering all exons, intron-exon boundaries, and the promoter region. Genetic variations have not been identified in 2–18% of patients [8]. Consistent gene defects in patients with mild and moderate FVIII deficiencies are not common, and full mutation analysis of *F8* by direct sequencing is usually necessary in these patients.

9.1.3   FVIII Inhibitors

The presence of neutralizing alloantibodies (inhibitors) to therapeutic FVIII is a serious complication in hemophilia A, occurring in approximately 30% of patients classed as severe [9]. In those individuals, the inhibitors usually develop during the first 20–30 days of exposure and appear to result from a multi-causal immune response involving both patient-related and treatment-related factors. A gene defect is thought to contribute to about 40% of the risk of inhibitor formation, although the immunology of inhibitor development remains to be fully understood. Large deletions and nonsense mutations seem to present the highest risk, whereas missense and splicing mutations mediate the lowest risk [10]. The most common acquired risk factor is the intensity of exposure to FVIII concentrate [11]. In non-severe hemophilia A patients and different from severe hemophilia A, inhibitors usually arise when the immune system is under intense stimulation or when exposure to FVIII concentrates is unusually high, independent of exposure days, in particular in

the postoperative period [12, 13]. Mutations leading to an abnormal conformation of FVIII are associated with a high risk of inhibitor development in mild hemophilia A [13], especially in those patients with mutations in the A2 and A3C1C2 domains (e.g., Arg593Cys and Arg2150His, respectively) [14, 15].

9.1.4 Treatment

Comprehensive guidelines for the treatment of hemophilia were endorsed as high standard by the International Society on Thrombosis and Hemostasis (ISTH) in 2012 (https://www.isth.org/?page=Published_Guidance) and were published in detail in 2013 by the World Federation of Hemophilia (WFH) working group [16]. The primary aim of hemophilia care is to prevent and treat hemorrhage using FVIII therapeutic products. An integrated support system, called comprehensive care, is highly recommended and includes specific therapy for prophylaxis or acute bleeding and also treatment for complications and drug-related side effects [17]. As noted above, however, the development of inhibitors presents many difficulties for the use of FVIII concentrates, and long-term complications, such as joint destruction with the need for synovectomy or joint replacement, together with infections transmitted by clotting factor concentrates are also important issues for maximally effective clinical management. Treatment for hemophilia A may not be straightforward, therefore, and depends overall on the severity of the FVIII defect, the presence of inhibitors, available resources, and clinical complications.

9.1.4.1 Replacement Therapy

Acute bleeding should be treated immediately with FVIII concentrate. Home infusion therapy has made it possible for patients to treat themselves quickly just after bleeding (and prophylactically). In the absence of an inhibitor, each unit of intravenously infused FVIII per kilogram of body weight will raise the plasma FVIII level by approximately 2 IU/dL [17]. The half-life of FVIII is 8–12 h, and optimal doses and duration of treatment are dependent on the site of bleeding and individual patient pharmacokinetics (Table 9.1) [16].

Hemarthrosis is a common complication in hemophilia patients, and repeated hemorrhage often occurs into the same joint, commonly called a target joint. In addition, compartment syndrome is a painful and potentially serious condition caused by bleeding or swelling within an enclosed bundle of muscles, and this poses a particular risk in hemophilia when soft tissue hemorrhage occurs [18]. The WFH strongly recommends the use of viral-inactivated plasma-derived (pd) or recombinant (r)FVIII concentrates in preference to cryoprecipitate or fresh frozen plasma for the treatment of hemophilia A [16]. rFVIII products especially have the theoretical advantage of being free from blood-borne pathogens. Recent research has also focused on inhibitor risk in patients treated with pd-FVIII or rFVIII, but the

Table 9.1 Summary of the desired plasma factor peak levels and duration of administration (modification of [16])

Type of hemorrhage	Hemophilia A		Hemophilia B	
	Desired level (IU/dL)	Duration (days)	Desired level (IU/dL)	Duration (days)
Joint	40–60	1–2	40–60	1–2
Superficial muscle	40–60	2–3	40–60	2–3
Iliopsoas and deep muscle				
Initial	80–100	1–2	60–80	1–2
Maintain	30–60	3–5	30–60	3–5
CNS/head				
Initial	80–100	1–7	60–80	1–7
Maintain	50	8–21	30	8–21
Throat and neck				
Initial	80–100	1–7	60–80	1–7
Maintain	50	7–14	30	7–14
Gastrointestinal				
Initial	80–100	7–14	60–80	7–14
Maintain	50		30	
Renal	50	3–5	40	3–5
Deep laceration	50	5–7	40	5–7
Surgery (major)				
Pre-op	80–100		60–80	
Post-op	60–80 (1–3 day), 40–60 (4–6 days), 30–50 (7–14 days)		40–60 (1–3 day), 30–50 (4–6 days), 20–40 (7–14 days)	
Surgery (minor)				
Pre-op	50–80		50–80	
Post-op	30–80	1–5, depend on the procedure	30–80	1–5, depend on the procedure

outcomes remain controversial. The Research of Determinants of Inhibitor Development (RODIN) study group has comprehensively examined the association between FVIII products and inhibitor development, and the only significant result determined was an unexpectedly higher risk of inhibitor development using second-generation full-length recombinant products compared with third-generation rFVIII products [19]. A more recent report [20] indicated that the risk of developing an inhibitor when using rFVIII products in previously untreated patients (PUPs) was significantly higher than when using pd-FVIII concentrates that contain von Willebrand factor (VWF), although this finding was not totally consistent with the earlier RODIN findings. Further research on inhibitor development is warranted to elucidate the precise immunogenicity of rFVIII.

Several clinical trials have been undertaken to investigate the use of rFVIII bio-engineered to extended half-life (EHL), including Fc-fusion, PEGylated, and glyco-PEGylated proteins. Fc-fusion proteins are designed to be recycled into the circulation through pH-dependent binding to neonatal Fc receptors (FcRn) that

delay lysosomal degradation [21], and one such preparation, rFVIIIFc, is currently available [22]. Moreover, other EHL-modified rFVIII products, including BAX 855 (PEGylation) [23] and N8-GP (glycoPEGylation) [24], have also completed Phase 3 trials. All of these novel products appeared to face to a "ceiling effect," however, and half-lives appear to be limited to approximately 1.5 to 1.6-fold of unmodified FVIII. This limitation could be related to the absence of VWF, the career protein.

For mild or moderately severe FVIII deficient patients, desmopressin (DDAVP) is an alternative option that rapidly increases FVIII:C by three- to sixfold from base-line levels [25]. DDAVP is generally administered intravenously at a dose of 0.3 μg/kg over 20–30 min and mediates elevated FVIII in 30–60 min that persists for 6–12 h. Tachyphylaxis occurs after repeated administration, however, due to depletion of endothelial stores [26].

9.1.4.2 Prophylaxis

Strategies for prophylaxis replacement therapy are based on evidence that moderately severe patients who have FVIII levels more than 1 IU/dL experience many fewer spontaneous bleeding events and have much better long-term joint function than those patients classed as severe [27]. In addition, randomized control studies have also convincingly demonstrated that prophylaxis with rFVIII can prevent joint damage and decrease the frequency of joint and other hemorrhages in young boys with severe hemophilia A [28]. Furthermore, even using prophylactic regimens that resulted in trough FVIII levels <1 IU/dL, episodes of joint hemorrhage were significantly decreased [29]. A range of different prophylactic protocols have been proposed, including 10–20 IU/kg or 25–40 IU/kg per dose 2–3 times a week, once a week for very young children [16], or temporal prophylaxis just before physical activity associated with a higher risk of injury [30]. It is clear, however, that the pharmacokinetics of FVIII is diverse, and the lowest effective level of FVIII should be determined individually for each patient considered for primary or secondary prophylaxis.

9.1.4.3 Adjunctive Therapy

Replacement therapy is the primary hemostatic treatment in patients with hemophilia. In addition, the concept of "RICE" (rest, ice, compression, and elevation) plays an important role as a first aid for acute bleeding in muscles and joints. Antifibrinolytics that enhance clot stability, such as tranexamic acid and epsilon aminocaproic acid, are also useful for mucosal and oral bleeding including dental extraction [31]. Tranexamic acid is commonly used at 25 mg/kg per dose every 6–8 h intravenously or orally. Physical fitness and the development of strong muscles are also recommended to protect joints. An appropriate exercise lifestyle should be encouraged, especially for children, to avoid overweight or obesity [32]. Noncontact sports such as swimming should be encouraged. In the absence of

thoroughly controlled prophylaxis, high-contact sports including football or high-velocity activities including skiing should be avoided due to the obvious potential for life-threatening injuries.

9.1.4.4 Inhibitors

Hemostatic Management

Anti-FVIII alloantibodies occur about 30% of patients with hemophilia A and remain a challenging clinical problem in congenital FVIII deficiency [9]. Acute bleeding in patients with inhibitors may be treated effectively with infusions of concentrates which "bypass" the intrinsic coagulation pathway. Two such bypassing agents are available, activated prothrombin complex concentrate (aPCC) and recombinant FVIIa (rFVIIa), and both are safe and efficient almost equally [33], despite the differences in their content, dosing, and mode of action. Preference often seems to be dependent on individual patient circumstances, and combined use of both drugs is sometimes required [34]. Prophylaxis with aPCC in both adult and pediatric patients with inhibitors has been reported to be effective in preventing joint bleeds and longer-term orthopedic damage [35]. This type of treatment could be an important alternative choice for inhibitor patients who have failed or are ineligible for ITI.

Immune Tolerance Induction (ITI)

Immune tolerance induction (ITI) utilizing frequent and long-term administration of FVIII-containing concentrates is presently the only strategy proven to eradicate persistent and high-responding inhibitors. Retrospective cohort studies have demonstrated similarly high success rates (60–80%) with various different ITI protocols [36]. Evidence from large-scale and methodologically rigorous trials is lacking, however, and the optimal ITI regimen and predictors of ITI outcome remain to be firmly established. A number of patient-related or treatment-related features have been analyzed as predictors of ITI outcome including the time interval to success, inhibitor titer prior to ITI start, historical inhibitor peak titer, time interval between inhibitor diagnosis and ITI start, and FVIII dose regimen [36, 37]. Meta-analyses of data from both the International Immune Tolerance Registry (IITR) [38] and the North American Immune Tolerance Registry (NAITR) [39] have indicated, however, that only a historical peak inhibitor titer <50 BU/mL and an inhibitor titer <10 BU/mL at the time of ITI start are reasonable predictors of success. In this context, an international ITI study in hemophilia A inhibitor patients with good risk factors indicated that the use of both high-dose FVIII (200 IU/kg per day) and low-dose FVIII (50 IU/kg every other day) were equally effective (approximately 70%), although the low-dose group had more bleeding events during the period of study [40].

9.1.4.5 Future Prospective Therapy for Hemophilia A

Although the treatment of hemophilia has advanced considerably in the last quarter century, several elegant studies have been described in an attempt to resolve remaining challenges. For example, the concept of rebalancing hemostasis by moderating natural anticoagulants rather than replacing FVIII is being explored. Hence, targeting antithrombin with small RNA interference agents [41] and monoclonal antibodies against tissue factor pathway inhibitor [42] are now undergoing clinical trials. In addition a bispecific antibody (anti-FIXa/FX antibody) designed to mimic FVIIIa has been developed [43], and Phase 1/2 clinical trials using this antibody (ACE910, emicizumab) has been completed. Prophylactic treatment with weekly subcutaneous administration was well tolerated and reduced the number of bleeding episodes in hemophilia A patients both with and without inhibitors [44]. The advantages of these novel therapeutic agents include the potential for less frequent administration (once per 1–4 weeks), subcutaneous injections (preferable for younger children), eliminating the risk of developing anaphylactic inhibitors, and effectiveness regardless of the presence of inhibitors.

9.2 Hemophilia B

9.2.1 Introduction

Hemophilia B is a bleeding disorder caused by mutations in the FIX gene (*F9*) leading to deficient or defective FIX, a pivotal serine protease in the intrinsic coagulation cascade. Hemophilia B (one per 30,000 male births) was first distinguished from the more prevalent hemophilia A (one per 5000 male births) in 1952 when it was noted that mixing plasma samples from two patients diagnosed with "hemophilia" mutually corrected the prolonged in vitro clotting times.

9.2.2 FIX Gene (F9) and FIX Protein

The *F9* coding sequence was partially described in the early 1980s and the full sequence was published in 1985 [45]. *F9* is located in the X chromosome at Xq27.1 and spans 33 kbp, including seven introns and eight exons that are transcribed into a 2802-bp mRNA (Fig. 9.3). Exons I and II code for a prepro leader sequence and a Gla domain, whereas exons IV and V each code for EGF domains. The last three exons code for the activation peptide and the catalytic domain. The 5′ sequence includes a LINE-1 element, and a major transcription initiation site appears to be at or near an adenine nucleotide at −176. The Factor IX Variant Database (http://www.factorix.org) identifies many *F9* mutations that can be attributed to changes in the CpG nucleotide (from CG to TG or CA). The majority of defects (64%) are point mutations, followed by small insertions or deletions (18%), splice site mutations
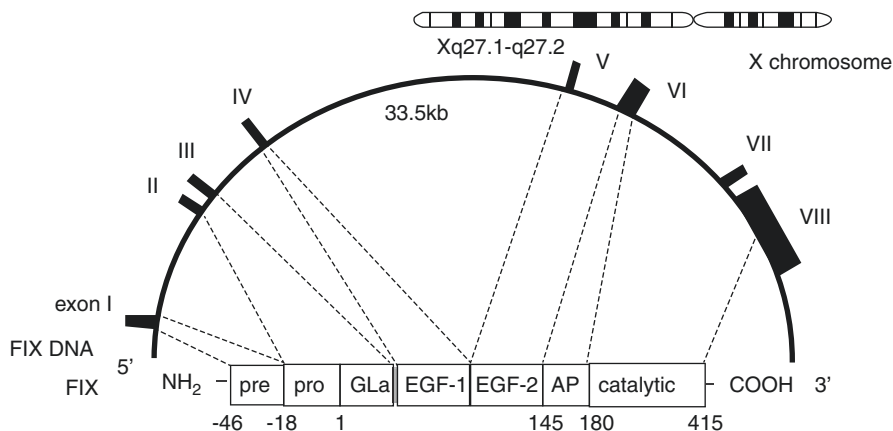
Fig. 9.3 Structure of *F9* and FIX peptide

(9%), large deletions or insertions (6%), and mutations in the promoter region. Large deletions, covering several tens of kb or an entire loss of total FIX, are thought to be associated with the development of anti-FIX inhibitors [46]. The *F9* Leyden phenotype is distinctively characterized by a severe deficiency at birth, but with FIX levels that start to increase during the second decade of life and achieve near normality during the third decade [47]. Normal or borderline-low levels are subsequently maintained. The pathogenesis of this unique abnormality appears to center on the promoter map where one site is partly overlapped by an androgen response element which regulates transcription levels following the hormonal changes that occur during puberty.

FIX is a single-chain glycoprotein composed of 415 amino acids [48]. It is a vitamin K-dependent protein containing 12 residues of γ-carboxyglutamic acid (Gla domain) located in the amino-terminal region of the molecule. This region of the protein contains two potential epidermal growth factor (EGF)-like domains, an activated peptide and a catalytic domain. Cotranslational translocation into the lumen of the endoplasmic reticulum (ER) occurs concomitantly with signal peptide cleavage. The addition of core high-mannose oligosaccharides to the polypeptide in the ER is followed by glucose trimming of the N-linked oligosaccharide structures and γ-carboxylation of the 12 amino-terminal glutamic acid residues. Upon transit into the Golgi compartment, additional modifications occur, which include (1) complex modification of N-linked oligosaccharides; (2) tyrosine sulfation at Tyr155; (3) Ser/Thr glycosylation at residues Ser61 and Ser53, as well as several Thr residues within the activation peptide; and (4) cleavage of the propeptide. In addition, FIX isolated from human plasma is phosphorylated at Ser158 within the activation peptide. A majority of the modifications within FIX occur within the activation peptide and may regulate activation of FIX. Appropriate γ-carboxylation and propeptide cleavage are essential for the functional secretion and activity of secreted FIX. During the coagulation cascade, FIX is cleaved at two internal peptide bonds (Ala145-Ala, Arg180-Val), resulting in the formation of a light chain and a heavy chain, held together by a disulfide bond. During the conversion to FIXa, an activation glycopeptide of 35 amino acid residues is released.

9.2.3 FIX Inhibitor

FIX inhibitors are relatively uncommon, being detected in 1–3% of patients with hemophilia B. Genetic factors play a major role, and specific mutations in *F9* appear to be associated with an increased incidence of inhibitor development. Large deletions and frameshift mutations leading to the loss of coding information are much more likely to be associated with inhibitor development. It has been reported that large deletions account for only 1–3% of all hemophilia B patients but account for 50% of inhibitor patients [49]. Thorland et al. [50] genotyped eight unrelated hemophilia B patients who had anaphylactic reactions to FlX-containing products, as well as an inhibitor, and compared their gene mutations with those of 550 other hemophilia B patients recorded in the hemophilia B database. Individuals with complete gene deletions were found to be at greatest risk for anaphylaxis. Anaphylaxis occurred more frequently in families with null mutations (large deletions, frameshift, or nonsense mutations) than in those with missense mutations. In addition, Astermark et al. [51] have recently reviewed the potential role of immune response genes, environmental factors, and other concurrent immune system challenges, noting especially the occurrence of a microsatellite polymorphism in the promoter region of the IL-10 gene which was previously shown to be highly associated with inhibitor formation in hemophilia A.

9.2.4 Treatment

The basic treatment principles described in the hemophilia A section above, including RICE and adjuvant therapy, can be also applied to hemophilia B. The only difference is that DDAVP does not affect FIX levels and has no value for the treatment of hemophilia B. As in hemophilia A, the main treatment for hemophilia B is replacement therapy, using FIX products either on demand or prophylactically. A number of new agents are being developed and are described below. The World Federation of Hemophilia Treatment working group has published detailed recommendations of an up-to-date list of various products currently available (see http://www.wfh.org).

9.2.4.1 FIX Products

Clotting factor concentrates are administrated intravenously to correct the FIX deficiency. Guidelines for target peak and trough levels are the same as in hemophilia A. Available FIX products include specific FIX concentrates that may be plasma derived or recombinant and multifactor concentrates that contain FII, FVII, FIX, and FX, known as prothrombin complex concentrates. The use of pure FIX products is always preferred. Similar to the novel FVIII proteins discussed above, extended half-life (EHL) products including fusion and PEGylated FIX have been recently introduced. The mean terminal half-life of the fusion proteins, rFIX-Fc and FIX-albumin (rIX-FP), appeared to be extended to 82.1 h [52] and 91.5 h [53], respectively, approximately 3–4 times that of conventional FIX therapy. In addition, the

site-directed glycoPEGylated protein (N9-GP) that covalently binds to FIX demonstrated a mean half-life of 93 h [54]. The Fc-fusion FIX protein is now commercially available, and the other EHL proteins are completing Phase 3 clinical trials.

9.2.4.2   Dosing (Table 9.1)

In vivo recovery of FIX (post-dose activity levels) is not as good as with FVIII products. FIX reversibly binds to endothelial cells and diffuses into extravascular tissues due to the smaller size of the molecule compared to FVIII. In principle, therefore, although 1 IU/kg of infused FIX could be expected to raise circulating levels by 1 IU/dL, the lower in vivo recovery moderates the response, and 1 IU/kg of FIX generally increases plasma FIX activity by approximately 0.7–0.8 IU/dL [55]. Both pd-FIX and rFIX have a longer half-life (16–17 h) [56] than FVIII (8–12 h) [57], however, and the dosing interval for FIX therapy is commonly 24 h with laboratory monitoring of FIX:C. EHL FIX products have three- to sixfold longer half-lives than unmodified materials, potentially reducing the frequency of the dosing. Peak levels and trough levels should be carefully assessed, however, to ensure the maintenance of adequate hemostasis in each individual's circumstance. EHL products may not always be consistent in this respect, and further studies are required to prepare guidelines of the most appropriate use of these modern therapies.

9.2.4.3   Prophylaxis

In common with hemophilia A, prophylaxis with the regular infusions of clotting factor to prevent bleeding and minimize the risk of joint destruction in advance is a highly effective treatment in patients with hemophilia B. The original concept in both hemophilia A and hemophilia B was to maintain levels of FVIII:C or FIX:C above 1 IU/dL, in order to ameliorate the phenotype from severe to moderate, and in particular to preserve joint function. Most prophylactic regimens use fixed doses, commonly administration of 25–40 IU/kg of FX twice a week. This approach is now being adapted, however, and specific dosing protocols are being adjusted to the requirements of individual patients.

9.2.4.4   Anaphylaxis and Inhibitors

Anaphylaxis and other allergic reactions are closely associated with FIX inhibitor development. Up to 50% of hemophilia B patients with inhibitors may have severe allergic reactions to FIX administration, including anaphylaxis. No evidence is available, however, to implicate any particular type of product as the primary cause of anaphylaxis and inhibitor development. These clinically severe events that occur after very few exposures to FIX have led to the important recommendation that all

infants and small children with severe hemophilia B, particularly those with a family history and/or with genetic defects predisposed to inhibitor development, should be closely monitored over their initial 10–20 exposure day to FIX concentrates in a facility equipped to treat anaphylactic shock. Reactions can occur later, but may be less severe [58, 59]. As discussed above, genotyping can provide vital data to identify any defect in *F9* (e.g., large gene deletions) known to be associated with a high risk of anaphylaxis and inhibitor development. Patients with an inhibitor of FIX and a history of anaphylaxis to FIX should be treated with rFVIIa.

9.2.4.5 ITI Therapy

ITI therapy is successful in the majority of patients with hemophilia A with inhibitor (approximately 70–80%), but the success rate is much lower (approximately 40%) in patients with hemophilia B. In addition, ITI in hemophilia B inhibitor patients with a history of severe allergic reactions to FIX may be associated with the development of nephrotic syndrome [38]. Those who develop nephrotic syndrome are often steroid resistant and ITI therapy is discontinued. Alternatively, ITI in conjunction with immunosuppression has been reported to be successful [60].

9.2.4.6 Future Prospective Therapy

Hemophilia B appears to offer an ideal model for gene therapy. The clinical disorder is a consequence of defects in the well-characterized FIX gene, with minimal influence from other genetic modifiers. In this context, a novel serotype 8 pseudotyped, self-complementary AAV (AAV8) vector expressing a codon-optimized factor IX transgene (scAAV2/8-LP1-hFIXco) was reported to be successful [61]. In a follow-up study of ten hemophilia B patients [62], FIX levels were shown to be consistently elevated to 5–7% of the normal value for 1–4.5 years. Other Phase 1/2 clinical trials on different strategies using AAV vectors are ongoing.

References

1. Rotblat F, Goodall AH, O'Brien DP, et al. Monoclonal antibodies to human procoagulant factor VIII. J Lab Clin Med. 1983;101:736–46.
2. Gitschier J, Wood WI, Goralka TM, et al. Characterization of the human factor VIII gene. Nature. 1984;312:326–30.
3. Toole JJ, Knopf JL, Wozney JM, et al. Molecular cloning of a cDNA encoding human antihaemophilic factor. Nature. 1984;312:342–7.
4. Eaton D, Rodriguez H, Vehar GA. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. Biochemistry. 1986;25:505–12.
5. Fay PJ. Activation of factor VIII and mechanisms of cofactor action. Blood Rev. 2004;18:1–15.

6. Lakich D, Kazazian Jr HH, Antonarakis SE, et al. Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. Nat Genet. 1993;5:236–41.

7. Bagnall RD, Waseem N, Green PM, et al. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood. 2002;99:168–74.

8. Peyvandi F, Garagiola I, Young G. The past and future of haemophilia: diagnosis, treatments, and its complications. Lancet. 2016;388(10040):187–97. doi:10.1016/S0140-6736(15)01123-X.

9. Hay CR. The epidemiology of factor VIII inhibitors. Haemophilia. 2006;12(Suppl 6):23–8.

10. Gouw SC, van den Berg HM, Oldenburg J, et al. F8 gene mutation type and inhibitor development in patients with severe hemophilia A: systematic review and meta-analysis. Blood. 2012;119:2922–34.

11. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. Blood. 2007;109:4648–54.

12. Sharathkumar A, Lillicrap D, Blanchette VS, et al. Intensive exposure to factor VIII is a risk factor for inhibitor development in mild hemophilia A. J Thromb Haemost. 2003;1:1228–36.

13. Eckhardt CL, van Velzen AS, Peters M, et al. Factor VIII gene (F8) mutation and risk of inhibitor development in nonsevere hemophilia A. Blood. 2013;122:1954–62.

14. Thompson AR, Murphy ME, Liu M, et al. Loss of tolerance to exogenous and endogenous factor VIII in a mild hemophilia A patient with an Arg593 to Cys mutation. Blood. 1997;90:1902–10.

15. Peerlinck K, Jacquemin MG, Arnout J, et al. Antifactor VIII antibody inhibiting allogeneic but not autologous factor VIII in patients with mild hemophilia A. Blood. 1999;93:2267–73.

16. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Treatment Guidelines Working Group on Behalf of The World Federation of Hemophilia. Guidelines for the management of hemophilia. Haemophilia. 2013; 19:e1–47.

17. Björkman S, Berntorp E. Pharmacokinetics of coagulation factors: clinical relevance for patients with haemophilia. Clin Pharmacokinet. 2001;40:815–32.

18. Naranja Jr RJ, Chan PS, High K, et al. Treatment of considerations in patients with compartment syndrome and an inherited bleeding disorder. Orthopedics. 1997;20:706–9.

19. Gouw SC, van der Bom JG, Ljung R, et al. Factor VIII products and inhibitor development in severe hemophilia A. N Engl J Med. 2013;368:231–9.

20. Peyvandi F, Mannucci PM, Garagiola I, et al. A randomized trial of factor VIII and neutralizing antibodies in hemophilia A. N Engl J Med. 2016;374:2054–64.

21. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? Nat Rev Immunol. 2013;13:176–89.

22. Mahlangu J, Powell JS, Ragni MV, et al. Phase 3 study of recombinant factor VIII Fc fusion protein in severe hemophilia A. Blood. 2014;123:317–25.

23. Konkle BA, Stasyshyn O, Chowdary P, et al. Pegylated, full-length, recombinant factor VIII for prophylactic and on-demand treatment of severe hemophilia A. Blood. 2015;126:1078–85.

24. Tiede A, Brand B, Fischer R, et al. Enhancing the pharmacokinetic properties of recombinant factor VIII: first-in-human trial of glycoPEGylated recombinant factor VIII in patients with hemophilia A. J Thromb Haemost. 2013;11:670–8.

25. Mannucci PM. Desmopressin (DDAVP) in the treatment of bleeding disorders treatment of hemophilia No 11. WFH; 2012.

26. Mannucci PM, Bettega D, Cattaneo M. Patterns of development of tachyphylaxis in patients with haemophilia and von Willebrand disease after repeated doses of desmopressin (DDAVP). Br J Haematol. 1992;82:87–93.

27. Fischer K, Van der Bom JG, Mauser-Bunschoten EP, et al. Changes in treatment strategies for severe haemophilia over the last 3 decades: effects on clotting factor consumption and arthropathy. Haemophilia. 2001;7:446–52.

28. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. N Engl J Med. 2007;357:535–44.

29. Petrini P. What factors should influence the dosage and interval of prophylactic treatment in patients with severe haemophilia A and B? Haemophilia. 2001;7:99–102.
30. Philpott J, Houghton K, Luke A. Physical activity recommendations for children with specific chronic health conditions: juvenile idiopathic arthritis, hemophilia, asthma and cystic fibrosis. Paediatr Child Health. 2010;15:213–25.
31. Djulbegovic B, Marasa M, Pesto A, et al. Safety and efficacy of purified factor IX concentrate and antifibrinolytic agents for dental extractions in hemophilia B. Am J Hematol. 1996;51:168–70.
32. Ross C, Goldenberg NA, Hund D, et al. Athletic participation in severe hemophilia: bleeding and joint outcomes in children on prophylaxis. Pediatrics. 2009;124:1267–72.
33. Astermark J, Donfield SM, DiMichele DM, et al. A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the FEIBA NovoSeven Comparative (FENOC) Study. Blood. 2007;109:546–51.
34. Schneiderman J, Rubin E, Nugent DJ, et al. Sequential therapy with activated prothrombin complex concentrates and recombinant FVIIa in patients with severe haemophilia and inhibitors: update of our previous experience. Haemophilia. 2007;13:244–8.
35. Leissinger C, Gringeri A, Antmen B, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. N Engl J Med. 2011;365:1684–92.
36. Wight J, Paisley S, Knight C. Immune tolerance induction in patients with haemophilia A with inhibitors: a systematic review. Haemophilia. 2003;9:436–63.
37. Mariani G, Kroner B, For the Immune Tolerance Study Group. Immune tolerance in haemophilia with inhibitors: predictors of success. Haematologica. 2001;86:1186–93.
38. DiMichele DM, Kroner BL. The North American Immune Tolerance Registry: practices, outcomes, outcome predictors. Thromb Haemost. 2002;87:52–7.
39. Kroner BL. Comparison of the international immune tolerance registry and the North American immune tolerance registry. Vox Sang. 1999;77(Suppl 1):33–7.
40. Hay CR, DiMichele DM. The principal results of the International Immune Tolerance Study: a randomized dose comparison. Blood. 2012;119:1335–44.
41. Sehgal A, Barros S, Ivanciu L, et al. An RNAi therapeutic targeting antithrombin to rebalance the coagulation system and promote hemostasis in hemophilia. Nat Med. 2015;21:492–7.
42. Chowdary P, Lethagen S, Friedrich U, et al. Safety and pharmacokinetics of anti-TFPI antibody (concizumab) in healthy volunteers and patients with hemophilia: a randomized first human dose trial. J Thromb Haemost. 2015;13:743–54.
43. Kitazawa T, Igawa T, Sampei Z, et al. A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model. Nat Med. 2012;18:1570–4.
44. Shima M, Hanabusa H, Taki M, Matsushita T, Sato T, Fukutake K, Fukazawa N, Yoneyama K, Yoshida H, Nogami K. Factor VIII-mimetic function of humanized bispecific antibody in hemophilia A. N Engl J Med. 2016;374:2044–53.
45. Yoshitake S, Schach BG, Foster DC, et al. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). Biochemistry. 1985;24:3736–50.
46. Tanimoto M, Kojima T, Kamiya T, et al. DNA analysis of seven patients with hemophilia B who have anti-factor IX antibodies: relationship to clinical manifestations and evidence that the abnormal gene was inherited. J Lab Clin Med. 1988;112:307–13.
47. Ljung R, Nilsson IM. Hemophilia B Leyden and a similar variant of hemophilia A. N Engl J Med. 1982;307:897–8.
48. Kurachi K, Davie EW. Isolation and characterization of a cDNA coding for human factor IX. Proc Natl Acad Sci U S A. 1982;79:6461–4.
49. High KA. Factor IX: molecular structure, epitopes, and mutations associated with inhibitor formation. Adv Exp Med Biol. 1995;386:79–86.
50. Thorland EC, Drost JB, Lusher JM, et al. Anaphylactic response to factor IX replacement therapy in haemophilia B patients: complete gene deletions confer the highest risk. Haemophilia. 1999;5:101–5.

51. Astermark J. Basic aspects of inhibitors to factors VIII and IX and the influence of non-genetic risk factors. Haemophilia. 2006;12(Suppl 6):8–13.
52. Shapiro AD, Ragni MV, Valentino LA, et al. Recombinant factor IX-Fc fusion protein (rFAC-TOR IXFc) demonstrates safety and prolonged activity in a phase 1/2a study in hemophilia B patients. Blood. 2012;119:666–72.
53. Santagostino E, Martinowitz U, Lissitchkov T, et al. Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: results of a phase 3 trial. Blood. 2016;127:1761–9.
54. Collins PW, Møss J, Knobe K, et al. Population pharmacokinetic modeling for dose setting of nonacog beta pegol (N9-GP), a glycoPEGylated recombinant factor IX. J Thromb Haemost. 2012;10:2305–12.
55. Collins PW, Young G, Knobe K, et al. Recombinant long-acting glycoPEGylated factor IX in hemophilia B: a multinational randomized phase 3 trial. Blood. 2014;124:3880–6.
56. Morfini M, Laguna P, Leissinger C. Factor IX pharmacokinetics: differences between plasma-derived and recombinant products and the clinical and economic implications: a meeting report. Haemophilia. 2008;14:873–5.
57. Poon MC, Aledort LM, Anderle K, et al. Comparison of the recovery and half-life of a high-purity factor IX concentrate with those of a factor IX complex concentrate. Transfusion. 1995;35:319–23.
58. Chitlur M, Warrier I, Rajpurkar M, et al. Inhibitors in factor IX deficiency a report of the ISTH-SSC international FACTOR IX inhibitor registry (1997-2006). Haemophilia. 2009;15:1027–31.
59. Recht M, Pollmann H, Tagliaferri A, et al. A retrospective study to describe the incidence of moderate to severe allergic reactions to factor IX in subjects with haemophilia B. Haemophilia. 2011;17:494–9.
60. Beutel K, Hauch H, Rischewski J, et al. ITI with high-dose FACTOR IX and combined immunosuppressive therapy in a patient with severe haemophilia B and inhibitor. Hamostaseologie. 2009;29:155–7.
61. Nathwani AC, Tuddenham EG, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med. 2011;365:2357–65.
62. Nathwani AC, Reiss UM, Tuddenham EG, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med. 2014;371:1994–2004.

Chapter 10
Thrombotic Disorders

Shouichi Ohga and Masataka Ishimura

Abstract Thromboembolism occurs as a consequence of the genetic predisposition, underlying diseases, and triggers of dehydration, infection, and injury. Thrombotic events are much rarer in infants and children than in adults. Aging is a potent risk factor for the development of venous thrombosis and stroke. The genetic effects on the hypercoagulability in pediatric patients are thus more carefully considered than in adult patients. The genetic predisposition of thromboembolism depends on the racial background. Factor V Leiden (G1691A) and factor II variant (G20210A) are the common thrombophilias in Caucasians, but not found in Asian ancestries. The incidence of pediatric thrombosis is increasing because of the advances in neonatal intensive care, cardiovascular surgery, and imaging techniques. There is an increasing number of reports on the molecular epidemiology of constitutional thrombophilias also in Asian countries. On the other hand, the treatment and prophylaxis of pediatric thrombosis have not been established. The advent of direct oral anticoagulants (DOACs) has opened a new era of anticoagulation for adult patients with thrombosis, while the management of heritable thrombophilia is under investigation. This chapter briefly introduces the thrombotic disorders in neonates, infants, and children, with special reference to the high-risk-inherited thrombophilia of natural anticoagulant deficiency.

Abbreviations

APC   Activated protein C
AT   Antithrombin
CVST   Cerebral venous sinus thrombosis
DVT   Deep vein thrombosis
ICTH   Intracranial thrombosis/hemorrhage

S. Ohga, M.D., Ph.D. (✉) • M. Ishimura
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University,
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: ohgas@pediatr.med.kyushu-u.ac.jp

| NETs	Neutrophil extracellular traps
OR	Odds ratio
PC	Protein C
PIVKAII	Protein induced by vitamin K absence or antagonists
PS	Protein S
PT	Prothrombin time
VTE	Venous thromboembolism

10.1 Introduction

Thromboembolisms occur as a consequence of genetic predispositions, underlying disorders, and various triggers including infection and injury. Research advances have recently characterized the cellular and molecular mechanisms of thrombosis that interdependently influence on the Virchow's triad, the vessel wall (endothelial damage), blood flow (stasis), and blood components (hypercoagulability). Stasis and hypercoagulability are crucial for the formation of venous thrombi that mainly consist of fibrin and red cells [1]. In contrast, platelets are essential for atherosclerosis and arterial thromboembolism, because of the pivotal role in primary hemostasis and repair of injured endothelium [2]. Neutrophil extracellular traps (NETs) are the scaffold of venous thrombosis in infection, because the network of extracellular fibers primarily composed of neutrophil DNA binds the pathogens [3]. Inflammation, lipids and the immune system, and their complex interplay with tissue factors are the determinants of arterial as well as venous thromboembolism.

Thromboembolic events are less commonly encountered in children than in adults. Newborn infants are at the highest risk, and adolescents are at the second highest risk of thrombosis until the adult life [4]. Circulating coagulant and anticoagulant factors attain the adult levels until the adolescence. The imbalance may in part explain the age-dependent thrombotic risks. However, the vascular and endothelial factors rather than the hematologic factors could contribute to the paucity of pediatric thrombosis.

Hereditary defects in the coagulant and anticoagulant pathways, but less fibrinolytic pathway, lead to the development of venous thromboembolism (VTE) with or without identifiable triggers. Acquired risk factors for systemic thromboses include diverse and heterogeneous conditions according to age (Fig. 10.1). Caucasian peoples were believed to have much higher risk of thrombosis than Asians. However, there is an exponential increase in the risk of both arterial and venous thrombotic events with age in the aging society. The incidence of pediatric thrombosis is also increasing in developed countries [5]. These trends reflect the medical progress in imaging techniques, neonatal intensive care (i.e., central venous lines), and pediatric cardiology (i.e., surgical and catheter interventions) [6, 7]. The established genetic risks of VTE include the deficiency of natural anticoagulants of protein C (PC), protein S (PS), and antithrombin (AT), as well as the variants of coagulation
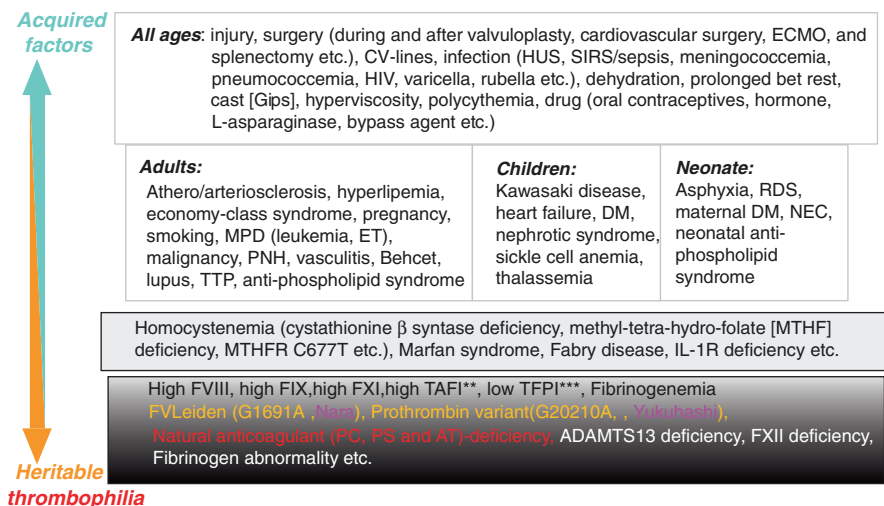
Fig. 10.1 Systemic thromboembolic conditions in children and adults. *TTP* thrombotic thrombocytopenic purpura, *ECMO* extracorporeal membrane oxygenation, *TAFI* thrombin-activatable fibrinolysis inhibitor, *TFPI* tissue factor pathway inhibitor

factors of factor V G1691A (FV Leiden) and prothrombin (FII) G20210A [8]. The high incidence of VTE in Caucasians accounts for the fact that FV Leiden and FII G20210A carriers are found in 20–60% of adult VTE patients in Caucasian but not Asian ancestries [9]. The deficiency of PC, PS, and AT is hard to diagnose in infants and young children without the genetic tests. Intensive efforts for whole exome/genome screenings identified additional genes with a relative contribution to thrombotic risk. "Omics" strategies will unveil the disease process and the targeted treatment for pediatric venous thromboembolism and arterial ischemic stroke [10]. We review herewith thrombophilia in infants and children.

10.2   Inherited Thrombophilia and Genetic Predisposition to Thrombosis

Thromboembolic events were less commonly reported in children than adults. The recent increase of pediatric thrombosis is attributed to advanced critical care and improved survival of high-risk infants. During three decades, neonatal managements have greatly improved the survival of preterm infants. Newborn infants are originally prone to thromboembolic events because of the physiologic *hypercoagulability* of the developing hemostatic system [11]. Sick preterm newborns all need central venous lines which run a high risk of VTE. The prothrombotic conditions may be augmented in the newborns harboring a heterozygous mutation of PC gene [12].

Thrombophilia is a disease entity to define severe congenital deficiency of PC, PS, and AT, for the analogy of *hemophilia*. On the other hand, persons having the heterogeneous mutation do not always experience thromboembolic events, and most of the patients develop thromboembolism over the mid-40s. Taken into account the distinct penetrance, *genetic predisposition/diathesis to thrombosis* is the precise nature of adult-onset but not pediatric-onset heritable thrombophilia.

10.2.1 Antithrombin (AT) Deficiency

AT is a plasma protein synthesized by the liver. A mutation in the AT gene (*SERPINC1*) located on chromosome 1q23-25 was identified in the first reported family with AT deficiency [13, 14]. The anticoagulant factor binds and neutralizes the serine proteases of thrombin, factor Xa, and factor IXa, which are generated by the coagulation cascade. The AT-mediated effects on the inhibition of these proteases are enhanced by binding to heparin. AT has two major active sites, the reactive center toward the carboxyl terminus and the heparin-binding site at the amino terminus. Thrombin cleaves the reactive site, and the inactive complex molecules are then cleared from circulation. Heterozygotes for AT deficiency are found in about 1% of patients having the first episode of deep venous thrombosis (DVT). The thrombotic risk of inherited AT-, PS-, or PC-deficient patients is higher than that of FV Leiden or PTII variant even in infants and children (Table 10.1). They have an eight- to tenfold higher risk for thrombosis than noncarriers [4, 15, 16]. More than 50% of the first-degree relatives of each deficient patients experience VTE until the first 25 years of life [17]. The first thrombotic episode of AT deficiency occurs in more than 40% of patients spontaneously. The remaining patients have some triggers including pregnancy, parturition, contraceptive use, surgery, or trauma.

Table 10.1 Reported risk of thromboembolism in thrombophilic children

| | Relative risk (odds ratio) | [95% conf. interval] | |
| | Cerebrovascular occlusion (first) | Deep vein thrombosis | |
Genetic predisposition		First attack	Recurrence
Protein C deficiency	9.3 [4.8–18.0]	7.7 [4.4–13.4]	2.4 [1.2–4.4]
Protein S deficiency	3.2 [1.2–8.4]	5.8 [3.0–11.0]	3.1 [1.5–6.5]
Antithrombin deficiency	7.1 [2.4–22.4]	9.4 [3.3–26.7]	3.0 [1.4–6.3]
Factor V G1691A	3.3 [2.6–4.1]	3.6 [3.8–4.8]	1.4 [0.4–1.2]
Factor II G20210A	2.4 [1.7–3.5]	2.6 [1.6–4.4]	2.1 [1.01–3.5]
Lipoprotein (a)	6.5 [4.5–8.7]	4.5 [3.3–6.2]	0.8 [0.5–1.4]
LA/aPL	6.6 [3.5–12.4]	4.9 [2.2–10.9]	
More than 3	11.9 [5.9–23.7]	9.5 [4.9–18.4]	4.5 [4.5–6.9]

LA/aPL lupus anticoagulants/antiphospholipid antibodies (modified from [4])

The odds ratio (OR) of thrombosis with AT deficiency is estimated to be 9.4, although the risk depends on the population selected (Table 10.1) [4]. The prevalence of AT deficiency was 1.1%, and the OR for thrombosis was 5.0 according to the Leiden Thrombophilia Study [18]. Heritable AT deficiency is an autosomal dominant disease with equal sex distribution. Common affected sites of thrombosis include deep veins in the legs, mesenteric veins, and pulmonary embolism. Complete AT defects due to null mutation lead to the fetal loss. Therefore, neonatal purpura fulminans is not the regular presentation of AT deficiency. Recurrent thromboses occur in approximately 60% of patients. AT deficiency may also be associated with perinatal, neonatal, and childhood arterial ischemic stroke. Cerebral venous sinus thrombosis (CVST) is the other presentation of AT deficiency. Although AT-deficient children are mostly asymptomatic before puberty, the substantial risk of thrombosis increases with age.

Heritable AT deficiency is classified into two types. Type I-deficient individuals show paralleled reduction of both antigen and activity levels of AT, representing a reduced synthesis of biologically normal protease inhibitor molecules. AT deficiency arises from mostly point mutations of *SERPINC1* and rarely a deletion encompassing the gene. More than 100 mutations have been reported in type I-deficient patients [19]. Type II-deficient subjects show reduced levels of plasma activity but normal antigen levels of AT, representing a discrete molecular defect. Type I deficiency is subdivided into Ia (normal) and Ib (reduced), based on the heparin affinity. Type II deficiency is subcategorized into three groups assessed by progressive AT assays and heparin-binding assays, the molecular abnormality in reactive site (RS, 393Arg-394Ser), heparin-binding site (helices A and D) (HBS), and PE (pleiotropic effect). Type Ib is now designated as type II PE, because of the *pleiotropic* abnormalities affecting the reactive site, the heparin-binding site, and plasma concentration.

Type II HBS variants are infrequently associated with thrombotic events. The homozygotes were, however, reported in young children with recurrent thromboses born to consanguineous healthy parents carrying type II HBS. The prevalence of type I AT deficiency, estimated to be 1 in 2000 adults [20], rises to 1 in 250–500 healthy blood donors screened by heparin cofactor activity [21]. The mean concentration of AT in normal pooled plasma is about 12.5 mg/dL. The standard value ranges between 75 and 120% of normal pooled plasma for AT-heparin cofactor determinations. Circulating AT levels decrease due to consumption in cases of sepsis, burns, and disseminated intravascular coagulation (DIC), but less acute thrombosis. Reduced AT levels are ascribed to impaired production and loss in liver disease and nephrotic syndrome, respectively. Hormonal effects (oral contraceptives or estrogens) and heparinization reduce plasma AT levels. The physiologically low AT levels in healthy newborns gradually reach the adult levels by 6 months of age [11, 22]. Preterm infants show much lower AT levels than term ones, the levels of which are further depressed in respiratory distress syndrome, sepsis, necrotizing enterocolitis, and DIC. The levels of alpha-2-macroglobulin, which is a second thrombin inhibitor and also an acute phase protein, are higher in children than adults. It reduces the chance of pediatric thrombosis especially in infection. The family members having affected proband need a correct diagnosis for genetic counseling, replacement therapy, and prophylaxis in daily life.

10.2.2 Protein C (PC) Deficiency

PC is a vitamin K-dependent anticoagulant factor, which is synthesized by the liver and circulates as a zymogen. It exerts the proper anticoagulant function after the cleavage of the molecule to the serine protease, activated PC (APC). This activation is mediated by thrombin alone, but occurs more efficiently when thrombin is bound to thrombomodulin on the endothelial cell receptor. APC inactivates FV and FVIII by cleaving the critical sites of these activated molecules. This reaction is enhanced by PS, FV, and lipid cofactors of lipoproteins and phospholipids. Reduced concentration of circulating PC fails to control the propagation of thrombin generation by FVa and FVIIIa, even if PC deficiency originates from the decreased production and/or increased consumption. APC has anti-inflammatory and cytoprotective functions [23]. These are directly driven by the endothelial and immunocompetent cells via the specific receptor on the given cells. The protective effect of APC in animal models of sepsis depends on its capacity to activate protease-activated receptor-1 (PAR1) and not on its anticoagulant properties. The pleiotropic effects were expected for the treatment of sepsis. However, clinical trials of recombinant APC were withdrawn for the risk of bleeding [24]. Refined molecules of APC are being explored for the effective treatment of sepsis and DIC [25].

Plasma levels of PC activity are assessed by chromogenic (amidolytic) or coagulometric (clotting) assays. The standard value in healthy adults ranges from 0.65 to 1.35 IU/mL (65–135% of normal). "Mild," "moderately severe," and "severe" PC deficiencies are defined as the range of >20% (>0.2 IU/mL), 1–20% (0.01–0.2 IU/mL), and <1% (<0.01 IU/mL), respectively [22]. PC gene (*PROC*) is located on chromosome 2q13-q14. The first report of PC deficiency was a 22-year-old Caucasian with recurrent thrombophlebitis and pulmonary embolism [26]. Heterozygous *PROC* mutation inherits "mild" or at most "moderately severe" PC deficiency in an autosomal dominant manner. The first presentation of PC deficiency includes the newborn-onset and the teens-onset modes. Neonatal purpura fulminans and stroke are the proper expression of PC deficiency [27, 28]. Thereafter, DVT in the legs and pulmonary thromboembolism are commonly affected sites. Approximately 70% of patients first present in teens spontaneously and the remaining 30% with the risk factors. Nonhemorrhagic arterial stroke is associated with pediatric PC deficiency. PC deficiency is the major heritable thrombophilia in Japanese children [29], while PS deficiency is that in the adult cases [30, 31].

Severe PC deficiency is inherited in an autosomal recessive fashion. The complete defects exclusively arise from biallelic *PROC* mutation, presenting neonatal purpura fulminans and/or intracranial hemorrhage. It is hard to differentiate in infancy between primary and secondary forms of PC deficiency, because of the physiologically low activity. Both antigen and activity levels increase after birth and reach the lower limit of adult references (~50 IU/dL) during 6 months to 1 year of age [11, 32]. PC levels increase in adolescence and further do 4% per decade in adults. Standard PC activity has wide ranges than PS activity during the first 7 days after birth. PC levels in preterm infants are much lower than those in term infants.

Acquired PC deficiency occurs in the settings of neonatal asphyxia, liver disease, sepsis, and DIC, along with acute thrombosis. Newborn infants are prone to vitamin K deficiency bleeding. For the genetic screening, PC activity levels should be followed concurrently with PS activity, protein induced by vitamin K absence or antagonists II (PIVKAII), D-dimer, and antiphospholipid antibodies, along with FVII activity. Unexplained dissociation between PC and PS activity levels portends a diagnosis of heritable PC deficiency [28, 33].

The prevalence of PC deficiency was initially estimated between 1 in 16,000 and 1 in 32,000 within the general population, based on the assumptions that the autosomal dominant disorder shows more than 50% of penetrance. However, the parents of infants with neonatal purpura fulminans rarely experience thrombotic events. Each parent has a heterozygous *PROC* mutation resulting in the modest effect on the thrombotic activity. The prevalence of heterozygosity for PC deficiency is considered to be 1/200–500 [34]. Heterozygous PC-deficient adults have a sevenfold increased risk for an initial episode of DVT compared with those having normal PC activity. On the other hand, asymptomatic carriers have low annual incidence of thrombosis at less than 1.0%. The OR of pediatric thrombosis with PC deficiency is estimated at 7.7 [4] (Table 10.1).

Heterozygous PC deficiency is classified into two types. The common form of type I represents an equally reduction in both immunologic and biologic activities. More than 200 mutations and rarely deletions were identified [35]. Type II-deficient subjects show normal antigen and decreased activity levels. However, several cases show normal levels of antigen and amidolytic activity, but with reduced levels of clotting activity. It may be ascribed to a reduced ability of APC to interact with the platelet membrane or its substrates of FVa and FVIIIa. The coagulant assay has then high sensitivity in screening for PC deficiency. Nevertheless, repeated studies are needed to clarify the mutation spectrum. Recently, K193del has been recognized as the most common variant in Chinese thromboembolisms [36]. On the other hand, it was considered as a polymorphism in Japan, because the heterozygotes often show normal activity for PC in resting conditions [37]. It was originally reported as PC-Tottori, in the homozygote that developed DVT for the first time at 28 years of age [38]. Based on our observations [12], age-dependent increase of PC activity seems to be delayed in the heterozygote infants for PC-Tottori. There is increasing number of reports in Asian countries on the patients with double mutations who escaped neonatal thromboses. In Caucasians, FV Leiden and FII variant might mask the effects of *PROC* variants on the thrombotic risk of patients [39].

PC activity is recommended to assess with the concurrent measurements of FVII, a vitamin K-dependent zymogen with a similar short plasma half-life, to exclude the effects of consumption coagulopathy. Discontinuation of oral warfarin therapy is needed to screen the baseline PC (and PS) activity. However, heparin is substituted in patients who cannot be stopped for anticoagulant therapy because of severe thrombotic diathesis. It occurs a few days after the start of warfarin therapy, in association with the initial loading doses. Erythematous macules progressively expand with the necrosis on the trunk and extremities over several hours. The clinical setting and histopathology (fibrin thrombi and interstitial hemorrhage) share

those of neonatal purpura fulminans. Even the initial standard dose of warfarin reduces PC activity to about half of the normal within 1 day. FVII levels decline followed by the decreased activity of other vitamin K-dependent factors according to the half-lives. The half-life of plasma PC (6–8 h) is shorter than that of PS and other procoagulant vitamin K-dependent factors. The enzymatic activity of PS depends on free PS concentration. Acute inflammation reduces PS activity due to binding with C4b. Warfarin-induced skin necrosis is a noticeable complication in PC- or PS-deficient cases with infection. On the other hand, neonatal purpura fulminans has been exclusively reported in PC deficiency. It may be explained by not only the shorter half-life of PC but also the low binding capacity of C4b to PS at birth. Replacement of PC concentrates is essential for the treatment of purpura fulminans [40–43].

PC and PS levels are influenced in diverse conditions throughout childhood [35]. Vitamin K antagonists reduce the levels of PC and PS activities. Vitamin K deficiency precipitates bleeding (i.e., hemorrhagic diseases of the newborn, vitamin K deficiency bleeding in infancy) and also thrombosis (i.e., warfarin-induced skin necrosis). Severe infections lower the plasma activity of natural anticoagulants. The mechanisms of "infectious purpura fulminans" are involved in antibody-mediated coagulopathy (i.e., post-varicella purpura fulminans) or toxic effects (i.e., meningo-coccemia purpura fulminans) associated with acquired PC/PS deficiency. PC or PS deficiency arises from the loss or consumption in patients with nephrotic syndrome, sepsis, and/or DIC and from the impaired synthesis in those with liver dysfunction. In contrast to AT, antigenic concentrations of vitamin K-dependent plasma proteins are elevated in nephrotic syndrome. Uremic patients have low anticoagulant activity, but normal amidolytic activity and antigen levels for PC, because of the interfering dialyzable moiety with clotting PC activity. Warfarin therapy reduces functional and, to a lesser extent, immunologic assays of PC. These conditions complicate the diagnosis of PC deficiency.

10.2.3   Protein S (PS) Deficiency

PS is a vitamin K-dependent protein that enhances the anticoagulant effect of APC. This coenzyme is primarily synthesized by hepatocytes and also by endothelial cells, megakaryocytes, and brain cells. It serves as a cofactor for APC in the setting of FVa and FVIIIla inactivation. The inactivation of FVa occurs, at first, in the rapid cleavage at Arg506 (FV Leiden, Gln506) of the molecules, followed by the slower cleavage at Arg306 (second binding site of PS with APC) and then Arg679 [44]. PS with APC increases the affinity for phospholipids to enhance FVa inactivation. Approximately 40% of circulating PS molecules is in the free form, and the remainder 60% is bound to C4b-binding protein (C4BP) not to interact with APC. Free PS levels are responsible for the direct anticoagulant effects.

PS deficiency was first described in 1984 in the families with low PS activity and recurrent thrombosis [45]. Heterozygous mutations in the gene encoding protein S

(*PROS1*) on chromosome 3q11 cause autosomal dominant thrombophilia. Homozygotes or compound heterozygotes for the mutation are quite rarely found as an autosomal dominant thrombophilia. However, most reported cases of neonatal purpura fulminans had PC but not PS deficiency [22, 28]. It may account for the relative increase of free PS concentrations (by physiologically low levels of C4BP) and the narrower ranged activity and shorter half-life of PS during the early neonatal period.

PS deficiency confers a risk of thrombosis similarly to PC deficiency. The clinical findings in the heterozygotes for PS deficiency are similar to those for AT or PC deficiency. Common affected sites are DVT and pulmonary emboli. The first thrombotic event in PS deficient patients occurs at approximately 25–30 years of age. More than half of the episodes were spontaneous, and the remainder were associated with identifiable factors. In pediatric cases, the OR for venous thrombosis due to PS deficiency is estimated to be 5.77 (Table 10.1) [4]. Several reports suggest an association between PS deficiency and arterial thromboses including ischemic stroke in infants and children [46].

On the other hand, adults with low PS levels but no family history of venous thrombosis have minimal risk of VTE. Low free PS or total PS levels (both <0.10th percentile) were not associated with an increased risk of VTE. Young patients with recurrent VTE are associated with double mutations of *PROS1*. Their parents were reportedly asymptomatic although they carried type I PS deficiency. PS deficiency is classified into three types, according to the levels of total and free antigens, along with functional activity. Type I deficiency shows about half levels of normal PS antigen, although free PS antigen and functional activity levels are greatly reduced [47]. Most patients have the missense mutations and base pair insertions. Type II is a qualitative deficiency with normal levels of total antigen and free PS, but impaired function. The rarity of type II-deficient patients indicates the insufficient screening power of functional assays. Type III is characterized by normal levels of total PS antigen, but reduced levels of free PS and functional activity. Plasma activity levels rather than antigen levels are then preferable for screening PS deficiency. Although the biologic basis of type III PS deficiency remains unclear, *PROS1* mutations were responsible for the type I deficiency, but not the type III phenotype of age-dependent free PS deficiency [48]. The prevalence of PS deficiency depends on the ethnicity. In Asian countries, PC and PS deficiencies are dominantly reported in adults and children with thrombosis [49–51]. PS-Tokushima K196E is found in one per 55 healthy Japanese adults as a common variant for thrombosis in Japan [52].

The lower limit of plasma levels of total and free PS (~65%) in heterozygous deficiency considerably overlaps the ranges of healthy controls [28]. PS levels are lower in female than male and increase with advanced age. PS, PC, and AT levels are reduced in inflammation. Because C4BP is an acute phase reactant, it shifts PS to the complexed inactive form leading to the reduced PS activity. On the other hand, PS but not PC levels decrease in pregnancy. In cases of nephrotic syndrome, the balance of elevated C4BP concentrations and loss of free PS reduce the functional activity levels despite the increased antigen levels. Repeat sampling, family studies, and genetic tests are required to make the correct diagnosis for pediatric thrombophilia.

10.2.4 Factor V Leiden

The resistance to APC in an activated partial thromboplastin time (APTT)-based clotting assay accounts for familial thrombophilia [53]. The major genotype of APC resistance is FV Leiden (Arg506Gln) [54], being found in more than 80% of APC resistant patients. In Western countries, FV Leiden was found in 20–50% of adult patients with VTE. FV Leiden is a risk factor for venous and arterial thrombosis. Retrospectively, heterozygosity for FV Leiden was identified in 12% of patients who had a first episode of DVT or pulmonary embolism and in 6% of controls. In the elderly (>60 years of age) who suffered from the first VTE without triggers, 26% were heterozygotes for FV Leiden. Pediatric studies found that FV Leiden heterozygosity conferred an odds ratio of 3.6 for a first episode of VTE and 1.4 for recurrent VTE in children [4] (Table 10.1). The OR for pediatric CVST in FV Leiden carriers was 2.74. On the other hand, the annual incidence of VTE in asymptomatic carriers of the FV Leiden is low (0.58%), which raises questions about the screening asymptomatic family members [55]. FV Leiden was not associated with the risk of myocardial infarction or stroke, but potentially perinatal and pediatric arterial ischemic stroke. FV Leiden homozygotes may have the similar high risk of VTE to the heterozygotes for AT, PC, or PS deficiency. As FV and AT genes are located on chromosome 1p, the coinheritance of FV Leiden and AT mutations leads to a more severe thrombotic diathesis.

Heritable thrombophilias including FV Leiden are associated with a tripling risk for the late fetal loss, but no risk for the first-trimester loss. High risk of thrombophilias was reported in association with obstetric complications other than preeclampsia and intrauterine growth retardation. The prevalence of heterozygosity for FV Leiden ranges 1–9% in Caucasians, but quite rare (<1%) in African blacks, Chinese, Japanese, or Native American ancestries. FV Leiden is more prevalent in northern Europe than the southern countries. A single founder allele was suggested among whites of differing ethnic backgrounds. Severe infections might shape the distribution of FV Leiden, because of the survival advantage inferred by the mice models. Co-segregation of heterozygous APC resistance due to FV Leiden and type I FV deficiency results in severe APC resistance in APTT assays, as found in homozygous FV Leiden patients (pseudohomozygous). Several polymorphisms in FV gene include a haplotype (HR2) containing the R2 polymorphism (Hisl299Arg) with mild APC resistance. Although FV Leiden was not found in Japan, FV Nara showing APC resistance has been recently identified in a Japanese family with recurrent thrombosis [56].

10.2.5 Prothrombin Variant

FII (prothrombin)-related thrombophilias are encountered in adults with VTE. Many individuals heterozygous and homozygous for FII G20210A develop no thrombosis [57]. Most patients of the heterozygotes also remain asymptomatic until adulthood.

On the other hand, the relative risk of thrombosis in the heterozygotes is increased two- to fivefold in adults and three- to fourfold in children. The recurrent risk for VTE is modest in FII G20210A carriers. Pregnant loss and obstetric complications in the carriers are similar to those in FV Leiden carriers. The diagnosis depends on the genetic study. The FII variant is not found in Asian ancestries, similarly in FV Leiden [39], although FII Yukuhashi with AT resistance has been recently identified in a Japanese family [58].

Because of the relatively low risk of thrombosis in FV Leiden and FII G20210A carriers, the routine screening is not recommended for healthy individuals as long as they have no positive family history of recurrent or young thromboses.

10.3   Other Thrombotic Predispositions

Thromboembolic events occur in association with inherited underlying diseases and/or acquired conditions. Increased FVIII levels raise the prothrombotic risk [59], although no causative genes for the predisposition have been identified. Elevated levels of FVIII and D-dimer could be predictors of poor outcomes of pediatric thrombosis [60]. High levels of other coagulation factors (von Willebrand factor, fibrinogen, FIX, and FXI) and low levels of anticoagulation factors (tissue factor pathway inhibitor, TFPI), along with dysregulation of or fibrinolytic factors (high thrombin-activatable fibrinolysis inhibitor, TAFI), have been suggested to confer a risk of thromboembolism. Hyperlipemia and inflammatory diseases causing vasculitis are involved in the development of thromboembolism (Fig. 10.1).

Hyperhomocysteinemia is an independent risk factor for venous and arterial thromboses, which resulting from the genetic and acquired abnormalities [61]. Homocystinuria is a rare autosomal recessive inborn error of metabolism presenting in childhood. Homozygous cystathionine β-synthase deficiency is the most common cause of homocystinuria (~1 in 250,000 of general population). Affected children show premature atherosclerosis and arterial and venous thromboembolism, along with mental retardation, ectopic lenses, and skeletal abnormalities. The mechanism of atherogenic and thrombogenic process remains elusive. Heterozygous cystathionine β-synthase deficiency is found in about 0.3% of the general population. However, a variant of methyltetrahydrofolate reductase (MTHFR) (p.Ala222Val, c.C677T polymorphism) is commonly found, the heterozygotes in 61% and the homozygotes in 1.4–15% of the population. It causes thermolability of MTHFR to reduce 50% of the specific activity. Mutated MTHFR genes impair the remethylation pathway to raise the homocysteine levels under fasting conditions. Acquired hyperhomocysteinemia also occurs in association with the deficiency of folate, vitamin B12 and vitamin B6. Although MTHFR C677T has a low impact on developing VTE [62] and vitamin supplementation did not reduce the risk of recurrent arterial thromboembolic disease [63], treatable high homocysteine conditions might occupy more than 10% of VTE patients [64].

Thrombotic microangiopathy (TMA) induces venous and arterial thromboses in children. TMA is a generic disease entity presenting the triad in order: (1) formation of the microangiopathic platelet thrombi, (2) destructive/consumptive thrombocytopenia, and (3) subsequent hemolytic anemia with fragmentation of erythrocytes (red cell fragmentation syndrome). Microcirculatory thrombi lead to the organ failure involving kidney and central nervous system. TMA is classified into thrombotic thrombocytopenic purpura (TTP) and hemolytic-uremic syndrome (HUS), both of which are hard to differentiate in practice. The classic pentad of TTP includes thrombocytopenia, hemolytic anemia with red cell fragmentation, diffuse and non-focal neurologic findings, renal failure, and fever. The triad of HUS consists of thrombocytopenia, microangiopathic hemolytic anemia (nonimmune, Coombs negative), and acute renal failure.

TMA (TTP/HUS) results from the genetic and acquired abnormalities. Chronic relapsing familial TTP (Upshaw-Schulman syndrome, USS) and atypical HUS belong to heritable TMA [65]. USS is caused by the mutation in *ADAMTS13* gene, which encodes the von Willebrand factor (VWF)-cleaving protease (VWFCP). USS is characterized by hemolytic disease of the newborn with thrombocytopenia, response to fresh plasma infusion, and frequent relapses. Thrombotic events are the noticeable complication in pregnant women with ADAMTS13 gene mutation [66]. Acquired TTP occurs in patients with autoimmune diseases, transplantation, and cardiac surgery. Various causes for HUS include infections, drugs, tumors, pregnancy, and systemic lupus erythematosus. The major cause of verotoxin-induced (diarrhea [D]-plus) HUS is shigella bacteria, most frequently the 0157:H7 strain. On the other hand, 10% of HUS patients suffer from aHUS, which is a group of monogenic diseases presenting with the recurrent attacks. More than half of patients with aHUS have genetically determined the alterations of complement system. Approximately 30% of aHUS patients have factor H disease. Factor H is one of the regulatory proteins of the complement system that protect blood vessels from injury. Factor H diseases arise from the loss of function mutations of *CFH* and *CHFR1-5*. On the other hand, anti-factor H autoantibodies are found in 6–10% of cases, mostly children homozygous for the deletion of the *CFHR1* and *CFHR3* genes. Other cases are associated with loss-of-function mutations in genes encoding other complement regulatory proteins, membrane cofactor protein, and factor I or with gain-of-function mutations in genes encoding the key complement proteins complement factor B and C3. Mutations in the gene encoding thrombomodulin, an endothelial anticoagulant glycoprotein exerting complement regulatory properties, and enhancing the function of APC, have been found in 3–5% of aHUS patients. aHUS children result in chronic renal failure after the recurrent attacks.

The presence of lupus anticoagulant with antiphospholipid antibody is one of the prothrombotic factors in adult patients with autoimmune diseases. Catastrophic antiphospholipid syndrome leads to multiple cerebral infarctions and amputation of the extremities in affected patients. On the other hand, antiphospholipid antibody is often produced in infants and children with or without apparent symptoms [67]. This condition tends to precipitate transient hemorrhagic diathesis rather than

thrombosis in children. Autoinflammatory disorders include Crohn disease and Behcet disease in a broad sense. The inflammatory diseases are closely associated with thrombotic events. Recently, pediatric thromboses have been reported in cases with autoinflammatory disorders of monogenic origin [68].

10.4 Managements

Standard management for the first acute thrombosis in adults consists of a course of low-molecular-weight heparin (LMWH) or fondaparinux and concurrent warfarin therapy for at least 5 days on the monitoring for international normalized ratio (INR). Rivaroxaban is approved for treatment of acute VTE and prevention of recurrence in adult cases. FXa inhibitors raise no chance of developing heparin-induced thrombocytopenia (HIT). However, the pediatric use of FXa inhibitors is challenging, because the practical monitoring is on controversy [69, 70]. The standard therapy using tissue plasminogen activator for arterial thrombosis has not been established in the newborn infants.

The concept of anticoagulation therapy depends on the potential risks for (1) VTE recurrence and (2) therapy-related bleeding. Individuals with a spontaneous thrombosis with no identifiable provoking factors and those with persistent risk factors are candidates for the long-term anticoagulation therapy. Three-month treatment is recommended for individuals with transient (reversible) risk factors such as surgery. Graduated compression stockings should be worn for at least 2 years following an acute DVT in adult cases.

No consensus for the use of FXa inhibitors alternates for conventional heparin, and warfarin therapy exists on the optimal management for pediatric patients with PC, PS, and AT deficiencies. Fresh frozen plasma should be sufficiently administered to keep at least the lower limit of standard ranges based on the age. PC levels may be required to exceed 20% levels for the effective use of recombinant thrombomodulin, although the effective application is limited to patients with sepsis-induced DIC [71]. The polymorphisms of thrombomodulin gene (*THBD*) have reportedly lacked the genetic predisposition for thrombophilia [72]. A gain-of-function mutation (c.1611C > A) has recently been found in bleeding but not thrombotic disorders [73]. Thrombomodulin therapy has not been then established in patients with defective PC-PS pathway.

Replacement therapy by using PC and antithrombin concentrates is useful for the treatment of acute thrombosis as well as the prophylaxis for recurrence. Anti-inflammatory and cytoprotective effects of APC concentrates on the treatment course in PC-deficient infants are needed to determine in the prospective study.

Sufficient replacement of fresh frozen plasma is required for the treatment of attacks as well as the renal outcome of USS patients [74]. Eculizumab, an inhibitor of the alternative complement pathway, is used as the first-line therapy for aHUS because of the rapid and sustained inhibition of the TMA process and significant improvements in long-term clinical outcomes.

10.5   Conclusion Remarks

Thrombosis occurs as a consequence of multifactorial disorder. Pediatric cases of thrombosis are rare but increasing in number. It is closely associated with the genetic predispositions of anticoagulant deficiency. Genetic contributions to developing arterial and venous thrombosis in individuals may be greater in the newborns and children than in adults. According to the age, ethnicity, and identifiable triggers, we have to consider the genetic cause of pediatric thrombophilia. No replacement of natural anticoagulants can be instituted for the treatment and prophylaxis for thrombosis without the diagnosis of heritable thrombophilia. Liver transplantation could be a curative therapy of heritable PC deficiency [75]. Future studies are directed not only to establish the optimal management of thrombosis in infants and children but also the curative regenerative medicine for inherited thrombophilias.

References

1. Spentzouris G, Scriven RJ, Lee TK, Labropoulos N. Pediatric venous thromboembolism in relation to adults. J Vasc Surg. 2012;55:1785–93.
2. Hagedorn I, Vögtle T, Nieswandt B. Arterial thrombus formation. Novel mechanisms and targets. Hamostaseologie. 2010;30:127–35.
3. Kimball AS, Obi AT, Diaz JA, Henke PK. The emerging role of NETs in venous thrombosis and immunothrombosis. Front Immunol. 2016;7:236.
4. Nowak-Göttl U, Janssen V, Manner D, Kenet G. Venous thromboembolism in neonates and children—update 2013. Thromb Res. 2013;131:S39–41.
5. Raffini L, Huang YS, Witmer C, et al. Dramatic increase in venous thromboembolism in children's hospitals in the United States from 2001 to 2007. Pediatrics. 2009;124:1001–8.
6. Ishola T, Kirk SE, Guffey D, et al. Risk factors and co-morbidities in adolescent thromboembolism are different than those in younger children. Thromb Res. 2016;141:178–82.
7. Tolbert J, Carpenter SL. Common acquired causes of thrombosis in children. Curr Probl Pediatr Adolesc Health Care. 2013;43:169–77.
8. Caspers M, Pavlova A, Driesen J, et al. Deficiencies of antithrombin, protein C and protein S—practical experience in genetic analysis of a large patient cohort. Thromb Haemost. 2012;108:247–57.
9. Roberts LN, Patel RK, Arya R. Venous thromboembolism and ethnicity. Br J Haematol. 2009;146:369–83.
10. Goldenberg NA, Everett AD, Graham D, Bernard TJ, Nowak-Göttl U. Proteomic and other mass spectrometry based "omics" biomarker discovery and validation in pediatric venous thromboembolism and arterial ischemic stroke: current state, unmet needs, and future directions. Proteomics Clin Appl. 2014;8:828–36.
11. Sosothikul D, Kittikalayawong Y, Aungbamnet P, Buphachat C, Seksarn P. Reference values for thrombotic markers in children. Blood Coagul Fibrinolysis. 2012;23:208–11.

12. Ichiyama M, Ohga S, Ochiai M, et al. Fetal hydrocephalus and neonatal stroke as the first presentation of protein C deficiency. Brain Dev. 2016;38:253–6.
13. Egeberg O. Inherited antithrombin deficiency causing thrombophilia. Thromb Diath Haemorrh. 1965;13:516–30.
14. Hultin MB, McKay J, Abildgaard U. Antithrombin Oslo. Type Ib classification of the first reported antithrombin-deficient family, with a review of hereditary antithrombin variants. Thromb Haemost. 1988;59:468–73.
15. Weingarz L, Schwonberg J, Schindewolf M, et al. Prevalence of thrombophilia according to age at the first manifestation of venous thromboembolism: results from the MAISTHRO registry. Br J Haematol. 2013;163:655–65.
16. Di Minno MN, Ambrosino P, Ageno W, et al. Natural anticoagulants deficiency and the risk of venous thromboembolism: a meta-analysis of observational studies. Thromb Res. 2015;135:923–32.
17. Holzhauer S, Goldenberg NA, Junker R, et al. Inherited thrombophilia in children with venous thromboembolism and the familial risk of thromboembolism: an observational study. Blood. 2012;120:1510–5.
18. van der Meer FJM, Koster T, Vandenbroucke JP, et al. The Leiden Thrombophilia Study (LETS). Thromb Haemost. 1997;78:631–5.
19. Perry DJ, Carrell RW. Molecular genetics of human antithrombin deficiency. Hum Mutat. 1996;7:7–22.
20. Rosenberg RD. Actions and interactions of antithrombin and heparin. N Engl J Med. 1975;292:146–51.
21. Harper PL, Luddington RJ, Daly M, et al. The incidence of dysfunctional antithrombin variants: four cases in 210 patients with thromboembolic disease. Br J Haematol. 1991;77:360–4.
22. Ohga S, Ishiguro A, Takahashi Y, et al. Protein C deficiency as the major cause of thrombophilias in childhood. Pediatr Int. 2013;55:267–71.
23. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C: biased for translation. Blood. 2015;125:2898–907.
24. Ranieri VM, Thompson BT, Barie PS, et al. Drotrecogin alfa (activated) in adults with septic shock. N Engl J Med. 2012;366:2055–64.
25. Quinn LM, Drakeford C, O'Donnell JS, Preston RJ. Engineering activated protein C to maximize therapeutic efficacy. Biochem Soc Trans. 2015;43:691–5.
26. Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, Wideman C. Deficiency of protein C in congenital thrombotic disease. J Clin Invest. 1981;68:1370–3.
27. Ohga S, Kang D, Kinjo T, et al. Paediatric presentation and outcome of congenital protein C deficiency in Japan. Haemophilia. 2013;19:378–84.
28. Ichiyama M, Ohga S, Ochiai M, et al. Age-specific onset and distribution of the natural anticoagulant deficiency in pediatric thromboembolism. Pediatr Res. 2016;79:81–6.
29. Ishiguro A, Ezinne CC, Michihata N, et al. Pediatric thromboembolism: a national survey in Japan. Int J Hematol. 2017;105:52–8.
30. Kinoshita S, Iida H, Inoue S, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. Clin Biochem. 2005;38:908–15.
31. Miyata T, Sato Y, Ishikawa J, et al. Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis. Thromb Res. 2009;124:14–8.
32. Takahashi Y, Yoshioka A. Hemostasis and its regulation system in childhood. Jpn J Pediatr Hematol. 1994;8:389–97.
33. Ishimura M, Saito M, Ohga S, et al. Fulminant sepsis/meningitis due to Haemophilus influenzae in a protein C-deficient heterozygote treated with activated protein C therapy. Eur J Pediatr. 2009;168:673–7.
34. Tait RC, Walker ID, Reitsma PH, et al. Prevalence of protein C deficiency in the healthy population. Thromb Haemost. 1995;73:87–93.

35. Bereczky Z, Kovács KB, Muszbek L. Protein C and protein S deficiencies: similarities and differences between two brothers playing in the same game. Clin Chem Lab Med. 2010;48: S53–66.
36. Tang L, Lu X, Yu JM, et al. PROC c.574_576del polymorphism: a common genetic risk factor for venous thrombosis in the Chinese population. J Thromb Haemost. 2012;10:2019–26.
37. Miyata T, Sakata T, Yasumuro Y, et al. Genetic analysis of protein C deficiency in nineteen Japanese families: five recurrent defects can explain half of the deficiencies. Thromb Res. 1998;92:181–7.
38. Iijima K, Nakamura A, Kurokawa H, Monobe S, Nakagawa M. A homozygous protein C deficiency (Lys 192 del) who developed venous thrombosis for the first time at adulthood. Thromb Res. 2010;125:100–1.
39. Yin T, Miyata T. Dysfunction of protein C anticoagulant system, main genetic risk factor for venous thromboembolism in northeast Asians. J Thromb Thrombolysis. 2014;37:56–65.
40. Sharma S, Anbazhagan J, Plakkal N. Neonatal purpura fulminans due to protein C deficiency. Arch Dis Child Fetal Neonatal Ed. 2015;100:F453.
41. Manco-Johnson MJ, Bomgaars L, Palascak J, et al. Efficacy and safety of protein C concentrate to treat purpura fulminans and thromboembolic events in severe congenital protein C deficiency. Thromb Haemost. 2016;116:58–68.
42. Price VE, Ledingham DL, Krümpel A, Chan AK. Diagnosis and management of neonatal purpura fulminans. Semin Fetal Neonatal Med. 2011;16:318–22.
43. Goldenberg NA, Manco-Johnson MJ. Protein C deficiency. Hemophilia. 2008;14:1214–21.
44. Hamasaki N, Kuma H, Tsuda H. Activated protein C anticoagulant system dysfunction and thrombophilia in Asia. Ann Lab Med. 2013;33:8–13.
45. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311:1525–8.
46. Kenet G, Liitkhoff LK, Albisetti M, et al. Impact of thrombophilia on risk of arterial ischemic stroke or cerebral sinovenous thrombosis in neonates and children: a systematic review and meta-analysis of observational studies. Circulation. 2010;121:1838–47.
47. Marlar RA, Gausman JN. Protein S abnormalities: a diagnostic nightmare. Am J Hematol. 2011;86:418–21.
48. Alhenc-Gelas M, Plu-Bureau G, Horellou MH, Rauch A, Suchon P, GEHT Genetic Thrombophilia Group. PROS1 genotype phenotype relationships in a large cohort of adults with suspicion of inherited quantitative protein S deficiency. Thromb Haemost. 2016;115: 570–9.
49. Sirachainan N, Sasanakul W, Visudibhan A, et al. Protein C deficiency in Thai children with thromboembolism: a report of clinical presentations and mutation analysis. Thromb Res. 2010;125:200–2.
50. Shen MC, Lin JS, Tsay W. Protein C and protein S deficiencies are the most important risk factors associated with thrombosis in Chinese venous thrombophilic patients in Taiwan. Thromb Res. 2000;99:447–52.
51. Tang L, Jian XR, Hamasaki N, et al. Molecular basis of protein S deficiency in China. Am J Hematol. 2013;88:899–905.
52. Ikejiri M, Wada H, Sakamoto Y, et al. The association of protein S Tokushima-K196E with a risk of deep vein thrombosis. Int J Hematol. 2010;92:302–5.
53. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to previously unrecognized mechanism characterized by poor anti-coagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A. 1993;90:1004–8.
54. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369(6475):64–7.
55. Middeldorp S, Meinardi JR, Koopman MM, et al. A prospective study of asymptomatic carriers of the factor V Leiden mutation to determine the incidence of venous thromboembolism. Ann Intern Med. 2001;135:322–7.
56. Nogami K, Shinozawa K, Ogiwara K, et al. Novel FV mutation (W1920R, FV Nara) associated with serious deep vein thrombosis and more potent APC resistance relative to FV Leiden. Blood. 2014;123:2420–8.

57. Poort SR, Rosendaal FR, Reitsma PH, et al. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated prothrombin levels and an increase in venous thrombosis. Blood. 1996;88:3698–703.

58. Miyawaki Y, Suzuki A, Fujita J, et al. Thrombosis from a prothrombin mutation conveying antithrombin resistance. N Engl J Med. 2012;366:2390–6.

59. Jenkins PV, Rawley O, Smith OP, O'Donnell JS. Elevated factor VIII levels and risk of venous thrombosis. Br J Haematol. 2012;157:653–63.

60. Goldenberg NA, Knapp-Clevenger R, Manco-Johnson MJ, et al. Elevated plasma factor VIII and D-dimer levels as predictors of poor outcomes of thrombosis in children. N Engl J Med. 2004;351:1081–8.

61. Moll S, Varga EA. Homocysteine and MTHFR mutations. Circulation. 2015;132:e6–9.

62. Bezemer ID, Doggen CJ, Vos HL, et al. No association between the common MTHFR677C——>T polymorphism and venous thrombosis: results from the MEGA study. Arch Intern Med. 2007;167:497–501.

63. Toole JF, Malinow MR, Chambless LE, et al. Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction and death. The vitamin intervention for stroke prevention (VISP) randomized controlled trial. JAMA. 2004;291:565–75.

64. Glueck CJ, Smith D, Gandhi N, et al. Treatable high homocysteine alone or in concert with five other thrombophilias in 1014 patients with thrombotic events. Blood Coagul Fibrinolysis. 2015;26:736–42.

65. Franchini M. Atypical hemolytic uremic syndrome: from diagnosis to treatment. Clin Chem Lab Med. 2015;53:1679–88.

66. Fujimura Y, Matsumoto M, Isonishi A, et al. Natural history of Upshaw-Schulman syndrome based on ADAMTS13 gene analysis in Japan. J Thromb Haemost. 2011;9(Suppl 1):283–301.

67. Sarker T, Roy S, Hollon W, Rajpurkar M. Lupus anticoagulant acquired hypoprothrombinemia syndrome in childhood: two distinct patterns and review of the literature. Haemophilia. 2015;21:754–60.

68. La Regina M, Orlandini F, Manna R. Autoinflammatory diseases: a possible cause of thrombosis? Thromb J. 2015;13:19.

69. Shah R, Ferreira P, Karmali S, Le D. Severe congenital protein C deficiency: practical aspects of management. Pediatr Blood Cancer. 2016;63:1488–90.

70. Tripodi A, Martinelli I, Chantarangkul V, et al. Thrombin generation and other coagulation parameters in a patient with homozygous congenital protein S deficiency on treatment with rivaroxaban. Int J Hematol. 2016;103:165–72.

71. Umemura Y, Yamakawa K, Ogura H, et al. Efficacy and safety of anticoagulant therapy in three specific populations with sepsis: a meta-analysis of randomized controlled trials. J Thromb Haemost. 2016;14:518–30.

72. Franchini M, Martinelli I, Mannucci PM. Uncertain thrombophilia markers. Thromb Haemost. 2016;115:25–30.

73. Dargaud Y, Jourdy Y, Le Quellec S, et al. Effect of five therapeutic strategies on the coagulation defect induced by the thrombomodulin c.1611C>A mutation. Br J Haematol. 2016;174:993–6.

74. Doi T, Ohga S, Ito N, et al. Limited renal prophylaxis in regular plasmatherapy for heritable ADAMTS13 deficiency. Pediatr Blood Cancer. 2013;60:1557–8.

75. Matsunami M, Ishiguro A, Fukuda A, et al. Successful living domino liver transplantation in a child with protein C deficiency. Pediatr Transplant. 2015;19:E70–4.

Part V
Histiocytic Disorders

Chapter 11
Langerhans Cell Histiocytosis

Akira Morimoto

Abstract Langerhans cell histiocytosis (LCH) is an inflammatory myeloid neoplasia characterized by accumulation of clonal CD1a-positive immature dendritic cells (LCH cells) accompanied by infiltration of various inflammatory cells such as eosinophils, lymphocytes, macrophages, and osteoclast-like multinuclear giant cells. Mutually exclusive oncogenic mutations in genes involved in the mitogen-activated protein kinase (MAPK) signaling pathway, such as *BRAF* and *MAP2K1*, are detected in LCH cells from more than two thirds of LCH patients, suggesting that LCH cells are neoplastic. Proinflammatory cytokines and chemokines play a role in the clinical presentation of LCH, indicating that LCH is also an inflammatory disorder. Several major issues regarding the treatment of childhood LCH remain: how do clinicians rescue patients with risk-organ involvement that do not respond to first-line therapy, and how do they reduce and treat central nervous system-related consequences such as central diabetes insipidus and neurodegeneration? More research is needed to resolve these treatment-related issues.

11.1 Introduction

Langerhans cell histiocytosis (LCH), previously termed histiocytosis X, is characterized by the accumulation of clonal CD1a-positive immature dendritic cells (DCs), so-called LCH cells, accompanied by eosinophils, lymphocytes, macrophages, and/or osteoclast-like multinucleated giant cells. Bone lesions are most common, followed by lesions of the skin and lymph nodes. LCH was originally described as three different entities: eosinophilic granuloma, Hand-Schüller-Christian disease, and Letterer-Siwe disease. However, at present, LCH is classified as a single-system (SS) versus multi-system (MS) disease. SS disease is classified

A. Morimoto
Department of Pediatrics, Jichi Medical University of Medicine,
3311-1, Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
e-mail: akira@jichi.ac.jp

as either unifocal or multifocal. MS disease is classified according to whether risk organs (RO), namely, the liver, spleen, and hematopoietic system, are involved. SS disease, RO-negative MS disease, and RO-positive MS disease are almost equivalent to eosinophilic granuloma, Hand-Schüller-Christian disease, and Letterer-Siwe disease, respectively. The clinical manifestations of LCH vary from a self-limiting single bone disease to a rapidly fatal RO-positive MS disease. However, LCH usually follows a chronic course and reactivations often occur. This can result in permanent consequences, such as orthopedic abnormalities, the development of central diabetes insipidus (CDI), and neurodegenerative central nervous system (ND-CNS) disease.

11.2 Epidemiology

The incidence of LCH in children aged less than 15 years is 4–5 cases per million per year, and the disease is diagnosed most often in this age group between the ages of 0 and 3 years [1–3]. It is estimated that 70 children are diagnosed with LCH every year in Japan [2]. LCH is also common in adolescents and young adults [4]. SS disease accounts for about 70% of cases, while nearly 10% are RO-positive MS disease. MS disease, especially RO-positive MS disease, is quite common in infants. The disease usually develops sporadically. However, genetic predisposition may play a role because about 1% of patients have relatives with LCH, monozygotic twin pairs are concordant for LCH [5], and single nucleotide polymorphism of cytokine genes is associated with the severity of LCH [6]. Patients with LCH are more likely to develop malignant disorders than the general population [7]. Acute lymphoblastic leukemia (ALL), particularly T cell type, rarely precedes LCH harboring the same oncogenic mutation harbored by ALL [8].

11.3 Pathology and Diagnosis

A diagnosis of LCH must be made by pathological examination of excisional biopsy specimens. After hematoxylin-eosin staining, LCH cells have a distinctive homogeneously stained pink cytoplasm. Nuclei appear twisted, with a longitudinal groove and a small nucleolus, a so-called "coffee bean" appearance. Immunohistochemical staining of S-100 protein is helpful for detecting LCH cells, which express CD14, CD68, and intracellular major histocompatibility complex class II antigen, but not Fascin, CD83, or CD86. A definitive diagnosis can be made when accumulated histiocytes are positive for CD1a or langerin (CD207) by immunohistochemistry [9]. The Birbeck granule is a Langerhans cell-specific tennis racquet-shaped organelle that can be detected by electron

microscopy. In terms of diagnostic value, the Birbeck granule has been replaced by langerin, which is a cell-surface receptor that induces formation of the Birbeck granule. Active LCH lesions contain granulomas caused by aggregation of LCH cells and a number of inflammatory cells. In the later stages of LCH, macrophages are more predominant in the lesions than LCH cells, and xanthomatous and fibrotic changes are often observed. In cases where aggregating histiocytes are negative for CD1a staining, clinicians may consider juvenile xanthogranuloma (JXG), Erdheim-Chester disease (ECD), or Rosai-Dorfman disease. LCH rarely precedes or co-occurs with JXG [10] or ECD [11]. Langerhans cell sarcoma should be ruled out when the CD1a-positive cells exhibit prominent dysmorphic features and mitotic figures [12, 13]. The differentiating characteristics of these histiocytoses are summarized in Table 11.1.

11.4   Pathogenesis

It is debatable whether LCH is a neoplasm or inflammation. Recently, LCH was defined as an inflammatory myeloid neoplasia [14]. Proliferation of LCH cells is neoplastic, although some clinical presentations are due to inflammation induced by infiltrating inflammatory cells, as well as LCH cells.

11.4.1   LCH and Neoplasia

11.4.1.1   Somatic Mutation of LCH Cells

LCH cells are monoclonal [15, 16] and sometimes show chromosomal deletion or gain [17]. However, recurrent genomic alteration was not detected for a long time due to the low percentage of LCH cells in the lesion [18]. However, a report published in 2010 revealed that LCH cells harbor an oncogenic mutation of the *BRAF* gene (V600E), which plays a role in the mitogen-activated protein kinase (MAPK) signaling pathway; it also showed that extracellular signal-regulated kinase (ERK), a molecule downstream of BRAF in this pathway, is phosphorylated constitutively in the presence or absence of this mutation [19]. This mutation is detected in about half of LCH patients, regardless of disease type [20]. The clinical significance of the *BRAF* V600E mutation is unclear, although it was reported recently that *BRAF* V600E mutation-positive patients may experience a higher rate of reactivation than mutation-negative patients [21]. More recently, oncogenic mutation of other genes within the MAPK signaling pathway, including *ERBB3*, *ARAF*, and *MAP2K1*, were identified in LCH cells [22–24] (Fig. 11.1). These MAPK signaling pathway-related mutations are mutually exclusive. Recurrent loss-of-function mutations in *MAP3K1* (which encodes MEKK1) in the c-Jun N-terminal kinase pathway were

Table 11.1 Differentiating characteristics of histiocytoses

Disease	Clinical characteristic	Histologic features									Others
		CD1a	CD14	CD68	CD163	CD207	S100	Factor XIIIa	Fascin	HLA-DR	
Langerhans cell histiocytosis	See text	+	–	+/–	–	+	+	–	–	+	Birbeck granules
Juvenile xanthogranuloma	Cutaneous type (75%) Mainly <2 years old, multiple nodular small papules	–	+	+	+	–	–	+	+	–	Touton giant cells (cutaneous type)
	Soft tissue type (15%) Mainly <2 years old, soft tissue mass lesions in subcutaneous fat or muscle										
	Systemic type (10%) Mainly <6 months old, mass lesions in visceral organs, subcutis, central nervous system, bone, liver, spleen, lung, and others										

Disease	Clinical features										Morphology
Erdheim-Chester disease	Mainly >40 years old. Symmetrical sclerotic lesions in the long bones. Nonosseous lesions—ureteral stricture associated with retroperitoneal fibrosis, xanthomatous skin lesions, central diabetes insipidus, and pulmonary fibrosis (50%)	−	+	+	+	−	−	+	+	−	Foamy histiocytes
Rosai-Dorfman disease	Children and young adults. Painless bilateral cervical lymphadenopathy (80%). Extranodal lesions—osteolytic bone lesions, maculopapular skin rash, orbital masses, and others (50%)	−	+	+	+	−	+	−	+	+	Emperipolesis
Langerhans cell sarcoma	Any age. Multiple organ involvements—lymph nodes, liver, spleen, bone, lung, and others	+	+	+	−	+	+	−	+/−	+	Nuclear pleomorphism and mitoses

Fig. 11.1 Somatic mutations in the mitogen-activated protein kinase signaling pathway in LCH cells

also identified [25]. These mutations of *MAP3K1* are not exclusive to the *BRAF* V600E mutation, and the mechanisms by which these variants promote neoplastic growth remain unknown.

11.4.1.2   Characteristic of Neoplastic LCH Cells

Gene expression analysis of purified LCH cells revealed that the LCH cell of origin is not a skin Langerhans cell but a myeloid DC [26]. Compared with normal Langerhans cells, LCH cells are more proliferative and have a lower antigen-presenting capacity. During maturation, LCH cells arrests in an activated state, possibly due to the action of transforming growth factor-β and interleukin (IL)-10 [27]. LCH cells are the only DCs that co-express Notch1 and its ligands Jagged-1 and -2 [28]. The JAG-mediated Notch signaling pathway may also play an important role in maintaining LCH cells in an immature state. Leukemic cells and LCH cells in patients who develop T cell acute lymphoblastic leukemia before LCH harbor the same activating Notch1 mutation and T cell receptor rearrangements [29, 30], further supporting the possibility that Notch1 may contribute to LCH pathogenesis. This is also interesting when considering the origin of LCH cells.

11.4.1.3 Detection of BRAF V600E-Positive Cells in Peripheral Blood and Bone Marrow

BRAF V600E mutations are detected in bone marrow hematopoietic progenitors, peripheral monocytes, and myeloid DCs in active high-risk (RO-positive MS) LCH patients harboring a *BRAF* V600E mutation. Conversely, the mutation is restricted to lesional LCH cells in low-risk patients [21].

11.4.1.4 Animal Model of LCH Generated by Forced BRAF V600E Expression in DCs

Forced expression of *BRAF* V600E in DCs is sufficient to drive LCH-like disease in mice [21]. Forced expression of *BRAF* V600E in CD11c$^+$ cells (DC progenitors) results in the formation of aggressive LCH lesions similar to those observed in humans with high-risk LCH, whereas *BRAF* V600E expression in langerin-positive cells (differentiated DCs) results in disease that resembles low-risk LCH without detectable mutations in circulating myeloid cells. These data suggest that the extent of LCH may be determined by whether ERK-activating mutations arise at any stage of DC differentiation.

11.4.2 LCH and Inflammation

11.4.2.1 Interaction Between LCH Cells and Inflammatory Cells That Infiltrate LCH Lesions

LCH lesions contain less than 10% (median value) LCH cells [21]; however, they contain many inflammatory cells, including eosinophils, T lymphocytes, plasma cells, macrophages, osteoclast (OC)-like multinucleated giant cells (MGCs), and neutrophils. Because LCH cells show immature DC characteristics, they may promote the expansion of regulatory T cells, which in turn results in the failure of the host immune system to eliminate LCH cells [31].

LCH cells express the immature DC marker CCR6 and secrete its ligand CCL20/MIP-3α, along with CCL5/RANTES (a CCR1/3/5 ligand) and CXCL11/I-TAC (a CXCR3 ligand) [32]. Interactions between these chemokine receptors and their ligands may play a role in the hematogenous migration of not only LCH cells but also of inflammatory cells into the lesions. These LCH cells and infiltrating cells in turn stimulate each other to produce cytokines such as GM-CSF, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-7, and IL-10 [33]. Moreover, LCH cells in lesions express high levels of CD40, whereas T cells express CD154 (the CD40 ligand): interactions between CD40 and CD154 are essential for activation of both antigen-presenting cells and T cells [34]. A recent report shows that LCH cells and lesional T cells express high levels

of the pleiotropic cytokine osteopontin (OPN) [26], which promotes the generation of T helper (Th)1 and Th17 cells, recruits histiocytes/monocytes, and activates osteoclasts [35].

We found that the serum levels of lesional inflammatory cytokines/chemokines and DC/macrophage-activating factors such as IL-18, M-CSF, and CCL2 are markers of disease dissemination and severity [36]. Marked increases in serum IL-18 and soluble tumor necrosis factor receptors are reported in patients at risk for developing hemophagocytic lymphohistiocytosis [37]. In addition, we found that patients with MS LCH have much higher serum OPN levels than patients with SS LCH [38].

11.4.2.2   Osteoclastogenesis in LCH Lesions

In LCH, OC-like MGCs are present not only in the bone but also in skin and lymph node lesions, and enzymes derived from OC-like MGCs may play a major role in chronic tissue destruction [39]. Both LCH cells and T cells in lesions produce the OC-inducing cytokines, receptor activator for nuclear factor kB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [39]. We found that patients with SS LCH and multiple bone lesions had significantly higher ratios of soluble RANKL and osteoprotegerin (OPG), a decoy receptor of RANKL, than patients with MS LCH; moreover, the soluble RANKL/OPG ratios correlated positively with the number of bone lesions [40].

Courey et al. showed that patients with active LCH have high serum levels of IL-17A, which is mainly produced by LCH cells [41]. In vitro, serum IL-17A causes fusion of immature DCs, which results in formation of OC-like MGCs [41]. This IL-17A-dependent fusion activity correlates with LCH activity. An IL-17A autocrine model of LCH was suggested. We also found that patients with LCH have higher serum levels of IL-17A than controls, although the serum IL-17A levels of patients with MS LCH and SS LCH did not differ significantly. However, we also observed that LCH cells in MS disease expressed higher levels of IL-17A receptor (IL-17RA) than those in SS disease and that IL-17RA expression levels help distinguish between LCH subclasses [42]. In addition, we found that the cleaved form of OPN plays a critical role in driving immature DC differentiation into OC-like MGCs in an autocrine and/or paracrine fashion [43].

11.4.2.3   Triggers of LCH

Merkel cell polyomavirus (MCPyV, a common dermotropic virus) sequences are detected in not only skin but also in bone and soft tissue lesions of LCH patients; also, the amount of MCPyV DNA in peripheral blood cells is increased in patients with high-risk (RO-positive MS) LCH but not in patients with low-risk LCH [44]. MCPyV infection may induce LCH cells with an oncogenic mutation to secrete IL-1; this leads to activation of LCH cells in an autocrine manner [45]. This suggests that MCPyV infection may trigger LCH.

Nicotine stimulation increases the production of both OPN and GM-CSF by alveolar macrophages. Bronchoalveolar lavage cells from patients with pulmonary LCH produce abundant amounts of OPN. Forced expression of OPN in rat lungs induces lesions similar to pulmonary LCH, with marked accumulation of Langerhans cells [46]. These findings suggest that increased OPN production upon stimulation by nicotine is responsible for pulmonary LCH.

11.5   Clinical Manifestations

Since LCH affects a number of different organs, clinical signs and symptoms can be extremely variable. In most pediatric patients with SS LCH, lesions occur in the bone, with either single-site or multifocal involvement; however, lesions can also occur in the skin, thymus, or lymph nodes [47]. The involved organ at diagnosis and the initial presentation of MS LCH are shown in Fig. 11.2. In patients
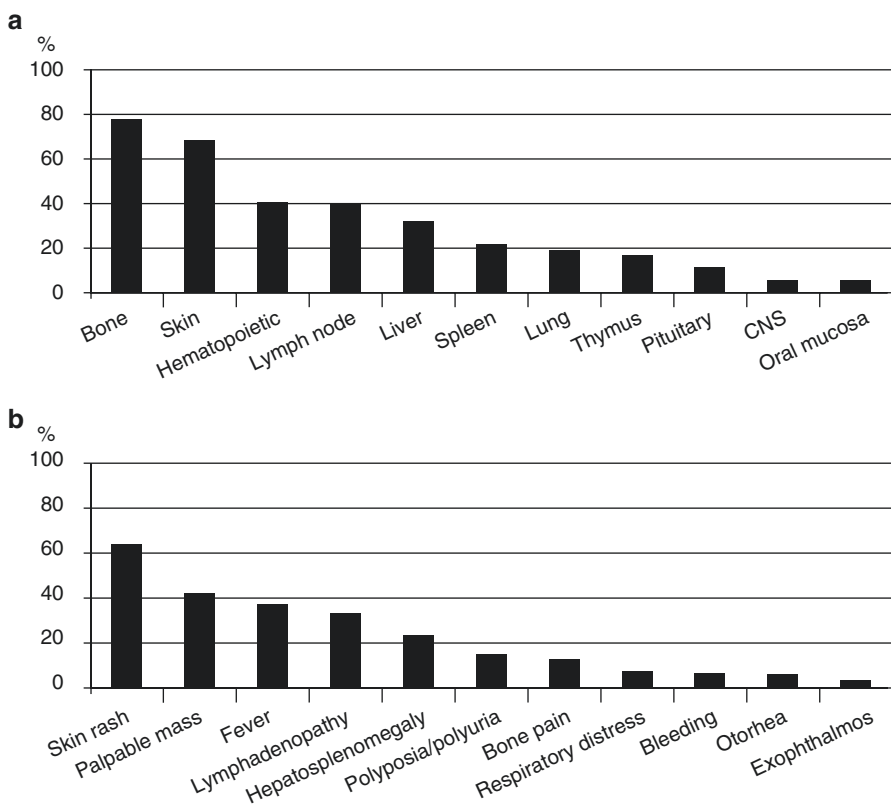
Fig. 11.2 (**a**) Involved organ and (**b**) Initial presentation of patients with multi-system Langerhans cell histiocytosis at the time of diagnosis (*n* = 206; JLSG-96/02 study)

with MS LCH, the organs involved are mainly the bone and skin, followed by the hematopoietic system, lymph nodes, liver, spleen, lung, and thymus. The most common initial manifestations are development of a skin rash, a soft tissue mass, fever, lymphadenopathy, hepatosplenomegaly, polyposia/polyuria, and bone pain. There are no specific laboratory markers for LCH. In many cases, LCH presents with nonspecific inflammatory signs that arise from chronic inflammation.

11.5.1   Bone Lesions

Bone is involved in about 80% of all LCH cases [48]. The skull is the most common location, followed by the spine, extremities, pelvic bone, and ribs [49]. X-rays typically show osteolytic "punched out" lesions with sharp margins. Fluorodeoxyglucose-positron emission tomography [50] and whole-body magnetic resonance imaging (MRI) [51] are also useful for detecting bone lesions and for evaluating their response to treatment. Bone lesions may be asymptomatic or accompanied by pain and soft tissue swelling. When LCH is localized solely to the bone, the clinical course is generally benign and sometimes resolves spontaneously over a period of months to years. In patients with RO-positive MS disease, lack of bone lesions at the time of diagnosis is associated with a poorer outcome [52]. Bone lesions can result in critical or irreversible symptoms, such as visual loss or exophthalmos (due to orbital involvement), conductive hearing loss (due to mastoid antrum lesions), loss of teeth (due to jaw disease), and spinal paralysis (due to vertebral lesions).

11.5.2   Skin Lesions

Skin involvement is seen in approximately half of all patients and is particularly common in infants. These patients present with various lesions, including erythema, papules, nodules, petechiae, vesicles, crusted plaques, and seborrhea-like eruptions. Ulcerative lesions in the genital or inguinal region may also be present [53]. It is essential that LCH patients with skin lesions undergo evaluation of other visceral organs because most of these patients have MS disease. Patients with skin involvement whom are aged older than 18 months are more likely to have lesions in organs other than the skin [54]. In neonatal infants, isolated skin lesions can regress spontaneously over weeks to months [55, 56]. However, some infants with isolated skin lesions at the time of diagnosis can develop MS disease, followed by a fatal clinical course [57].

Table 11.2 Definition of risk-organ involvement (modified from [60])

Hematopoietic involvement (with or without bone marrow involvement)
Both of the following should be present:
– Hemoglobin less than 10 g/dL (not due to other causes, e.g., iron deficiency)
– Thrombocytopenia, with a platelet count less than 100,000/mm³
Liver involvement (the patient can show a combination of these symptoms):
– Enlargement >3 cm below the costal margin at the midclavicular line, confirmed by ultrasound
– Dysfunction documented by hyperbilirubinemia >3 times normal hypoalbuminemia (<30 g/dL); γ GT increased >2 times normal; ALT/AST >3 times normal; ascites or edema
– Intrahepatic nodular mass
Spleen involvement
– Enlargement >3 cm below the costal margin at the midclavicular line, confirmed by ultrasound

11.5.3 Risk Organs (Hematopoietic System, Liver, and Spleen)

Because disease involving the hematopoietic system, liver, or spleen is associated with a higher risk of death, these organs are defined as "risk organs". Pulmonary involvement is no longer considered an independent prognostic variable [58, 59]. The recent definition of risk-organ involvement is shown in Table 11.2 [60].

Hematopoietic system involvement is defined by cytopenia, either with or without bone marrow infiltration of LCH cells, and is seen exclusively in MS disease. In severe cases, serious anemia and thrombocytopenia may develop and are often associated with a secondary hemophagocytic syndrome, followed by a fatal course. In particular, anemia with thrombocytopenia, with or without leukopenia and hypoalbuminemia, is associated with a worse prognosis in children with MS LCH [61].

Liver and/or spleen involvement is characterized by organomegaly and/or liver dysfunction and is observed exclusively in MS disease. Histological examination of the liver reveals a portal infiltrate, which can lead to destruction of bile ducts and periportal fibrosis (sclerosing cholangitis), culminating in biliary cirrhosis with portal hypertension and, ultimately, secondary hypersplenism, which can cause cytopenias. A liver transplant is the treatment of choice for patients with sclerosing cholangitis due to LCH [62].

11.5.4 Lung Lesions

In children, pulmonary involvement is usually associated with MS disease. Lung pathology is associated with cough, dyspnea, pleural effusion, and recurring pneumothorax. High resolution-computed tomography may reveal reticular or

micronodular opacity, as well as large nodules and honeycombing. KL-6 may be a useful marker for pulmonary involvement [63]. Severe pulmonary hypertension is a common feature in patients with end-stage pulmonary LCH. Lung transplantation is a therapeutic option in this setting [64].

11.5.5   CNS Lesions

LCH may involve the CNS, presenting as space-occupying mass lesions, hypothalamic-pituitary disease, or neurodegenerative disease.

11.5.5.1   Mass Lesions

Intracranial tumorous lesions may occur in the meninges, choroid plexus, and brain parenchyma; these lesions can mimic brain tumors [65]. Cladribine (2-CdA), which penetrates the blood–brain barrier, may be an effective treatment for these lesions [66].

11.5.5.2   Hypothalamic-Pituitary Disease

Infiltration and dysfunction of the pituitary gland and/or adjacent hypothalamus is observed in 25% of all patients with LCH and in 50% of patients with MS disease [65]. The most frequent manifestation is CDI, which usually follows (but some-times precedes or co-occurs with) other symptoms and signs of the disease. The cumulative incidence of CDI continues to increase, even 10 years after a diagnosis of LCH [67]. CDI occurs more often in patients with craniofacial bone lesions (with the exception of the vault), ear, eye, and/or oral lesions [68]; thus these lesions are known as CNS-risk lesions. Patients with MS disease that includes bone lesions are more likely to have CNS-risk lesions than patients with bone lesions alone [49]. LCH involvement of the pituitary gland can be shown by MRI, namely, pituitary stalk thickening and loss of the physiological high-intensity signal in the posterior pituitary lobe on T1-weighted images. Appropriate systemic chemotherapy may effectively inhibit the development of CDI [67, 68]. Systemic chemotherapy rarely cures CDI, but LCH patients with newly diagnosed CDI should be treated with systemic chemotherapy to prevent anterior pituitary hormone loss and neurodegen-erative disease. About half of patients with CDI develop anterior pituitary hormone deficiencies and/or ND-CNS disease during follow-up, usually after a disease course of several years [69]. Patients with hypothalamic-pituitary lesions may pres-ent with non-endocrine hypothalamic dysfunction, such as eating disorder, thirst, fatigue, autonomic disturbance, temperature instability, memory deficit, and dis-turbed consciousness [70]. Hypothalamic lesions are observed on MRI as gadolinium-enhanced masses [71].

11.5.5.3   Neurodegenerative CNS Disease

ND-CNS disease may develop during the years after disease onset, often when the disease is considered quiescent [72, 73]. Three years after a diagnosis of LCH, more than half of patients with CNS-risk lesions and/or CDI demonstrate abnormities on MRI (radiological ND-CNS disease), including bilateral symmetrical lesions in the cerebellar white matter and/or basal ganglia [74]. These brain MRI abnormalities gradually deteriorate and are irreversible. One quarter of patients with brain MRI abnormalities present with neurological symptoms (neurological ND-CNS disease), which includes ataxia, tremor, dysarthria, dysphagia, and hyperreflexia, within several years, which is followed by further deterioration. Patients with neurological ND-CNS disease eventually become bedridden. Histologically, ND-CNS disease is characterized by the presence of a CD8-positive T cell infiltrate, activation of microglia, gliosis, and neuronal and axonal destruction with secondary demyelination, as observed in autoimmune encephalitis. CD1a-positive LCH cells may be absent [75]. Currently, there is no established therapy for ND-CNS disease, although promising evidence suggests that intravenous immunoglobulin [76, 77] can prevent progression. Although first-line therapies for LCH that include cytarabine (Ara-C) cannot prevent development of ND-LCH completely [78], treatment with Ara-C might be effective for ND-CNS disease [79].

11.5.6   Other Lesions

The most commonly affected lymph nodes are the cervical nodes, followed by axillary and inguinal nodes [80]. Rarely, lymph nodes can become massive and cause upper airway obstruction. Thymic involvement mainly occurs in young children and is diagnosed when a CT indicates thymus enlargement and calcification. Patients with thymic involvement sometimes have profound T lymphocytopenia and (albeit rarely) suffer superior vena cava syndrome or asphyxiation [81]. Infiltration of the oral mucosa may appear as ulceration or swelling of the gingiva [82]. Infiltration of the gastrointestinal tract rarely occurs, but can cause vomiting, abdominal pain, constipation, intractable diarrhea with blood and/or mucus [83], and malabsorption of nutrients [84]. Occasionally, the thyroid gland [85], pancreas, and kidneys [86] are involved.

11.6   New Clinical Markers of LCH

In patients with various solid tumors, cell-free DNA (cfDNA) in the peripheral blood contains cancer-derived genomic DNA and can be tested for using a noninvasive diagnostic procedure called a "liquid biopsy." cfDNA *BRAF* V600E in plasma may serve as a noninvasive biomarker of treatment response in high-risk LCH patients [87, 88].

Acute-phase serum levels of inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4), whose production is up-regulated by IL-6, may represent disease activity or extent of LCH [89].

11.7 Treatment and Outcome

The clinical course of LCH varies widely depending on the extent of organ involvement. The mortality rate of patients with SS disease, or RO-negative MS disease, is less than 1%. However, children with RO-positive MS disease have a worse prognosis: these children often have fatal outcomes despite intensive treatment and mortality rates are 10–50% [90]. Treatment of LCH should be planned according to the clinical presentation and the extent of organ involvement. Etoposide (VP-16) is no longer considered a reasonable first-line therapeutic agent because it is no more efficacious than vinblastine [91] and causes therapy-related acute myeloid leukemia (t-AML) [92]. Radiation is rarely used in children because of the increased risk of secondary malignancies, particularly brain tumors, in irradiated areas [93].

11.7.1 Single-System LCH

In SS LCH, the main aim of treatment is to reduce the symptoms and the chances of permanent sequelae. In patients with localized, unifocal bone disease, LCH may resolve spontaneously. In the case of a single bone lesion in a region other than the craniofacial area, or in those with no symptoms, a wait-and-see approach or diagnostic curettage is the standard method of care. Local injection of corticosteroid may be used for these bone lesions [94]. Patients with CNS-risk lesions with compressing soft tissue extension of bone lesions or multifocal bone disease should receive systemic chemotherapy with vinca alkaloids and corticosteroids for more than 6 months to prevent late sequelae such as CDI and nerve compression [95]. A nationwide survey in Japan suggests that appropriate chemotherapy for patients with a single bone lesion results in an excellent prognosis [47]. Radical resection of large bone lesions is discouraged as this often results in disfigurement. In cases of skin involvement only, the best option is to take a wait-and-see approach, with careful attention paid to progression to MS disease. Alternatively, patients can be treated with topical corticosteroids.

11.7.2 Multi-System LCH

In MS LCH, the main aims of treatment are to increase survival and to reduce the incidence of late sequelae. The results of recent clinical studies are summarized in Table 11.3. The most commonly used regimen in countries other than Japan is systemic chemotherapy resorting primarily to vinblastine (VBL) and corticosteroid for 12 months. Three international clinical trials (LCH-I, -II, and -III) conducted by the

Table 11.3 Clinical studies involving pediatric patients with multisystem Langerhans cell histiocytosis [58, 96–98]

	RO+ group				RO− group		
	Japan LCH Study Group*1		Histiocyte Society*2		Japan LCH Study Group*1		Histiocyte Society*2
	JLSG-96*3	JLSG-02*4	LCH-II*5	LCH-III*6	JLSG-96*3	JLSG-02*4	LCH-III*6
No. of patients	41	84	133	227	18	63	179
Duration of therapy (weeks)	30	54	30	52 or 64	30	54	25 or 52
Rate of responders at 6 weeks	68%	76%	62%	66%	94%	94%	87%
Mortality rate	7%	8%	30%	15%	0%	0%	1%
Probable OS at 5 years	93 ± 4%	92 ± 3%	69 ± 4%	84 ± ND%	100%	100%	99%
Responders at 6 weeks Poor responders at 6 weeks	96 ± 4% 85 ± 10%	98 ± 2% 70 ± 10%	ND ND	95 ± ND% 72 ± ND%	100% 100%	100% 100%	ND ND
Probable EFS at 5 years	27 ± 7%	46 ± 5%	ND	ND	39 ± 12%	70 ± 6%	ND

RO+ risk-organ involvement positive, *RO−* risk-organ involvement negative, *OS* overall survival, *EFS* event-free survival, *ND* not described

*1, adopting a cytarabine/vincristine/prednisolone-based regimen; *2, adopting a vinblastine/prednisolone-based regimen; *3, enrolled in 1996–2001; *4, enrolled in 2002–2009; *5, enrolled in 1996–2001; *6, enrolled in 2002–2008

Histiocyte Society have tested this regimen [91, 96, 97] and showed that it yields excellent survival rates in risk-organ-negative patients, even in infants. However, despite this treatment, the mortality rates of risk-organ-positive patients remain high at 15–30%. The 6 weeks of induction chemotherapy comprising Ara-C, vincristine (VCR), and prednisolone introduced by the Japan LCH Study Group (the JLSG-96/-02 protocol) yields more favorable survival rates, with high initial response rates (68–76%) for RO-positive patients: the overall mortality rate is less than 10% [58, 98]. A JLSG-02 protocol in which treatment was prolonged from 30 to 54 weeks achieved a lower reactivation rate than the original JLSG-96 protocol [58].

11.7.3   Salvage Therapy and New Treatments for Refractory Patients

A major prognostic factor is a patient's response to the first 6 weeks of systemic multi-agent chemotherapy: RO-positive patients who exhibit progressive disease during combination chemotherapy have an extremely high rate of mortality (between 30 and

80%) [58, 96–99]. For these patients, more aggressive salvage therapy should be considered. In the Japanese JLSG-96/-02 protocol, the salvage regimen (comprising an intensive combination of doxorubicin, cyclophosphamide, VCR, and PSL) is beneficial for those who respond poorly to the 6-week induction treatment [58, 98]. Myeloablative therapy using a combination of 2-CdA/high-dose Ara-C [100] and clofarabine [101] is also effective for poor responders. However, profound and prolonged myelosuppression should be anticipated when using these therapies. A combination of 2-CdA/Ara-C at reduced doses may also show a good response in these refractory patients, with manageable toxicity [102]. Allogeneic hematopoietic stem cell transplantation is another promising salvage therapy for children with refractory LCH [103, 104]. A reduced-intensity conditioning regimen may be sufficient to induce a cure because of its immunomodulatory effect [105]. A recent report showed that a pediatric patient with refractory RO-positive MS disease harboring a *BRAF* V600E mutation was successfully treated with vemurafenib, with no severe toxicity [106].

11.8 Reactivation

Nearly half of patients with MS disease exhibit reactivation, which occurs in an unpredictable manner [107, 108]. Some patients experience several episodes of reactivation. Most cases of initial reactivation occur within 2 years of diagnosis. Reactivations occur most frequently in bone, but rarely in RO. Reactivated lesions may sometimes resolve spontaneously; indeed, most patients with reactivation undergo "second disease resolution" and survive. However, they have an increased risk of permanent sequelae.

For patients who show reactivation in non-RO while they are off therapy, treatment with the same first-line therapy, pamidronate combined with a Cox2 inhibitor [109] or 2-CdA monotherapy [110, 111] may be effective. Careful attention is required to prevent adverse effects when using 2-CdA, namely, the development of severe and persistent bone marrow failure and myelodysplastic syndrome [112, 113].

11.9 Permanent Sequelae

Many LCH patients suffer permanent sequelae [114], which most often result from the infiltrative nature of the disease itself, which causes tissue destruction and granulomatous fibrosis or gliosis of various tissues. Seventy percent of patients with MS disease and 25% of those with SS disease develop one or more life-long sequelae, including CDI, orthopedic problems, hearing loss, neurological problems, growth hormone deficiency, loss of teeth, pulmonary fibrosis, and biliary cirrhosis with portal hypertension. The most common and serious of these involve the CNS (e.g., CDI and ND-CNS disease); these sequelae are particularly common in MS disease and

correlate with an increased frequency of CNS-risk lesions [49, 65]. The incidence of these sequelae increases over follow-up. More research is needed to resolve these issues.

11.10   Conclusions

There is no optimal treatment for LCH, although some progress has been made. There are several major issues that remain to be resolved. First, it is unclear how we rescue patients with RO-positive MS disease who do not respond to first-line therapy. Second, novel therapeutic measures are required to reduce and treat CNS-related consequences.

References

1. Guyot-Goubin A, Donadieu J, Barkaoui M, et al. Descriptive epidemiology of childhood Langerhans cell histiocytosis in France, 2000–2004. Pediatr Blood Cancer. 2008;51:71–5.
2. Horibe K, Saito AM, Takimoto T, et al. Incidence and survival rates of hematological malignancies in Japanese children and adolescents (2006–2010): based on registry data from the Japanese Society of Pediatric Hematology. Int J Hematol. 2013;98:74–88.
3. Salotti JA, Nanduri V, Pearce MS, et al. Incidence and clinical features of Langerhans cell histiocytosis in the UK and Ireland. Arch Dis Child. 2009;94:376–80.
4. Aricò M, Girschikofsky M, Généreau T, et al. Langerhans cell histiocytosis in adults. Report from the International Registry of the Histiocyte Society. Eur J Cancer. 2003;39:2341–8.
5. Aricò M, Danesino C. Langerhans' cell histiocytosis: is there a role for genetics? Haematologica. 2001;86:1009–14.
6. De Filippi P, Badulli C, Cuccia M, et al. Specific polymorphisms of cytokine genes are associated with different risks to develop single-system or multi-system childhood Langerhans cell histiocytosis. Br J Haematol. 2006;132:784–7.
7. Egeler RM, Neglia JP, Aricò M, et al. The relation of Langerhans cell histiocytosis to acute leukemia, lymphomas, and other solid tumors. The LCH-Malignancy Study Group of the Histiocyte Society. Hematol Oncol Clin North Am. 1998;12:369–78.
8. Kato M, Seki M, Yoshida K, et al. Genomic analysis of clonal origin of Langerhans cell histiocytosis following acute lymphoblastic leukaemia. Br J Haematol. 2016;175(1):169–72.
9. Jaffe R, Weiss LM, Facchetti F. Tumours derived from Langerhans cells. In: Swerdlow SH, Campo E, Harris NL, et al., editors. WHO classification of tumours, tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: IARC; 2008. p. 358–60.
10. Bains A, Parham DM. Langerhans cell histiocytosis preceding the development of juvenile xanthogranuloma: a case and review of recent developments. Pediatr Dev Pathol. 2011;14:480–4.
11. Kim S, Lee M, Shin HJ, et al. Coexistence of intracranial Langerhans cell histiocytosis and Erdheim-Chester disease in a pediatric patient: a case report. Childs Nerv Syst. 2016;32:893–6.
12. Lau SK, Chu PG, Weiss LM. Immunohistochemical expression of Langerin in Langerhans cell histiocytosis and non-Langerhans cell histiocytic disorders. Am J Surg Pathol. 2008;32:615–9.
13. Weitzman S, Whitlock J. Uncommon histiocytic disorder: the non-Langerhans cell histiocytoses. In: Weitzman S, Egeler RM, editors. Histiocytic disorders of children and adults. Cambridge: Cambridge University Press; 2005. p. 293–320.

14. Berres ML, Merad M, Allen CE. Progress in understanding the pathogenesis of Langerhans cell histiocytosis: back to Histiocytosis X? Br J Haematol. 2015;169:3–13.

15. Willman CL, Busque L, Griffith BB, et al. Langerhans'-cell histiocytosis (histiocytosis X)—a clonal proliferative disease. N Engl J Med. 1994;331:154–60.

16. Yu RC, Chu C, Buluwela L, et al. Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. Lancet. 1994;343:767–8.

17. Murakami I, Gogusev J, Fournet JC, et al. Detection of molecular cytogenetic aberrations in Langerhans cell histiocytosis of bone. Hum Pathol. 2002;33:555–60.

18. da Costa CE, Szuhai K, van Eijk R, et al. No genomic aberrations in Langerhans cell histiocytosis as assessed by diverse molecular technologies. Genes Chromosomes Cancer. 2009;48:239–49.

19. Badalian-Very G, Vergilio JA, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. Blood. 2010;116:1919–23.

20. Bubolz AM, Weissinger SE, Stenzinger A, et al. Potential clinical implications of BRAF mutations in histiocytic proliferations. Oncotarget. 2014;5:4060–70.

21. Berres ML, Lim KP, Peters T, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. J Exp Med. 2014;211: 669–83.

22. Brown NA, Furtado LV, Betz BL, et al. High prevalence of somatic MAP2K1 mutations in BRAF V600E-negative Langerhans cell histiocytosis. Blood. 2014;124:1655–8.

23. Chakraborty R, Hampton OA, Shen X, et al. Mutually exclusive recurrent somatic mutations in MAP2K1 and BRAF support a central role for ERK activation in LCH pathogenesis. Blood. 2014;124:3007–15.

24. Nelson DS, Quispel W, Badalian-Very G, et al. Somatic activating ARAF mutations in Langerhans cell histiocytosis. Blood. 2014;123:3152–5.

25. Nelson DS, van Halteren A, Quispel WT, et al. MAP2K1 and MAP3K1 mutations in Langerhans cell histiocytosis. Genes Chromosomes Cancer. 2015;54:361–8.

26. Allen CE, Li L, Peters TL, et al. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. J Immunol. 2010;184:4557–67.

27. Geissmann F, Geissmann F, Lepelletier Y, et al. Differentiation of Langerhans cells in Langerhans cell histiocytosis. Blood. 2001;97:1241–8.

28. Hutter C, Kauer M, Simonitsch-Klupp I, et al. Notch is active in Langerhans cell histiocytosis and confers pathognomonic features on dendritic cells. Blood. 2012;120:5199–208.

29. Rodig SJ, Payne EG, Degar BA, et al. Aggressive Langerhans cell histiocytosis following T-ALL: clonally related neoplasms with persistent expression of constitutively active NOTCH1. Am J Hematol. 2008;83:116–21.

30. Yokokawa Y, Taki T, Chinen Y, et al. Unique clonal relationship between T-cell acute lymphoblastic leukemia and subsequent Langerhans cell histiocytosis with TCR rearrangement and NOTCH1 mutation. Genes Chromosomes Cancer. 2015;54:409–17.

31. Senechal B, Elain G, Jeziorski E, et al. Expansion of regulatory T cells in patients with Langerhans cell histiocytosis. PLoS Med. 2007;4:e253.

32. Annels NE, Annels NE, Da Costa CE, et al. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. J Exp Med. 2003;197:1385–90.

33. Egeler RM, Favara BE, van Meurs M, et al. Differential in situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. Blood. 1999;94:4195–201.

34. Egeler RM, Favara BE, Laman JD, et al. Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions. Eur J Cancer. 2000;36: 2105–10.

35. Uede T. Osteopontin, intrinsic tissue regulator of intractable inflammatory diseases. Pathol Int. 2011;61:265–80.

36. Morimoto A, Nakamura S, Shioda Y et al. Comprehensive analysis of serum cytokine/chemokine and growth factors in pediatric patients with Langerhans cell histiocytosis. Pediatr Blood Cancer 2011a;56:696 [abstract].
37. Ikawa Y, Nishimura R, Araki R, et al. Pathognomonic serum cytokine profiles identify life-threatening Langerhans cell histiocytosis. Br J Haematol. 2017;176(3):495–7.
38. Oh Y, Morimoto A, Shioda Y, et al. High serum osteopontin levels in pediatric patients with high risk Langerhans cell histiocytosis. Cytokine. 2014a;70:194–7.
39. da Costa CE, Annels NE, Faaij CM, et al. Presence of osteoclast-like multinucleated giant cells in the bone and nonostotic lesions of Langerhans cell histiocytosis. J Exp Med. 2005;201:687–93.
40. Ishii R, Morimoto A, Ikushima S, et al. High serum values of soluble CD154, IL-2 receptor, RANKL and osteoprotegerin in Langerhans cell histiocytosis. Pediatr Blood Cancer. 2006;47:194–9.
41. Coury F, Annels N, Rivollier A, et al. Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. Nat Med. 2008;14:81–7.
42. Murakami I, Morimoto A, Oka T, et al. IL-17A receptor expression differs between subclasses of Langerhans cell histiocytosis, which might settle the IL-17A controversy. Virchows Arch. 2013;462:219–28.
43. Oh Y, Oh I, Morimoto J, et al. Osteopontin has a crucial role in osteoclast-like multinucleated giant cell formation. J Cell Biochem. 2014b;115:585–95.
44. Murakami I, Matsushita M, Iwasaki T, et al. Merkel cell polyomavirus DNA sequences in peripheral blood and tissues from patients with Langerhans cell histiocytosis. Hum Pathol. 2014;45:119–26.
45. Murakami I, Matsushita M, Iwasaki T, et al. Interleukin-1 loop model for pathogenesis of Langerhans cell histiocytosis. Cell Commun Signal. 2015a;13:13.
46. Prasse A, Stahl M, Schulz G, et al. Essential role of osteopontin in smoking-related interstitial lung diseases. Am J Pathol. 2009;174:1683–91.
47. Morimoto A, Ishida Y, Suzuki N, et al. Nationwide survey of single-system single site LCH in Japan. Pediatr Blood Cancer. 2010;54:98–102.
48. Weitzman S, Egeler RM. Langerhans cell histiocytosis of bone. In: Weitzman S, Egeler RM, editors. Histiocytic disorders of children and adults. Cambridge: Cambridge University Press; 2005. p. 154–73.
49. Imashuku S, Kinugawa N, Matsuzaki A, et al. Langerhans cell histiocytosis with multifocal bone lesions: comparative clinical features between single and multi-systems. Int J Hematol. 2009;90:506–12.
50. Phillips M, Allen C, Gerson P, et al. Comparison of FDG-PET scans to conventional radiography and bone scans in management of Langerhans cell histiocytosis. Pediatr Blood Cancer. 2009;52:97–101.
51. Goo HW, Yang DH, Ra YS, et al. Whole-body MRI of Langerhans cell histiocytosis: comparison with radiography and bone scintigraphy. Pediatr Radiol. 2006;36:1019–31.
52. Aricò M, Astigarraga I, Braier J, et al. Lack of bone lesions at diagnosis is associated with inferior outcome in multisystem Langerhans cell histiocytosis of childhood. Br J Haematol. 2015;169:241–8.
53. Krafchik B, Pope E, Walsh S. Histiocytosis of the skin in children and adults. In: Weitzman S, Egeler RM, editors. Histiocytic disorders of children and adults. Cambridge: Cambridge University Press; 2005. p. 130–53.
54. Simko SJ, Garmezy B, Abhyankar H, et al. Differentiating skin-limited and multisystem Langerhans cell histiocytosis. J Pediatr. 2014a;165:990–6.
55. Battistella M, Fraitag S, Teillac DH, et al. Neonatal and early infantile cutaneous Langerhans cell histiocytosis: comparison of self-regressive and non-self-regressive forms. Arch Dermatol. 2010;146:149–56.
56. Minkov M, Prosch H, Steiner M, et al. Langerhans cell histiocytosis in neonates. Pediatr Blood Cancer. 2005;45:802–7.

57. Lau L, Krafchik B, Trebo MM, et al. Cutaneous Langerhans cell histiocytosis in children under one year. Pediatr Blood Cancer. 2006;46:66–71.

58. Morimoto A, Shioda Y, Imamura T, et al. Intensified and prolonged therapy comprising cytarabine, vincristine and prednisolone improves outcome in patients with multisystem Langerhans cell histiocytosis: results of the Japan Langerhans Cell Histiocytosis Study Group-02 Protocol Study. Int J Hematol. 2016;104:99–109.

59. Ronceray L, Pötschger U, Janka G, et al. Pulmonary involvement in pediatric-onset multisystem Langerhans cell histiocytosis: effect on course and outcome. J Pediatr. 2012;161:129-33. e1–3.

60. Haupt R, Minkov M, Astigarraga I, et al. Langerhans cell histiocytosis (LCH): guidelines for diagnosis, clinical work-up, and treatment for patients till the age of 18 years. Pediatr Blood Cancer. 2013;60:175–84.

61. Braier JL, Rosso D, Latella A, et al. Importance of multi-lineage hematologic involvement and hypoalbuminemia at diagnosis in patients with "risk-organ" multi-system Langerhans cell histiocytosis. J Pediatr Hematol Oncol. 2010;32:e122–5.

62. Braier J, Ciocca M, Latella A, et al. Cholestasis, sclerosing cholangitis, and liver transplantation in Langerhans cell histiocytosis. Med Pediatr Oncol. 2002;38:178–82.

63. Matsubayashi T, Miwa Y, Saito I, et al. KL-6: marker for pulmonary involvement in Langerhans cell histiocytosis in infants. J Pediatr Hematol Oncol. 2004;26:584–6.

64. Dauriat G, Mal H, Thabut G, et al. Lung transplantation for pulmonary Langerhans' cell histiocytosis: a multicenter analysis. Transplantation. 2006;81:746–50.

65. Grois N, Fahrner B, Arceci RJ, et al. Central nervous system disease in Langerhans cell histiocytosis. J Pediatr. 2010;156:873–81.

66. Dhall G, Finlay JL, Dunkel IJ, et al. Analysis of outcome for patients with mass lesions of the central nervous system due to Langerhans cell histiocytosis treated with 2-chlorodeoxyadenosine. Pediatr Blood Cancer. 2008;50:72–9.

67. Shioda Y, Adachi S, Imashuku S, et al. Analysis of 43 cases of Langerhans cell histiocytosis (LCH)-induced central diabetes insipidus registered in the JLSG-96 and JLSG-02 studies in Japan. Int J Hematol. 2011;94:545–51.

68. Grois N, Potschger U, Prosch H, et al. Risk factors for diabetes insipidus in Langerhans cell histiocytosis. Pediatr Blood Cancer. 2006;46:228–33.

69. Donadieu J, Rolon MA, Thomas C, et al. Endocrine involvement in pediatric-onset Langerhans' cell histiocytosis: a population-based study. J Pediatr. 2004;144:344–50.

70. Grois N, Prayer D, Prosch H, et al. Course and clinical impact of magnetic resonance imaging findings in diabetes insipidus associated with Langerhans cell histiocytosis. Pediatr Blood Cancer. 2004;43:59–65.

71. Imashuku S, Kudo N, Kaneda S, et al. Treatment of patients with hypothalamic-pituitary lesions as adult-onset Langerhans cell histiocytosis. Int J Hematol. 2011;94:556–60.

72. Imashuku S, Ishida S, Koike K, et al. Cerebellar ataxia in pediatric patients with Langerhans cell histiocytosis. J Pediatr Hematol Oncol. 2004;26:735–9.

73. Wnorowski M, Prosch H, Prayer D, et al. Pattern and course of neurodegeneration in Langerhans cell histiocytosis. J Pediatr. 2008;153:127–32.

74. Prosch H, Grois N, Wnorowski M, et al. Long-term MR imaging course of neurodegenerative Langerhans cell histiocytosis. Am J Neuroradiol. 2007;28:1022–8.

75. Grois N, Prayer D, Prosch H, et al. Neuropathology of CNS disease in Langerhans cell histiocytosis. Brain. 2005;128(Pt 4):829–38.

76. Imashuku S, Shioda Y, Kobayashi R, et al. Neurodegenerative central nervous system disease as late sequelae of Langerhans cell histiocytosis. Report from the Japan LCH Study Group. Haematologica. 2008;93:615–8.

77. Imashuku S, Fujita N, Shioda Y, et al. Follow-up of pediatric patients treated by IVIG for Langerhans cell histiocytosis (LCH)-related neurodegenerative CNS disease. Int J Hematol. 2015;101:191–7.

78. Imashuku S, Shioda Y, Tsunematsu Y. VCR/AraC chemotherapy and ND-CNS-LCH. Pediatr Blood Cancer. 2010;55:215–6.

79. Ehrhardt MJ, Karst J, Donohoue PA, et al. Recognition and treatment of concurrent active and neurodegenerative Langerhans cell histiocytosis: a case report. J Pediatr Hematol Oncol. 2015;37:e37–40.

80. Edelweiss M, Medeiros LJ, Suster S, et al. Lymph node involvement by Langerhans cell histiocytosis: a clinicopathologic and immunohistochemical study of 20 cases. Hum Pathol. 2007;38:1463–9.

81. Ducassou S, Seyrig F, Thomas C, et al. Thymus and mediastinal node involvement in childhood Langerhans cell histiocytosis: long-term follow-up from the French National Cohort. Pediatr Blood Cancer. 2013;60:1759–65.

82. Madrigal-Martínez-Pereda C, Guerrero-Rodríguez V, Guisado-Moya B, et al. Langerhans cell histiocytosis: literature review and descriptive analysis of oral manifestations. Med Oral Patol Oral Cir Bucal. 2009;14:E222–8.

83. Yadav SP, Kharya G, Mohan N, et al. A. Langerhans cell histiocytosis with digestive tract involvement. Pediatr Blood Cancer. 2010;55:748–53.

84. Shima H, Takahashi T, Shimada H. Protein-losing enteropathy caused by gastrointestinal tract-involved Langerhans cell histiocytosis. Pediatrics. 2010;125:e426–32.

85. Burnett A, Carney D, Mukhopadhyay S, et al. Thyroid involvement with Langerhans cell histiocytosis in a 3-year-old male. Pediatr Blood Cancer. 2008;50:726–7.

86. Goyal R, Das A, Nijhawan R, et al. Langerhans cell histiocytosis infiltration into pancreas and kidney. Pediatr Blood Cancer. 2007;49:748–50.

87. Hyman DM, Diamond EL, Vibat CR, et al. Prospective blinded study of BRAFV600E mutation detection in cell-free DNA of patients with systemic histiocytic disorders. Cancer Discov. 2015;5:64–71.

88. Kobayashi M, Tojo A. The BRAF-V600E mutation in circulating cell-free DNA is a promising biomarker of high-risk adult Langerhans cell histiocytosis. Blood. 2014;124:2610–1.

89. Murakami I, Oh Y, Morimoto A, et al. Acute-phase ITIH4 levels distinguish multi-system from single-system Langerhans cell histiocytosis via plasma peptidomics. Clin Proteomics. 2015b;12:16.

90. Donadieu J, Egeler RM, Pritchard J. Langerhans cell histiocytosis: a clinical update. In: Weitzman S, Egeler RM, editors. Histiocytic disorders of children and adults. Cambridge: Cambridge University Press; 2005. p. 95–129.

91. Gadner H, Grois N, Arico M, et al. A randomized trial of treatment for multisystem Langerhans' cell histiocytosis. J Pediatr. 2001;138:728–34.

92. Kudo K, Yoshida H, Kiyoi H, et al. Etoposide-related acute promyelocytic leukemia. Leukemia. 1998;12:1171–5.

93. Haupt R, Nanduri V, Egeler RM. Late effects of Langerhans cell histiocytosis and its association with malignancy. In: Weitzman S, Egeler RM, editors. Histiocytic disorders of children and adults. Cambridge: Cambridge University Press; 2005. p. 272–92.

94. Egeler RM, Thompson Jr RC, Voûte PA, et al. Intralesional infiltration of corticosteroids in localized Langerhans' cell histiocytosis. J Pediatr Orthop. 1992;12:811–4.

95. Minkov M, Grois N, McClain K et al. Standard of care LCH treatment guidelines. 2009. http://www.histiocytesociety.org/document.doc?id=290. Accessed 10 Jun 2016.

96. Gadner H, Grois N, Pötschger U, et al. Improved outcome in multisystem Langerhans cell histiocytosis is associated with therapy intensification. Blood. 2008;111:2556–62.

97. Gadner H, Minkov M, Grois N, et al. Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis. Blood. 2013;121:5006–14.

98. Morimoto A, Ikushima S, Kinugawa N, et al. Improved outcome in the treatment of pediatric multifocal Langerhans cell histiocytosis: results from the Japan Langerhans Cell Histiocytosis Study Group-96 protocol study. Cancer. 2006;107:613–9.

99. Minkov M, Grois N, Heitger A, et al. Response to initial treatment of multisystem Langerhans cell histiocytosis: an important prognostic indicator. Med Pediatr Oncol. 2002;39:581–5.

100. Donadieu J, Bernard F, van Noesel M, et al. Cladribine and cytarabine in refractory multisystem Langerhans cell histiocytosis: results of an international phase 2 study. Blood. 2015;126:1415–23.

101. Simko SJ, Tran HD, Jones J, et al. Clofarabine salvage therapy in refractory multifocal histiocytic disorders, including Langerhans cell histiocytosis, juvenile xanthogranuloma and Rosai-Dorfman disease. Pediatr Blood Cancer. 2014b;61:479–87.
102. Rosso DA, Amaral D, Latella A, et al. Reduced doses of cladribine and cytarabine regimen was effective and well tolerated in patients with refractory-risk multisystem Langerhans cell histiocytosis. Br J Haematol. 2016;172:287–90.
103. Kudo K, Ohga S, Morimoto A, et al. Improved outcome of refractory Langerhans cell histiocytosis in children with hematopoietic stem cell transplantation in Japan. Bone Marrow Transplant. 2010;45:901–6.
104. Veys PA, Nanduri V, Baker KS, et al. Haematopoietic stem cell transplantation for refractory Langerhans cell histiocytosis: outcome by intensity of conditioning. Br J Haematol. 2015;169:711–8.
105. Steiner M, Matthes-Martin S, Attarbaschi A, et al. Improved outcome of treatment-resistant high-risk Langerhans cell histiocytosis after allogeneic stem cell transplantation with reduced-intensity conditioning. Bone Marrow Transplant. 2005;36:215–25.
106. Héritier S, Jehanne M, Leverger G, et al. Vemurafenib use in an infant for high-risk Langerhans cell histiocytosis. JAMA Oncol. 2015;1:836–8.
107. Minkov M, Steiner M, Pötschger U, et al. Reactivations in multisystem Langerhans cell histiocytosis: data of the international LCH registry. J Pediatr. 2008;153(5):700.
108. Morimoto A, Kobayashi R, Maeda M, et al. Impact of reactivation on the sequelae of multisystem Langerhans cell histiocytosis patients. Pediatr Blood Cancer. 2008;50:931–2.
109. Morimoto A, Shioda Y, Imamura T, et al. Nationwide survey of bisphosphonate therapy for children with reactivated Langerhans cell histiocytosis in Japan. Pediatr Blood Cancer. 2011b;56:110–5.
110. Imamura T, Sato T, Shiota Y, et al. Outcome of pediatric patients with Langerhans cell histiocytosis treated with 2 chlorodeoxyadenosine: a nationwide survey in Japan. Int J Hematol. 2010;91:646–51.
111. Weitzman S, Braier J, Donadieu J, et al. 2′-Chlorodeoxyadenosine (2-CdA) as salvage therapy for Langerhans cell histiocytosis (LCH). Results of the LCH-S-98 protocol of the Histiocyte Society. Pediatr Blood Cancer. 2009;53:1271–6.
112. Suzuki D, Kobayashi R, Sano H, et al. Spontaneous remission of 2-chlorodeoxyadenosine (2-CdA)-related secondary myelodysplastic syndrome in a patient with refractory Langerhans cell histiocytosis. Int J Hematol. 2013;97:782–5.
113. Yamada K, Yasui M, Sawada A, et al. Severe persistent bone marrow failure following therapy with 2-chlorodeoxyadenosine for relapsing juvenile xanthogranuloma of the brain. Pediatr Blood Cancer. 2012;58(2):300.
114. Haupt R, Nanduri V, Calevo MG, et al. Permanent consequences in Langerhans cell histiocytosis patients: a pilot study from the Histiocyte Society-Late Effects Study Group. Pediatr Blood Cancer. 2004;42:438–44.

Chapter 12
Primary Hemophagocytic Lymphohistiocytosis

Takahiro Yasumi, Hirofumi Shibata, Saeko Shimodera, and Toshio Heike

Abstract   Hemophagocytic lymphohistiocytosis (HLH) is a rare but fatal syndrome of dysregulated immune reaction that mostly affects infants and young children. HLH represents the extreme end of uncontrolled inflammatory reaction and can occur in various clinical settings. It is classified into primary and secondary forms based on the underlying etiology. Since the first report of causative gene for primary HLH in 1999, it has been clarified that a defect in lymphocyte cytotoxic function leads to abnormal activation of T cells and subsequent release of inflammatory cytokines. Herein, recent advances in genetics, pathophysiology, diagnostic procedure, and clinical management of primary HLH are reviewed.

12.1   Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a rare but life-threatening syndrome of excessive inflammation caused by dysregulated immune activation. It is characterized clinically by persistent fever, splenomegaly, cytopenia, hypofibrinogenemia, hypertriglyceridemia, increased levels of serum ferritin and soluble interleukin-2 receptor (sIL-2R), and histologically by the presence of benign hemophagocytic macrophages [1, 2]. It most often manifests in young infants but also affects children and adults of all ages. HLH is classified into primary or secondary forms according to the underlying etiology. Most forms of primary HLH are caused by defects in genes involved in the cytolytic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), and dysregulated activation of these lymphocytes is central to the pathogenesis. Secondary HLH occurs in association with infections, autoimmunity, auto-inflammatory disorders, and malignancies. HLH can also infrequently associate with immune deficiency conditions such as chronic granulomatous diseases and combined immunodeficiency disorders [3]. These cases are generally recognized as secondary HLH complicating other genetic disorders and

T. Yasumi (✉) • H. Shibata • S. Shimodera • T. Heike
Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan
e-mail: yasumi@kuhp.kyoto-u.ac.jp

not as primary HLH. The mechanism leading to secondary HLH development needs to be unraveled, but recent reports suggest distinct pathophysiology from that of primary HLH. In this chapter, we focus on primary forms of HLH and review their genetics, pathophysiology, diagnostic procedure, and clinical management.

12.2   Subtypes of Primary HLH (Table 12.1)

The granule-dependent cytotoxic pathway is a major immune effector mechanism employed by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. The pathway involves a series of steps including delivery of the lysosomal granules to the immunological synapse, exocytosis of lytic proteins such as perforin and granzymes, and induction of apoptosis in the target cells (Fig. 12.2). This pathway plays a central role in the defense against intracellular infections and in tumor immunity, and also an important role in the regulation of immune homeostasis. A defect in genes involved in granule-dependent cytotoxic pathway is the cause of familial hemophagocytic lymphohistiocytosis (FHL), the prototypic primary HLH syndrome [4].

Based on genetics, FHL has been categorized into five subtypes. The causative gene of FHL1 is yet to be identified, and it is unknown if the patients had any defect in the lymphocyte cytolytic activities. FHL2-5 are caused by mutations in the genes encoding perforin (*PRF1*; FHL2), Munc13-4 (*UNC13D*; FHL3), syntaxin-11 (*STX11*; FHL4), and syntaxin-binding protein 2 (also known as Munc18-2) (*STXBP2*; FHL5) [5–9]. Perforin is an effector molecule contained in cytolytic granules. Munc13-4,

Table 12.1 Subtypes of primary HLH

Disease	Defective gene	Protein	Inheritance	Protein function
FHL syndromes without hypopigmentation				
FHL2	*PRF1*	Perforin	AR	Pore formation
FHL3	*UNC13D*	Munc13-4	AR	Vesicle priming
FHL4	*STX11*	Syntaxin11	AR	Vesicle fusion
FHL5	*STXBP2*	Munc18-2	AR, AD	Vesicle fusion
FHL syndromes with hypopigmentation				
Chediak-Higashi syndrome	*CHS1/LYST*	LYST	AR	Vesicle sorting
Griscelli syndrome type 2	*RAB27A*	Rab27a	AR	Vesicle docking
Hermansky-Pudlak syndrome type 2	*AP3B1*	AP-3 complex β3A subunit	AR	Vesicle polarization
Hermansky-Pudlak syndrome type 9	*PLDN*	Pallidin	AR	Vesicle polarization
X-linked lymphoproliferative disease				
XLP type 1	*SH2D1A*	SAP	XL	SLAM family receptor signaling
XLP type 2	*XIAP/BIRC4*	XIAP	XL	Inhibition of cell death and inflammasome activation

AR autosomal recessive, *AD* autosomal dominant, *XL* X-linked

syntaxin-11, and Munc18-2 are involved in intracellular trafficking or the fusion of those granules to the plasma membrane and the delivery of their contents into target cells (Fig. 12.1). As a result, defective cytotoxic activity of CTLs and NK cells is the hallmark findings of FHL. Because the trafficking of melanosomes shares machinery with that of cytotoxic granules, primary HLH includes disorders characterized by oculocutaneous albinism and impaired lymphocyte cytotoxicity (Fig. 12.1). Chediak-Higashi syndrome (CHS) is caused by mutations in *LYST*, Griscelli syndrome (GS) type 2 by mutations in *RAB27A*, and Hermansky-Pudlak syndrome (HPS) type 2 by mutations in *AP3B1* [10–13]. Majority of patients with CHS and GS type 2 develop symptom of HLH; however, few cases of HLH in HPS type 2 have been described in literature [14–16]. *PLDN* mutations have recently been identified to cause HPS type 9, but it is not clear if full-blown HLH accompanies this disorder [17].

Two X-linked disorders associated with Epstein-Barr virus (EBV)-driven HLH without direct evidence of impairment in lymphocyte cytotoxicity are X-linked lymphoproliferative syndrome (XLP) type 1 and type 2. XLP type 1 is caused by a mutation in gene coding signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) (*SH2D1A*) and XLP type 2 by mutations in gene coding X-linked inhibitor of apoptosis (XIAP) (*BIRC4*) [18–20]. Recently, mutations in genes including *ITK*, *CD27*, and *MAGT1* are reported to cause EBV-associated lymphoproliferation and lymphoma; however, HLH seems to be an infrequent complication in these diseases [21].
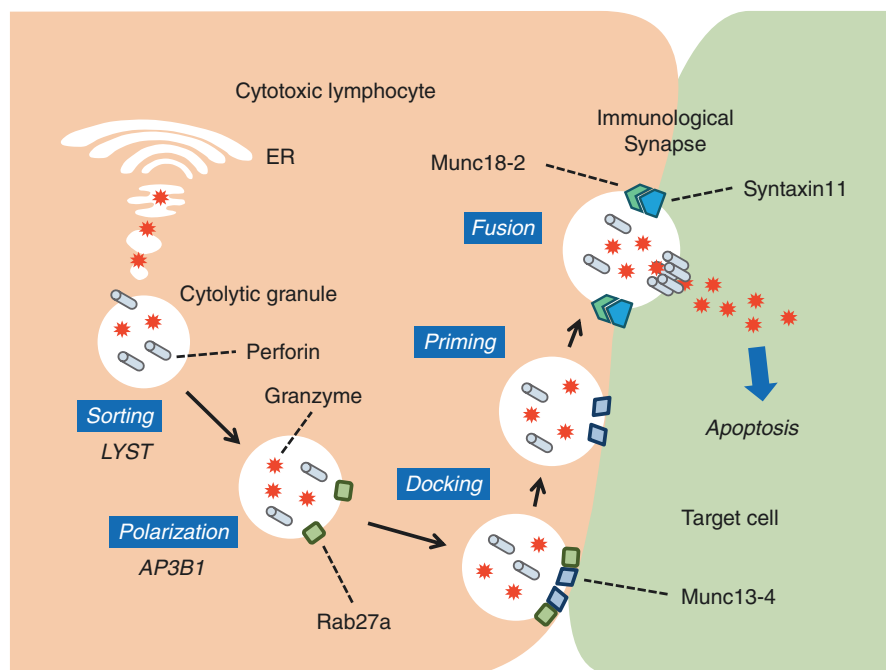
Fig. 12.1 Overview of the granule-dependent cytotoxic pathway. The granule-dependent cytotoxic pathway is a major immune effector mechanism employed by CTLs and NK cells. The pathway involves a series of steps including delivery of the lysosomal granules to the immunological synapse, exocytosis of lytic proteins such as perforin and granzymes, and induction of apoptosis in the target cells

12.3   Pathophysiology

In addition to its central role in inducing apoptosis in targets such as cells infected with intracellular pathogens and tumor cells, the granule-dependent cytotoxic pathway is involved in inducing apoptosis in antigen-presenting cells (APCs) and possibly in CTLs themselves. In healthy individuals, CTL reaction subsides with a small number of residual memory cells as the antigen is cleared. In FHL patients, impaired clearance of antigens and defective suppression of antigen presentation, together with the inhibition of apoptosis in CTLs themselves, result in hyperactivation of CTLs and excessive production of inflammatory cytokines such as interferon-γ (IFN-γ) (Fig. 12.2). This leads to secondary activation of other cells including macrophages to produce additional inflammatory cytokines and results in "cytokine-storm" state characteristic of HLH.
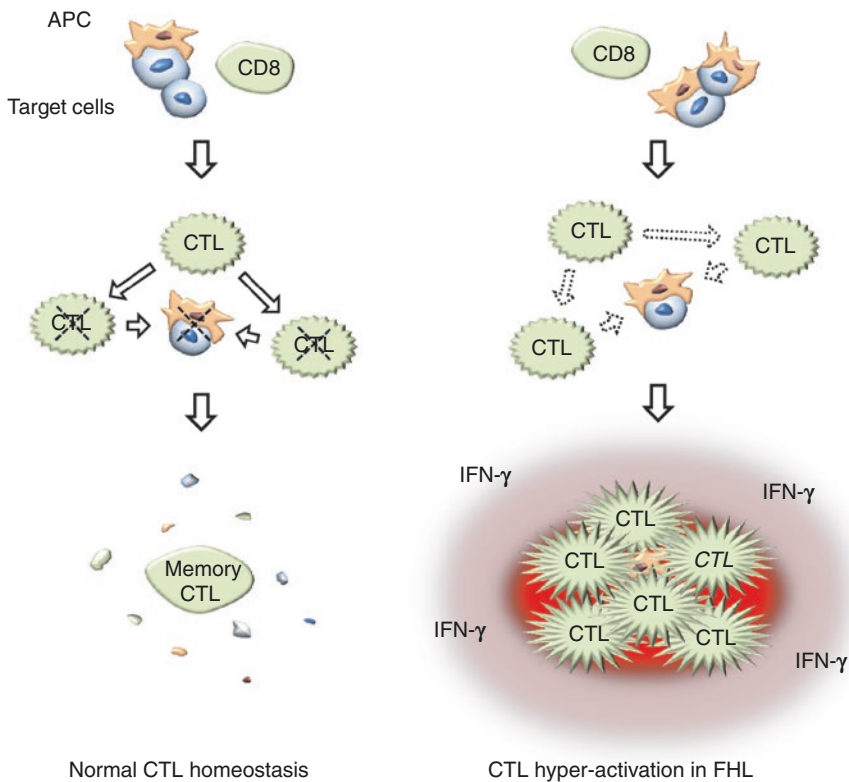
Fig. 12.2 Defective cytotoxic activity causes dysregulated CTL activation. In addition to its central role in inducing apoptosis in target cells, the granule-dependent cytotoxic pathway is involved in inducing apoptosis in APCs and in CTLs themselves. In healthy individuals, CTL reaction subsides with a small number of residual memory cells as the antigen is cleared. In FHL patients, impaired clearance of targets and defective suppression of antigen presentation, together with the inhibition of apoptosis in CTLs themselves, result in hyperactivation of CTLs and excessive production of inflammatory cytokines such as IFN-γ

In XLP type 1 patients, HLH development is often associated with EBV-infection. SAP-deficient CTLs from patients with XLP type 1 display a specific lytic defect against autologous and allogeneic EBV-infected B cells, as well as an impairment in activation-induced apoptosis of their own [22, 23]. These observations suggest that the pathogenesis of HLH development after EBV-infection in XLP type 1 is similar to that of FHL syndromes (Fig. 12.3).

In contrast to XLP type 1, no evidence of cytolytic defect is reported for CTLs from XLP type 2 patients, and XIAP deficiency is associated with increased apoptosis of activated CTLs. Therefore, the mechanism behind HLH development in XLP type 2 seems to differ from that of FHL and XLP type 1. Recently, XIAP is shown to suppress inflammasome activity, and sustained elevation of serum IL-18 is reported in patients with XLP type 2 [24–26]. It is possible that pathophysiology of HLH in XLP type 2 may be similar to that of secondary HLH complicating auto-inflammatory disorders.
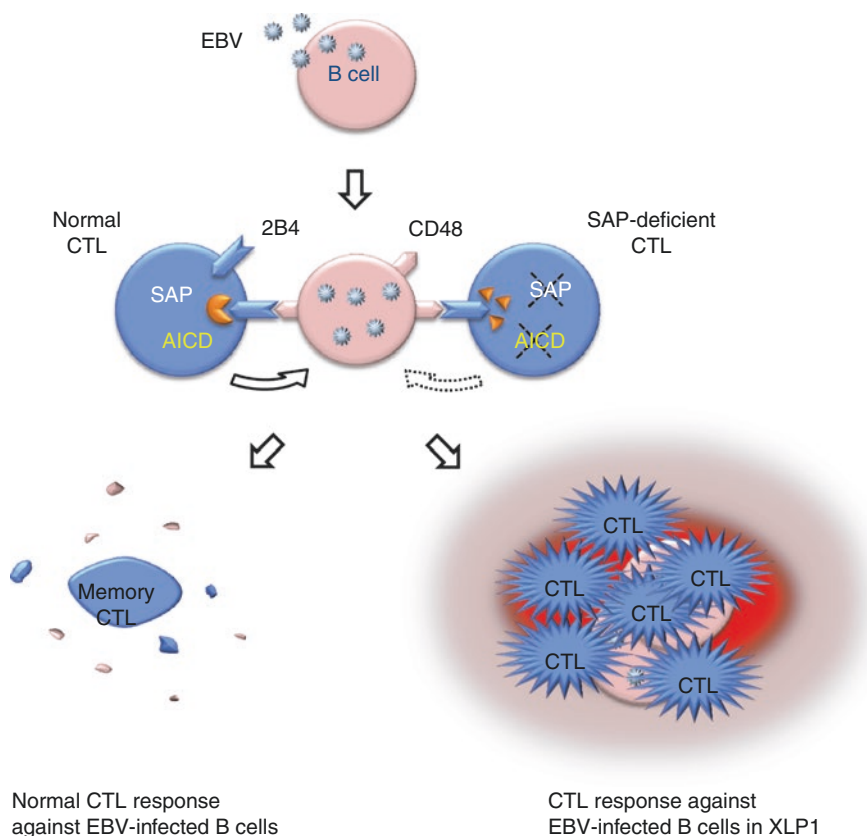
Fig. 12.3 Hyperactivation of CTLs against EBV-infected B cells in SAP-deficient patients. SAP-deficient CTLs from patients with XLP type 1 display a specific lytic defect against EBV-infected B cells, as well as an impairment in their own activation-induced apoptosis

12.4 Clinical Manifestations

Primary HLH predominantly manifests in young infants but can potentially affect all age groups. Rare cases with fetal and elderly onset have been reported. Clinical presentation in these cases may be atypical and the diagnosis is often delayed [27, 28].

Classical manifestations of HLH include prolonged fever, progressive cytopenias, hepatosplenomegaly, liver dysfunction, and coagulopathy. Central nervous system (CNS) symptoms can be found in one- to two-thirds of FHL patients and include decreased level of consciousness, cranial nerve palsies, and seizures [29, 30]. FHL3 patients are more prone to have CNS symptoms compared to FHL2 [31]. FHL4 and XLP type 2 patients are reported to have milder and recurrent episodes of HLH compared to patients with other forms of primary HLH. It has recently been proposed that graded defects in lymphocyte cytotoxicity determine the severity of HLH associated with each subtype [32].

Beside symptoms of HLH, each subtype of primary HLH is characterized by specific symptoms. Colitis is seen in a significant proportion of patients with FHL5 and XLP type 2, and sensorineural hearing deficit is a common finding in FHL5 patients [33, 34]. Hypogammaglobulinemia is reported in patients with FHL5 and XLP types 1 and 2 [33, 35]. Lymphoma develops in one-third of XLP type 1 but not in type 2 patients [35]. Partial albinism accompanies CHS, GS type 2, and HPS type 2, and recurrent pyogenic infections are frequently observed in these patients. Bleeding diathesis is commonly seen in patients with CHS and HPS type 2 but usually requires no specific treatment. Progressive motor and sensory neuropathies and low cognitive abilities develop in CHS patients who survive into adulthood [36].

12.5 Diagnosis

Diagnostic criteria for HLH was proposed and updated by the Histiocyte Society based on studies of FHL syndromes [2]. The criteria include two clinical symptoms and six laboratory findings. Fever is the first criterion and is a common symptom of HLH; however, it is often absent in neonates. Splenomegaly with or without hepatomegaly is also on the list. Laboratory findings include cytopenias affecting at least two of three lineages in the peripheral blood; hypertrigryceridemia and/or hypofibrinogenemia; hemophagocytosis in the bone marrow, spleen, or lymph nodes; low or absent NK-cell activity; hyperferritinemia; and high levels of sIL-2R. HLH is considered present if at least five out of eight features are positive.

Leukocyte count at diagnosis is variable among patients; however, neutropenia and thrombocytopenia are typical findings of FHL patients from the early course of the disease. Analysis of bone marrow aspirates is commonly performed for diagnosing HLH, but hemophagocytosis is detected in only half to two-thirds of patients with FHL. Thus, a negative result should not preclude initiation of therapy when there is high clinical suspicion of FHL. Serum levels of sIL-2R and ferritin are

elevated in HLH and primarily reflect activation of T lymphocytes and macrophages, respectively. Since the activation of CTLs precedes that of macrophages in FHL, sIL-2R elevation is more prominent compared to that of ferritin in early course of the disease [37]. Low or absent NK cell activity is a common finding of FHL. However, transient impairment of NK cell activity is also a frequent finding in patients with secondary HLH [38].

Several rapid screening tests are available to identify FHL patients (Fig. 12.4). Detection of perforin expression in NK cells with flow cytometry is a reliable method to screen for FHL2 in a given patient [39, 40]. FHL3 can be screened by detecting intra-platelet expression of Munc13-4 protein [41]. XLP types 1 and 2 can be identified by detecting the intracellular expression of SAP and XIAP proteins, respectively [42–44]. Surface expression of CD107a on NK cells and CTLs reflects the integrity of cytotoxic granular release and is useful for identification of FHL3-5, CHS, GS type 2, and HPS type 2 [38, 45]. Some patients with secondary HLH may have a defect in resting NK cell degranulation; however, these patients show normal degranulation of NK cells after culturing with IL-2 [38, 45].
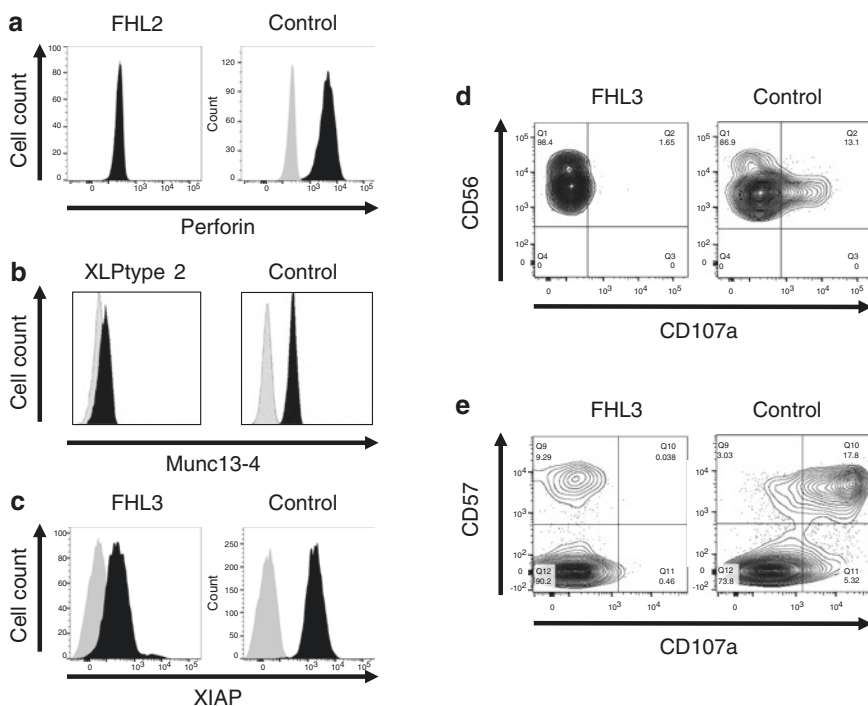
Fig. 12.4 Flow cytometry-based screening tests to identify patients with primary HLH. (**a**) FHL2 screening by detecting perforin expression in NK cells. (**b**) Screening of XLP type 2 patients by detecting XIAP expression in lymphocytes (Courtesy of Dr. Hirokazu Kanegane). (**c**) Intra-platelet Munc13-4 expression assay identifies FHL3 patients. Lysosomal degranulation assay using (**d**) NK cells and (**e**) CD57 + CTLs

Genetic analysis confirms the diagnosis of primary HLH. The incidents of gene defects responsible for primary HLH subtypes vary among countries and ethnic groups. Yet, up to 70% of cases are due to mutations in two genes, *PRF1* and *UNC13D* [46, 47].

FHL2 accounts for 20–50% of all FHL cases depending on the cohort studies. It is most common among African-Americans and mutation c.50delT is found very frequently. FHL2 is also the most common subtype of primary HLH in Japan, and c.1090-1091delCT mutation has been reported only in Japanese patients [48]. FHL3 covers 30–40% of FHL patients based on geographic and ethnic groups. FHL3 is the most common form of primary HLH in Korea and among Caucasians in the US [46, 49]. In Japan, it accounts for one-third of FHL cases [48]. It has become evident in 2011 that deep intronic mutation c.118-308C>T and the 253-kb inversion affecting the 3′-end of *UNC13D* transcript abolish Munc13-4 expression and account for significant proportion of FHL3 in northern European populations [50]. Original reports of FHL4 were restricted to families of Turkish/Kurdish origin, but patients of different origin have been reported recently [51, 52]. FHL4 patients seem to have a late onset and a milder clinical presentation compared to FHL2 and FHL3. FHL5 is reported to account for 20% of FHL cases in German cohort and seem to be found in every geographic region [33]. Clinical findings of FHL5 include colitis, bleeding tendency, and hypogammaglobulinemia in addition to typical presentation of HLH. A recent study described two unrelated FHL5 patients with mono-allelic dominant-negative mutations, c.193C>T and c.194G>A, in STXBP2 gene [53].

Incidence of primary HLH syndromes with albinism is much lower than that of FHL [47]. In Japan, only one or two CHS patients are newly diagnosed every year [54]. The pathognomonic feature of CHS is the presence of giant lysosomes and related organelles in various cell types. Decreased pool of platelet dense granules is associated with bleeding diathesis, while a defect in antimicrobial activity is due to the decrease of neutrophil enzymes. In neuronal cells, giant granule formation might have a role in the pathophysiology of neurological manifestations. CHS clinical phenotypes correlate with molecular genotypes; nonsense or frameshift mutations that cause early truncation of the protein are associated with early-onset disease characterized by severe infections and HLH while missense mutations are associated with milder, late-onset course of disease with progressive neuropathies [55]. In most patients with GS type 2, HLH develops between 6 and 12 months of age, later than in patients with FHL2 but earlier than in patients with FHL4 and CHS [32]. Neurological manifestations in GS type 2 patients occur only in association with the development of HLH because *RAB27A* gene is not expressed in neuronal cells. In 2015, GS type 2 patients without albinism were reported. Rab27a protein interacts with Munc13-4 and melanophilin. *RAB27A* mutations identified in these patients selectively disrupted the interaction of Rab27a protein with Munc13-4 but not with melanophilin [56]. HPS is a group of at least nine genetic disorders characterized by oculocutaneous albinism and bleeding diathesis. Of these, HPS types 2 and 9 are associated with cytotoxicity

impairment. However, only a few HPS type 2 patients with HLH development are reported in the literature [13, 16].

XLP is a rare inherited immunodeficiency affecting approximately one in 1,000,000 males. *SH2D1A* and *BIRC4* genes, responsible for XLP types 1 and 2, respectively, are both located on Xq25 in close vicinity. However, it is now evident that two disorders are not functionally related despite their clinical similarities at first sight. *SH2D1A* mutation is responsible for 60–70% of XLP cases. XLP type 1 patients typically exhibit EBV-induced HLH, progressive hypogammaglobulinemia, and malignant lymphoma. *BIRC4* mutation is found in 20–30% of XLP cases, and XLP type 2 patients typically present with EBV-triggered HLH, transient hypogammaglobulinemia during HLH episodes, hemorrhagic colitis, but not with malignant lymphoma [21, 57, 58].

12.6   Treatment

Current management of primary HLH involves a two-step approach. First step is a short-term immunosuppressive therapy aiming at controlling the inflammatory state. Second step aims at correcting the underlying genetic defect by allogeneic hematopoietic stem cell transplantation (HSCT). Because most HLH patients are critically ill at the time of HLH diagnosis, timely introduction of immunosuppressive therapy is required before determining if a given patient has primary or secondary HLH. Molecular screening for primary HLH is usually performed in parallel with the treatment.

Initial therapy of HLH focuses on stopping the activation of CTLs and macrophages and on blocking excessive production of inflammatory cytokines. It is also important to search for and treat underlying infectious trigger of HLH development. Early use of γ-globulin and/or corticosteroid is sometimes useful for transiently controlling the HLH activity; however, an aggressive immunosuppressive therapy is warranted for most cases.

The first international treatment protocol for HLH (HLH-94) was organized by the Histiocyte Society in 1994 mainly aiming at treating primary HLH patients by HSCT. This protocol included an 8-week induction therapy with dexamethasone, etoposide, and intrathecal methotrexate, followed by maintenance therapy with pulse dexamethasone, etoposide, and cyclosporine A, which is intended as a bridging therapy to HSCT. The 5-year survival rate was $54 \pm 6\%$ in all patients, $66 \pm 8\%$ for those who received HSCT, and the outcomes of HSCT were better in patients with adequately controlled HLH activity before HSCT conditioning [59]. Genetic information was unavailable in HLH-94 study because the protocol started 5 years before the first FHL causative gene was reported. Intensification of induction therapy was indicated because most of the patients who did not receive HSCT and who died during the first year had active signs of HLH. Thus, the second international trial HLH-2004 was opened with major modification in

induction therapy; cyclosporine A was used from the beginning and hydrocortisone was added to intrathecal therapy [2]. The result of the study is yet to be published, but cases of early death during the first 2-month period has been reduced. As a second line therapy for HLH refractory to etoposide-based therapy, Alemtuzumab, a monoclonal antibody to CD52, is reported to have induced partial response in 64% of the treated patients [60]. Beside etoposide-based regimens, an antithymocyte globulin (ATG) used in combination with corticosteroids as an initial treatment of primary HLH has been reported with comparable survival rate and acceptable toxicity [61].

For the most of patients with primary HLH, correction of genetic defects by HSCT is warranted after controlling the hyper-inflammatory state. Myeloablative conditioning (MAC) using busulfan, cyclophosphamide, and etoposide with or without ATG has long been the standard of HSCT for HLH patients [2]. However, MAC-based HSCT is associated with high treatment-related mortality [59, 62]. Recent reports suggest that reduced-intensity conditioning (RIC) can improve the outcome of HLH patients undergoing HSCT [63–65]. Still, a high incidence of mixed donor chimerism is associated with RIC-HSCT and long-term follow-up is needed to ascertain the outcome of the treatment.

The indication of HSCT for XLP type 2 depends on the clinical picture, because patients with XIAP deficiency were shown to have poor tolerance to MAC [66]. Recently reported patients survived RIST-HSCT with improvement of refractory inflammatory colitis [58, 67], and the control of posttransplant HLH with etoposide and corticosteroid seems to be an important factor for favorable outcomes [68]. The precise indication for HSCT remains also unclear for CHS patients because some patients have milder phenotype and survive into adulthood and because HSCT cannot prevent neurological dysfunction. Recent reports showed that the differences in the cytotoxic and degranulation capacity of CTLs may be predictive for the risk of HLH development [69]. Preemptive HSCT is not indicated for patients with HPS type 2 because it confers low risk of HLH [16].

12.7 Future Directions

Since the identification of first genetic defect for primary HLH in 1999, remarkable progress has been made in clinical practice and basic research. Detailed characterization of patients with primary HLH has contributed to our understanding of the granule-dependent cytotoxic pathway as well as pathophysiology of HLH. Aggressive immunosuppressive therapy followed by HSCT has greatly improved the prognosis of the patients; however, substantial risk of mortality remains. Novel therapeutic approaches with the use of ATG, Alemuzumab, and anti-IFN-γ monoclonal antibody are currently explored. Recently, *PRF1* gene transfer into hematopoietic stem cells was shown to improve immune dysregulation observed in murine model of FHL2 [70]. It is possible that a gene therapy option may become available for primary HLH in the near future.

References

1. Janka GE, Lehmberg K. Hemophagocytic syndromes—an update. Blood Rev. 2014;28(4):135–42. doi:10.1016/j.blre.2014.03.002.

2. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007;48(2):124–31. doi:10.1002/pbc.21039.

3. Bode SF, Ammann S, Al-Herz W, Bataneant M, Dvorak CC, Gehring S, et al. The syndrome of hemophagocytic lymphohistiocytosis in primary immunodeficiencies: implications for differential diagnosis and pathogenesis. Haematologica. 2015;100(7):978–88. doi:10.3324/haematol.2014.121608.

4. Sieni E, Cetica V, Hackmann Y, Coniglio ML, Da Ros M, Ciambotti B, et al. Familial hemophagocytic lymphohistiocytosis: when rare diseases shed light on immune system functioning. Front Immunol. 2014;5:167. doi:10.3389/fimmu.2014.00167.

5. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286(5446):1957–9.

6. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003;115(4):461–73.

7. zur Stadt U, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Hum Mol Genet. 2005;14(6):827–34. doi:10.1093/hmg/ddi076.

8. Cote M, Menager MM, Burgess A, Mahlaoui N, Picard C, Schaffner C, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. J Clin Invest. 2009;119(12):3765–73. doi:10.1172/JCI40732.

9. zur Stadt U, Rohr J, Seifert W, Koch F, Grieve S, Pagel J, et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. Am J Hum Genet. 2009;85(4):482–92. doi:10.1016/j.ajhg.2009.09.005.

10. Nagle DL, Karim MA, Woolf EA, Holmgren L, Bork P, Misumi DJ, et al. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. Nat Genet. 1996;14(3):307–11. doi:10.1038/ng1196-307.

11. Barbosa MD, Nguyen QA, Tchernev VT, Ashley JA, Detter JC, Blaydes SM, et al. Identification of the homologous beige and Chediak-Higashi syndrome genes. Nature. 1996;382(6588):262–5. doi:10.1038/382262a0.

12. Menasche G, Pastural E, Feldmann J, Certain S, Ersoy F, Dupuis S, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat Genet. 2000;25(2):173–6. doi:10.1038/76024.

13. Enders A, Zieger B, Schwarz K, Yoshimi A, Speckmann C, Knoepfle EM, et al. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. Blood. 2006;108(1):81–7. doi:10.1182/blood-2005-11-4413.

14. Introne W, Boissy RE, Gahl WA. Clinical, molecular, and cell biological aspects of Chediak-Higashi syndrome. Mol Genet Metab. 1999;68(2):283–303. doi:10.1006/mgme.1999.2927.

15. Meeths M, Bryceson YT, Rudd E, Zheng C, Wood SM, Ramme K, et al. Clinical presentation of Griscelli syndrome type 2 and spectrum of RAB27A mutations. Pediatr Blood Cancer. 2010;54(4):563–72. doi:10.1002/pbc.22357.

16. Jessen B, Bode SF, Ammann S, Chakravorty S, Davies G, Diestelhorst J, et al. The risk of hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type 2. Blood. 2013;121(15):2943–51. doi:10.1182/blood-2012-10-463166.

17. Badolato R, Prandini A, Caracciolo S, Colombo F, Tabellini G, Giacomelli M, et al. Exome sequencing reveals a pallidin mutation in a Hermansky-Pudlak-like primary immunodeficiency syndrome. Blood. 2012;119(13):3185–7. doi:10.1182/blood-2012-01-404350.

18. Sayos J, Wu C, Morra M, Wang N, Zhang X, Allen D, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature. 1998;395(6701):462–9. doi:10.1038/26683.

19. Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, et al. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. Nat Genet. 1998;20(2):129–35. doi:10.1038/2424.

20. Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, Soulas P, et al. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature. 2006;444(7115):110–4. doi:10.1038/nature05257.

21. Veillette A, Perez-Quintero LA, Latour S. X-linked lymphoproliferative syndromes and related autosomal recessive disorders. Curr Opin Allergy Clin Immunol. 2013;13(6):614–22. doi:10.1097/ACI.0000000000000008.

22. Dupre L, Andolfi G, Tangye SG, Clementi R, Locatelli F, Arico M, et al. SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. Blood. 2005;105(11):4383–9. doi:10.1182/blood-2004-08-3269.

23. Snow AL, Marsh RA, Krummey SM, Roehrs P, Young LR, Zhang K, et al. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. J Clin Invest. 2009;119(10):2976–89. doi:10.1172/JCI39518.

24. Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, et al. Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity. 2012;36(2):215–27. doi:10.1016/j.immuni.2012.01.012.

25. Yabal M, Muller N, Adler H, Knies N, Gross CJ, Damgaard RB, et al. XIAP restricts TNF- and RIP3-dependent cell death and inflammasome activation. Cell Rep. 2014;7(6):1796–808. doi:10.1016/j.celrep.2014.05.008.

26. Wada T, Kanegane H, Ohta K, Katoh F, Imamura T, Nakazawa Y, et al. Sustained elevation of serum interleukin-18 and its association with hemophagocytic lymphohistiocytosis in XIAP deficiency. Cytokine. 2014;65(1):74–8. doi:10.1016/j.cyto.2013.09.007.

27. Rohr J, Beutel K, Maul-Pavicic A, Vraetz T, Thiel J, Warnatz K, et al. Atypical familial hemophagocytic lymphohistiocytosis due to mutations in UNC13D and STXBP2 overlaps with primary immunodeficiency diseases. Haematologica. 2010;95(12):2080–7. doi:10.3324/haematol.2010.029389.

28. Zhang K, Jordan MB, Marsh RA, Johnson JA, Kissell D, Meller J, et al. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial HLH. Blood. 2011;118(22):5794–8. doi:10.1182/blood-2011-07-370148.

29. Horne A, Trottestam H, Arico M, Egeler RM, Filipovich AH, Gadner H, et al. Frequency and spectrum of central nervous system involvement in 193 children with haemophagocytic lymphohistiocytosis. Br J Haematol. 2008;140(3):327–35. doi:10.1111/j.1365-2141.2007.06922.x.

30. Deiva K, Mahlaoui N, Beaudonnet F, de Saint BG, Caridade G, Moshous D, et al. CNS involvement at the onset of primary hemophagocytic lymphohistiocytosis. Neurology. 2012;78(15):1150–6. doi:10.1212/WNL.0b013e31824f800a.

31. Ueda I, Ishii E, Morimoto A, Ohga S, Sako M, Imashuku S. Correlation between phenotypic heterogeneity and gene mutational characteristics in familial hemophagocytic lymphohistiocytosis (FHL). Pediatr Blood Cancer. 2006;46(4):482–8. doi:10.1002/pbc.20511.

32. Jessen B, Kogl T, Sepulveda FE, de Saint Basile G, Aichele P, Ehl S. Graded defects in cytotoxicity determine severity of hemophagocytic lymphohistiocytosis in humans and mice. Front Immunol. 2013;4:448. doi:10.3389/fimmu.2013.00448.

33. Pagel J, Beutel K, Lehmberg K, Koch F, Maul-Pavicic A, Rohlfs AK, et al. Distinct mutations in STXBP2 are associated with variable clinical presentations in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL5). Blood. 2012;119(25):6016–24. doi:10.1182/blood-2011-12-398958.

34. Latour S, Aguilar C. XIAP deficiency syndrome in humans. Semin Cell Dev Biol. 2015;39:115–23. doi:10.1016/j.semcdb.2015.01.015.

35. Pachlopnik Schmid J, Canioni D, Moshous D, Touzot F, Mahlaoui N, Hauck F, et al. Clinical similarities and differences of patients with X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) versus type 2 (XLP-2/XIAP deficiency). Blood. 2011;117(5):1522–9. doi:10.1182/blood-2010-07-298372.

36. Dotta L, Parolini S, Prandini A, Tabellini G, Antolini M, Kingsmore SF, et al. Clinical, laboratory and molecular signs of immunodeficiency in patients with partial oculo-cutaneous albinism. Orphanet J Rare Dis. 2013;8:168. doi:10.1186/1750-1172-8-168.

37. Yasumi T, Hori M, Hiejima E, Shibata H, Izawa K, Oda H, et al. Laboratory parameters identify familial haemophagocytic lymphohistiocytosis from other forms of paediatric haemophagocytosis. Br J Haematol. 2015;170(4):532–8. doi:10.1111/bjh.13461.

38. Bryceson YT, Pende D, Maul-Pavicic A, Gilmour KC, Ufheil H, Vraetz T, et al. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. Blood. 2012;119(12):2754–63. doi:10.1182/blood-2011-08-374199.

39. Kogawa K, Lee SM, Villanueva J, Marmer D, Sumegi J, Filipovich AH. Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. Blood. 2002;99(1):61–6.

40. Abdalgani M, Filipovich AH, Choo S, Zhang K, Gifford C, Villanueva J, et al. Accuracy of flow cytometric perforin screening for detecting patients with FHL due to PRF1 mutations. Blood. 2015;126(15):1858–60. doi:10.1182/blood-2015-06-648659.

41. Murata Y, Yasumi T, Shirakawa R, Izawa K, Sakai H, Abe J, et al. Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4 protein. Blood. 2011;118(5):1225–30. doi:10.1182/blood-2011-01-329540.

42. Marsh RA, Villanueva J, Zhang K, Snow AL, Su HC, Madden L, et al. A rapid flow cytometric screening test for X-linked lymphoproliferative disease due to XIAP deficiency. Cytometry B Clin Cytom. 2009;76(5):334–44. doi:10.1002/cyto.b.20473.

43. Marsh RA, Bleesing JJ, Filipovich AH. Using flow cytometry to screen patients for X-linked lymphoproliferative disease due to SAP deficiency and XIAP deficiency. J Immunol Methods. 2010;362(1–2):1–9. doi:10.1016/j.jim.2010.08.010.

44. Zhao M, Kanegane H, Kobayashi C, Nakazawa Y, Ishii E, Kasai M, et al. Early and rapid detection of X-linked lymphoproliferative syndrome with SH2D1A mutations by flow cytometry. Cytometry B Clin Cytom. 2011;80(1):8–13. doi:10.1002/cyto.b.20552.

45. Hori M, Yasumi T, Shimodera S, Shibata H, Hiejima E, Oda H, et al. A CD57+ CTL degranulation assay effectively identifies familial hemophagocytic lymphohistiocytosis type 3 patients. J Clin Immunol. 2017;37(1):92–9. doi:10.1007/s10875-016-0357-3.

46. Jordan MB, Allen CE, Weitzman S, Filipovich AH, McClain KL. How I treat hemophagocytic lymphohistiocytosis. Blood. 2011;118(15):4041–52. doi:10.1182/blood-2011-03-278127.

47. Cetica V, Sieni E, Pende D, Danesino C, De Fusco C, Locatelli F et al. Genetic predisposition to hemophagocytic lymphohistiocytosis: report on 500 patients from the Italian registry. J Allergy Clin Immunol. 2016;137(1):188–96, e4. doi:10.1016/j.jaci.2015.06.048.

48. Nagai K, Yamamoto K, Fujiwara H, An J, Ochi T, Suemori K, et al. Subtypes of familial hemophagocytic lymphohistiocytosis in Japan based on genetic and functional analyses of cytotoxic T lymphocytes. PLoS One. 2010;5(11):e14173. doi:10.1371/journal.pone.0014173.

49. Yoon HS, Kim HJ, Yoo KH, Sung KW, Koo HH, Kang HJ, et al. UNC13D is the predominant causative gene with recurrent splicing mutations in Korean patients with familial hemophagocytic lymphohistiocytosis. Haematologica. 2010;95(4):622–6. doi:10.3324/haematol.2009.016949.

50. Meeths M, Chiang SC, Wood SM, Entesarian M, Schlums H, Bang B, et al. Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) caused by deep intronic mutation and inversion in UNC13D. Blood. 2011;118(22):5783–93. doi:10.1182/blood-2011-07-369090.

51. Rudd E, Goransdotter Ericson K, Zheng C, Uysal Z, Ozkan A, Gurgey A, et al. Spectrum and clinical implications of syntaxin 11 gene mutations in familial haemophagocytic lymphohistiocytosis: association with disease-free remissions and haematopoietic malignancies. J Med Genet. 2006;43(4):e14. doi:10.1136/jmg.2005.035253.

52. Marsh RA, Satake N, Biroschak J, Jacobs T, Johnson J, Jordan MB, et al. STX11 mutations and clinical phenotypes of familial hemophagocytic lymphohistiocytosis in North America. Pediatr Blood Cancer. 2010;55(1):134–40. doi:10.1002/pbc.22499.
53. Spessott WA, Sanmillan ML, McCormick ME, Patel N, Villanueva J, Zhang K, et al. Hemophagocytic lymphohistiocytosis caused by dominant-negative mutations in STXBP2 that inhibit SNARE-mediated membrane fusion. Blood. 2015;125(10):1566–77. doi:10.1182/blood-2014-11-610816.
54. Nagai K, Ochi F, Terui K, Maeda M, Ohga S, Kanegane H, et al. Clinical characteristics and outcomes of Chediak-Higashi syndrome: a nationwide survey of Japan. Pediatr Blood Cancer. 2013;60(10):1582–6. doi:10.1002/pbc.24637.
55. Karim MA, Suzuki K, Fukai K, Oh J, Nagle DL, Moore KJ, et al. Apparent genotype-phenotype correlation in childhood, adolescent, and adult Chediak-Higashi syndrome. Am J Med Genet. 2002;108(1):16–22.
56. Cetica V, Hackmann Y, Grieve S, Sieni E, Ciambotti B, Coniglio ML et al. Patients with Griscelli syndrome and normal pigmentation identify RAB27A mutations that selectively disrupt MUNC13-4 binding. J Allergy Clin Immunol. 2015;135(5):1310–8, e1. doi:10.1016/j.jaci.2014.08.039.
57. Booth C, Gilmour KC, Veys P, Gennery AR, Slatter MA, Chapel H, et al. X-linked lymphoproliferative disease due to SAP/SH2D1A deficiency: a multicenter study on the manifestations, management and outcome of the disease. Blood. 2011;117(1):53–62. doi:10.1182/blood-2010-06-284935.
58. Speckmann C, Lehmberg K, Albert MH, Damgaard RB, Fritsch M, Gyrd-Hansen M, et al. X-linked inhibitor of apoptosis (XIAP) deficiency: the spectrum of presenting manifestations beyond hemophagocytic lymphohistiocytosis. Clin Immunol. 2013;149(1):133–41. doi:10.1016/j.clim.2013.07.004.
59. Trottestam H, Horne A, Arico M, Egeler RM, Filipovich AH, Gadner H, et al. Chemoimmunotherapy for hemophagocytic lymphohistiocytosis: long-term results of the HLH-94 treatment protocol. Blood. 2011;118(17):4577–84. doi:10.1182/blood-2011-06-356261.
60. Marsh RA, Allen CE, McClain KL, Weinstein JL, Kanter J, Skiles J, et al. Salvage therapy of refractory hemophagocytic lymphohistiocytosis with alemtuzumab. Pediatr Blood Cancer. 2013;60(1):101–9. doi:10.1002/pbc.24188.
61. Mahlaoui N, Ouachee-Chardin M, de Saint Basile G, Neven B, Picard C, Blanche S, et al. Immunotherapy of familial hemophagocytic lymphohistiocytosis with antithymocyte globulins: a single-center retrospective report of 38 patients. Pediatrics. 2007;120(3):e622–8. doi:10.1542/peds.2006-3164.
62. Ouachee-Chardin M, Elie C, de Saint Basile G, Le Deist F, Mahlaoui N, Picard C, et al. Hematopoietic stem cell transplantation in hemophagocytic lymphohistiocytosis: a single-center report of 48 patients. Pediatrics. 2006;117(4):e743–50. doi:10.1542/peds.2005-1789.
63. Marsh RA, Vaughn G, Kim MO, Li D, Jodele S, Joshi S, et al. Reduced-intensity conditioning significantly improves survival of patients with hemophagocytic lymphohistiocytosis undergoing allogeneic hematopoietic cell transplantation. Blood. 2010;116(26):5824–31. doi:10.1182/blood-2010-04-282392.
64. Nishi M, Nishimura R, Suzuki N, Sawada A, Okamura T, Fujita N, et al. Reduced-intensity conditioning in unrelated donor cord blood transplantation for familial hemophagocytic lymphohistiocytosis. Am J Hematol. 2012;87(6):637–9. doi:10.1002/ajh.23190.
65. Sawada A, Ohga S, Ishii E, Inoue M, Okada K, Inagaki J, et al. Feasibility of reduced-intensity conditioning followed by unrelated cord blood transplantation for primary hemophagocytic lymphohistiocytosis: a nationwide retrospective analysis in Japan. Int J Hematol. 2013;98(2):223–30. doi:10.1007/s12185-013-1391-z.
66. Marsh RA, Rao K, Satwani P, Lehmberg K, Muller I, Li D, et al. Allogeneic hematopoietic cell transplantation for XIAP deficiency: an international survey reveals poor outcomes. Blood. 2013;121(6):877–83. doi:10.1182/blood-2012-06-432500.

67. Lehmberg K, Albert MH, Beier R, Beutel K, Gruhn B, Kroger N, et al. Treosulfan-based conditioning regimen for children and adolescents with hemophagocytic lymphohistiocytosis. Haematologica. 2014;99(1):180–4. doi:10.3324/haematol.2013.094730.
68. Ono S, Okano T, Hoshino A, Yanagimachi M, Hamamoto K, Nakazawa Y, et al. Hematopoietic stem cell transplantation for XIAP deficiency in Japan. J Clin Immunol. 2017;37(1):85–91. doi:10.1007/s10875-016-0348-4.
69. Jessen B, Maul-Pavicic A, Ufheil H, Vraetz T, Enders A, Lehmberg K, et al. Subtle differences in CTL cytotoxicity determine susceptibility to hemophagocytic lymphohistiocytosis in mice and humans with Chediak-Higashi syndrome. Blood. 2011;118(17):4620–9. doi:10.1182/blood-2011-05-356113.
70. Carmo M, Risma KA, Arumugam P, Tiwari S, Hontz AE, Montiel-Equihua CA, et al. Perforin gene transfer into hematopoietic stem cells improves immune dysregulation in murine models of perforin deficiency. Mol Ther. 2015;23(4):737–45. doi:10.1038/mt.2014.242.

Chapter 13
Hemophagocytic Lymphohistiocytosis, Secondary

Ryu Yanagisawa and Yozo Nakazawa

Abstract Secondary hemophagocytic lymphohistiocytosis (HLH) is a group of diseases characterized by fever, splenohepatomegaly, pancytopenia, hypertriglyceridemia, hypofibrinogenemia, decreased natural killer cell activity, high ferritin value, high soluble interleukin-2 receptor value, and hemophagocytosis, which are induced or associated by infections, malignant tumors, or autoimmune diseases. In Eastern Asia, primary Epstein–Barr virus (EBV) infection is the most common cause of secondary HLH. Other common causes of secondary HLH are non-EBV viral infections (cytomegalovirus, adenovirus, herpes simplex virus, varicella–zoster virus, parvovirus B19, parainfluenza virus, influenza virus, etc.), malignant lymphoma, and juvenile idiopathic arthritis. Secondary HLH can develop in a wide range of age groups starting from children to seniors. Autoimmune diseases and infection-related HLH are common in children, whereas lymphoma-related HLH is common in seniors. Although EBV-HLH is a heterogeneous disorder with various symptoms ranging from mild to severe, early initiation of immunochemotherapy consisting of dexamethasone, cyclosporin A, and etoposide has been recommended for patients with moderate to severe EBV-HLH.

13.1 Introduction

Hemophagocytic lymphohistiocytosis or hyperinflammatory lymphohistiocytosis (HLH) is a group of diseases characterized by clinical symptoms such as fever and splenohepatomegaly and abnormal test findings such as pancytopenia, hypertriglyceridemia, hypofibrinogenemia, decrease in natural killer (NK) cell activity, high ferritin, hyperlipidemia, and high soluble interleukin-2 receptor (sIL-2R) values. HLH is also referred to as hemophagocytic syndrome (HPS). Histologically,

R. Yanagisawa, M.D., Ph.D.
Division of Blood Transfusion, Shinshu University Hospital, Matsumoto, Japan

Y. Nakazawa, M.D., Ph.D. (✉)
Department of Pediatrics, Shinshu University School of Medicine,
3-1-1 Asahi, Matsumoto 390-8621, Japan
e-mail: yxnakaza@shinshu-u.ac.jp

infiltration of histiocytes accompanied by lymphocytes and a clinical image of hemophagocytosis into the reticuloendothelial system, bone marrow, or central nervous tissue is confirmed [1, 2].

HLH is classified into primary [familial HLH (FHL), X-linked lymphoproliferative disorder, etc.] and secondary HLH. FHL is mainly an autosomal recessive inheritance with an onset in infancy, whereas secondary HLH follows infections, malignant tumors, or autoimmune diseases. Secondary HLH can develop in a wide range of age groups starting from children to seniors. Autoimmune diseases and infection-related HLH are common in children, whereas lymphoma-related HLH is common in seniors [1, 2].

In Eastern Asia, prevalence of Epstein–Barr virus (EBV)-associated HLH due to initial EBV infection is high, and at least 25 cases of EBV-HLH occur in Japan each year [1]. A Japanese national survey of 567 HLH cases (both children and adults) showed that about half of the cases were EBV- (28.7%) or infection-associated HLH that followed other infections (24.3%) [2]. Many cases of EBV-HLH were children aged 15 years or younger, and 120 out of 163 cases (74%) were initial EBV infection with a positive viral capsid antigen (VCA)-IgM test result or a negative EB nuclear antigen (EBNA) test result. Causative viruses other than EBV are cytomegalovirus, adenovirus, herpes simplex virus, varicella–zoster virus, parvovirus B19, parainfluenza virus, and influenza virus [2]. In general, infection-associated, non-EBV-HLH shows a mild clinical course with lower serum levels of interferon (IFN)-γ and soluble interleukin (IL)-2 receptor (sIL2-R) compared to EBV-HLH [3]. However, neonatal HLH due to HSV or enterovirus (echovirus and coxsackievirus), which occurs within 4 weeks after birth, can become seriously ill [4, 5]. Other causes were lymphoma-associated HLH, which accounted for about one-fifth of patients (19.0%). The major disease subtype was B-cell or T/NK-cell lymphoma. Other malignancy-associated HLH cases (acute leukemia, myelodysplastic syndrome, Langerhans cell histiocytosis, renal or liver carcinoma, and others) were also diagnosed in 4.2% of the patients. Autoimmune-associated HLH was observed in about one-tenth (9.3%) of the patients. Majority of the causes were juvenile idiopathic arthritis, systemic lupus erythematosus, and others. Post-allogeneic stem cell transplantation (SCT)-related HLH can develop in the early phase of allogeneic SCT around the time of engraftment and overlap with hyperacute graft vs. host disease or engraftment syndrome. In rare cases, drug hypersensitivity syndrome is associated with HLH. Here we focus on EBV-HLH and discuss the details below.

13.2 Pathogenesis of EBV-HLH

In normal initial EBV infection, EBV infects B cells, and to remove this infection, mainly CD8-positive T cells present an EBV-specific immune response followed by elevated levels of tumor necrosis factor α and IL-6 (Fig. 13.1a) [6, 7]. Depending on the intensity of this immune response, symptoms may vary from asymptomatic to infectious mononucleosis (IM). On the other hand, EBV infection is confirmed mainly in CD8-positive T cells at the time of onset of EBV-HLH [8], but the route
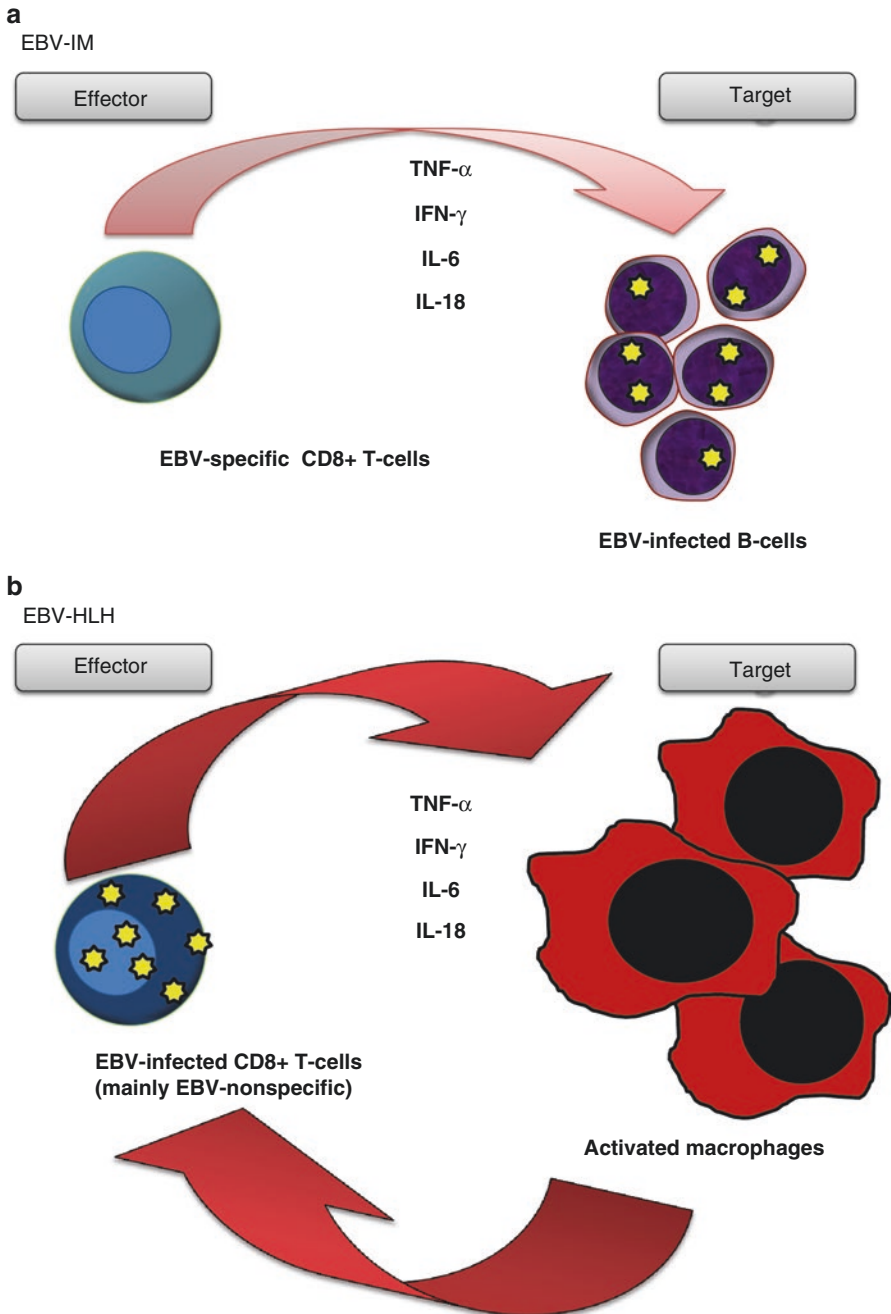
Fig. 13.1 Pathophysiology of infectious mononucleosis (**a**) and hemophagocytic lymphohistiocytosis (**b**) due to primary EB virus infection. *EBV-IM* EB virus-associated infectious mononucleosis, *EBV-HLH* EB virus-associated hemophagocytic lymphohistiocytosis, *INFγ* interferon, *IL-6* interleukin-6, *IL-18* interleukin-18, *TNFα* tumor necrosis factor-α

of infection to T cells has not been elucidated [1, 9]. CD8-positive T cells infected by EBV release excessive IFNγ, which activate macrophages. These activated macrophages release abundant pro-inflammatory cytokines (IL-6, IL-18, etc.) and cause hemophagocytosis (Fig. 13.1b) [7]. Unlike IM, which generally recovers by itself, the prognosis of EBV-HLH is poor without early and appropriate treatment [1, 9]. In EBV-HLH, EBV infections of NK cells or B cells are minimal or absent, whereas spontaneous growth of EBV-positive lymphoblastoid B cells can be seen in the recovery or remission stages, indicating that EBV infection of B cells is delayed but occurs during the course of disease [9]. It has been proposed that in cases of EBV-HLH, EBV-infected B cells may induce an abnormal immune response in CD8-positive T cells [10, 11] and that they may be the source of EBV in T cells [12, 13].

13.3   Diagnostic Findings in EBV-HLH

The HLH-2004 criteria have been widely used for the diagnosis of HLH [14]. However, their sensitivity and specificity should be validated before application of these diagnostic criteria is suitable for EBV-HLH. Because these criteria focus on FHL, some of them, including impaired NK cell activity, may not be suitable for EBV-HLH [2]. Increased titers of EBV-specific antibodies are not reliable for the diagnosis of EBV-HLH [2]. Although EBV-HLH sets in following the first EBV infection, there are few cases in which onset is brought on by the reactivation of EBV accompanied by chronic active EBV infection. In addition, there are few cases of FHL onset triggered by EBV infection. The real-time polymerase chain reaction (PCR) to quantify EBV DNA in the peripheral blood is a useful method for diagnosis of EBV-HLH. Patients with EBV-HLH show a significantly higher viral load compared to IM in both plasma and peripheral blood mononuclear cells on real-time PCR [15]. In addition, CD5 downregulation in CD8-positive T cells may be rapidly discriminated between EBV-HLH and EBV-IM [16].

The monoclonality of EBV-infected T lymphocytes is confirmed in EBV-HLH. The monoclonality of EBV-infected T cells can be evaluated by assessing the uniformity of the EBV genome and/or the monoclonality of T-cell receptor (TCR) gene rearrangement using Southern blotting and conventional PCR analyses [17, 18]. Recently, the multiplex PCR for detecting clonal immunoglobulin and TCR gene rearrangement was established to detect clonally proliferating EBV-containing T-cells in EBV-HLH [19]. The combination of these genetic analyses is useful in the diagnosis of EBV-HLH.

13.4   Prognostic Factors for EBV-HLH

Kogawa et al. [18] reported that factors for poor prognosis were 7 days or more until diagnosis, a neutrophil count of 1700/μL or higher, a prothrombin time/international normalized ratio of 1.68 or higher, an activated partial thromboplastin time (aPTT)

of 69 s or more, a total bilirubin (T.Bil) content of 1.8 mg/dL or higher, and lactate dehydrogenase (LDH) level of 4310 IU/L or higher. Prognosis for cases with a T.Bil of 1.8 mg/dL and higher and a ferritin level of 20,300 ng/mL or higher was especially poor. Another study reported that hyperbilirubinemia, hyperferritinemia, and cerebrospinal fluid pleocytosis at diagnosis and thrombocytopenia and hyperferritinemia 2 weeks after the initiation of treatment adversely affect the outcome of HLH [20]. Imashuku et al. [21] reported that despite the high prevalence of abnormal test findings that are found in EBV-HLH and are similar to those in tumors such as EBV clonality, T-cell clonality, and chromosomal abnormality, only chromosomal abnormality can be a factor for poor prognosis. Another study in children and adults with EBV-HLH reported that an adult age, EBV reactivation, and multidrug chemotherapy were associated with a poor clinical outcome in patients with EBV-HLH [2]. Although several clinical factors provide useful information for treatment of EBV-HLH, a larger prospect study is needed.

13.5 Treatment for EBV-HLH and Prognosis

In FHL, etoposide is considered necessary in controlling the disease [22]. Imashuku et al. [23] showed that the overall survival (OS) of the group that used etoposide within 4 weeks of diagnosis reached 90% and that the early use of etoposide is a prognostic factor (use within 4 weeks resulted in an OS of 90.2 ± 6.9% and after 4 weeks resulted in an OS of 56.5 ± 12.6%). Based on the above results, an 8-week treatment with etoposide (HLH-94/HLH2004) has been used in EBV-HLH as the standard treatment. However, other reports indicate that about half of the patients with EBV-HLH can be treated without etoposide. Shiraishi et al. [24] reported that 14 out of 22 cases of EBV-HLH (64%) recovered without etoposide [24]. Those cases that recovered without etoposide had a short period of fever prior to the treatment or had significantly low LDH and sIL-2R values. In a retrospective national survey, 37 out of 93 cases (40%) recovered from EBV-HLH without using etoposide. Three-year OS and event-free survival rates for all EBV-HLH cases were 91.2% and 79.3%, respectively, and there was no difference between etoposide and non-etoposide groups [18]. Therefore, with an etoposide-containing regimen, care should be taken to avoid overtreatment of some EBV-HLH patients. In addition, onset of secondary leukemia has been reported in a case of EBV-HLH that was treated with etoposide [25]; thus, minimizing the administration dose of etoposide is a major challenge.

Allogeneic SCT has decreased the mortality in EBV-HLH patients, but there is insufficient evidence to suggest that SCT is better than immunochemotherapy in children with EBV-HLH [26]. When the toxicity is considered, HSCT should be available only for EBV-HLH patients who relapse or show resistance to immunochemotherapy. In one retrospective study, a total of 14 patients with EBV-HLH underwent allogeneic SCT, including cord blood transplantation (CBT) in half of the cases [27]. The 10-year OS was 85.7 ± 9.4%, and the survival of CBT recipients was >65%. However, SCT with myeloablative conditioning is associated with high

transplantation-related mortality [28]. Recently, reduced-intensity conditioning, which is less toxic and has a low incidence of long-term sequelae, has been used for primary HLH. Further studies are needed to determine the optimal SCT regimen and establish a comprehensive strategy for the cure of EBV-HLH.

Based on the hypothesis that the removal of EBV-infected B cells contribute to an improved prognosis of EBV-HLH, B-cell targeting monoclonal antibody rituximab may be another concept of treatment. Balamuth et al. [11] reported that adding rituximab to the HLH-2004 treatment protocol improved the efficacy of the treatment regimen. The EBV-HLH Rituximab Study Group [13] in the USA retrospectively examined 42 cases of EBV-HLH in whom rituximab was administered. In 18 cases (43%), clinical symptoms improved, and EBV and ferritin levels significantly decreased after the administration of rituximab; the survival rate was high in cases that showed such a decrease. Rituximab may be a possible therapeutic option during the course of therapy or at the end of initial therapy to remove proliferating EBV-infected B cells.

References

1. Imashuku S, Hibi S. Clinical features and treatment strategies of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. Crit Rev Oncol Hematol. 2002;44(3):259–72.
2. Ishii E, Ohga S, Imashuku S, Yasukawa M, Tsuda H, Miura I, et al. Nationwide survey of hemophagocytic lymphohistiocytosis in Japan. Int J Hematol. 2007;86(1):58–65.
3. Imashuku S, Hibi S, Tabata Y, Sako M, Sekine Y, Hirayama K, et al. Biomarker and morphological characteristics of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis. Med Pediatr Oncol. 1998;31(3):131–7.
4. Imashuku S, Ueda I, Teramura T, Mori K, Morimoto A, Sako M, et al. Occurrence of haemophagocytic lymphohistiocytosis at less than 1 year of age: analysis of 96 patients. Eur J Pediatr. 2005;164(5):315–9.
5. Suzuki N, Morimoto A, Ohga S, Kudo K, Ishida Y, Ishii E, HLH/LCH Committee of the Japanese Society of Pediatric Hematology. Characteristics of hemophagocytic lymphohistiocytosis in neonates: a nationwide survey in Japan. J Pediatr. 2009;155(2):235–238.e1.
6. Wright-Browne V, Schnee AM, Jenkins MA, Thall PF, Aggarwal BB, Talpaz M, et al. Serum cytokine levels in infectious mononucleosis at diagnosis and convalescence. Leuk Lymphoma. 1998;30(5–6):583–9.
7. Wada T, Muraoka M, Yokoyama T, Toma T, Kanegane H, Yachie A. Cytokine profiles in children with primary Epstein-Barr virus infection. Pediatr Blood Cancer. 2013;60(7):E46–8.
8. Kasahara Y, Yachie A, Takei K, Kanegane C, Okada K, Ohta K, et al. Differential cellular targets of Epstein-Barr virus (EBV) infection between acute EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. Blood. 2001;98(6):1882–8.
9. Kasahara Y, Yachie A. Cell type specific infection of Epstein-Barr virus (EBV) in EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. Crit Rev Oncol Hematol. 2002;44(3):283–94.
10. Al Asad O, Salam A, Mannem S, Ninan M, Markowitz A, Jana B. Alternative therapy for epstein-barr virus related hemophagocytic lymphohistiocytosis. Case Rep Oncol Med. 2015;2015:508387.
11. Balamuth NJ, Nichols KE, Paessler M, Teachey DT. Use of rituximab in conjunction with immunosuppressive chemotherapy as a novel therapy for Epstein Barr virus-associated hemophagocytic lymphohistiocytosis. J Pediatr Hematol Oncol. 2007;29(8):569–73.

12. Beutel K, Gross-Wieltsch U, Wiesel T, Stadt UZ, Janka G, Wagner HJ. Infection of T lymphocytes in Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children of non-Asian origin. Pediatr Blood Cancer. 2009;53(2):184–90.

13. Chellapandian D, Das R, Zelley K, Wiener SJ, Zhao H, Teachey DT, et al. Treatment of Epstein Barr virus-induced haemophagocytic lymphohistiocytosis with rituximab-containing chemo-immunotherapeutic regimens. Br J Haematol. 2013;162(3):376–82.

14. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007;48(2):124–31.

15. Kimura H, Hoshino Y, Hara S, Nishikawa K, Sako M, Hirayama M, et al. Viral load in Epstein-Barr virus-associated hemophagocytic syndrome. Microbiol Immunol. 2002;46(8):579–82.

16. Toga A, Wada T, Sakakibara Y, Mase S, Araki R, Tone Y, et al. Clinical significance of cloned expansion and CD5 down-regulation in Epstein-Barr virus (EBV)-infected CD8+ T lymphocytes in EBV-associated hemophagocytic lymphohistiocytosis. J Infect Dis. 2010;201(12):1923–32.

17. Owen G, Webb DK. Evidence of clonality in a child with haemophagocytic lymphohistiocytosis. Br J Haematol. 1995;89(3):681–2.

18. Kogawa K, Sato H, Asano T, Ohga S, Kudo K, Morimoto A, et al. Prognostic factors of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children: report of the Japan Histiocytosis study group. Pediatr Blood Cancer. 2014;61(7):1257–62.

19. Matsuda K, Nakazawa Y, Yanagisawa R, Honda T, Ishii E, Koike K. Detection of T-cell receptor gene rearrangement in children with Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis using the BIOMED-2 multiplex polymerase chain reaction combined with GeneScan analysis. Clin Chim Acta. 2011;412(17–18):1554–8.

20. Trottestam H, Berglof E, Horne A, Onelov E, Beutel K, Lehmberg K, et al. Risk factors for early death in children with haemophagocytic lymphohistiocytosis. Acta Paediatr. 2012;101(3):313–8.

21. Imashuku S, Hibi S, Tabata Y, Itoh E, Hashida T, Tsunamoto K, et al. Outcome of clonal hemophagocytic lymphohistiocytosis: analysis of 32 cases. Leuk Lymphoma. 2000;37(5–6):577–84.

22. Su IJ, Wang CH, Cheng AL, Chen RL. Hemophagocytic syndrome in Epstein-Barr virus-associated T-lymphoproliferative disorders: disease spectrum, pathogenesis, and management. Leuk Lymphoma. 1995;19(5–6):401–6.

23. Imashuku S, Kuriyama K, Teramura T, Ishii E, Kinugawa N, Kato M, et al. Requirement for etoposide in the treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. J Clin Oncol. 2001;19(10):2665–73.

24. Shiraishi A, Ohga S, Doi T, Ishimura M, Takimoto T, Takada H, et al. Treatment choice of immunotherapy or further chemotherapy for Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2012;59(2):265–70.

25. Imashuku S, Teramura T, Kuriyama K, Kitazawa J, Ito E, Morimoto A, et al. Risk of etoposide-related acute myeloid leukemia in the treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. Int J Hematol. 2002;75(2):174–7.

26. Qin Q, Xie Z, Shen Y, Yang S, Liu C, Huang Z, et al. Assessment of immunochemotherapy and stem cell transplantation on EBV-associated hemophagocytic lymphohistiocytosis in children: a systematic review and meta analysis. Eur Rev Med Pharmacol Sci. 2012;16(5):672–8.

27. Ohga S, Kudo K, Ishii E, Honjo S, Morimoto A, Osugi Y, et al. Hematopoietic stem cell transplantation for familial hemophagocytic lymphohistiocytosis and Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in Japan. Pediatr Blood Cancer. 2010;54(2):299–306.

28. Chandrakasan S, Filipovich AH. Hemophagocytic lymphohistiocytosis: advances in pathophysiology, diagnosis, and treatment. J Pediatr. 2013;163(5):1253–9.