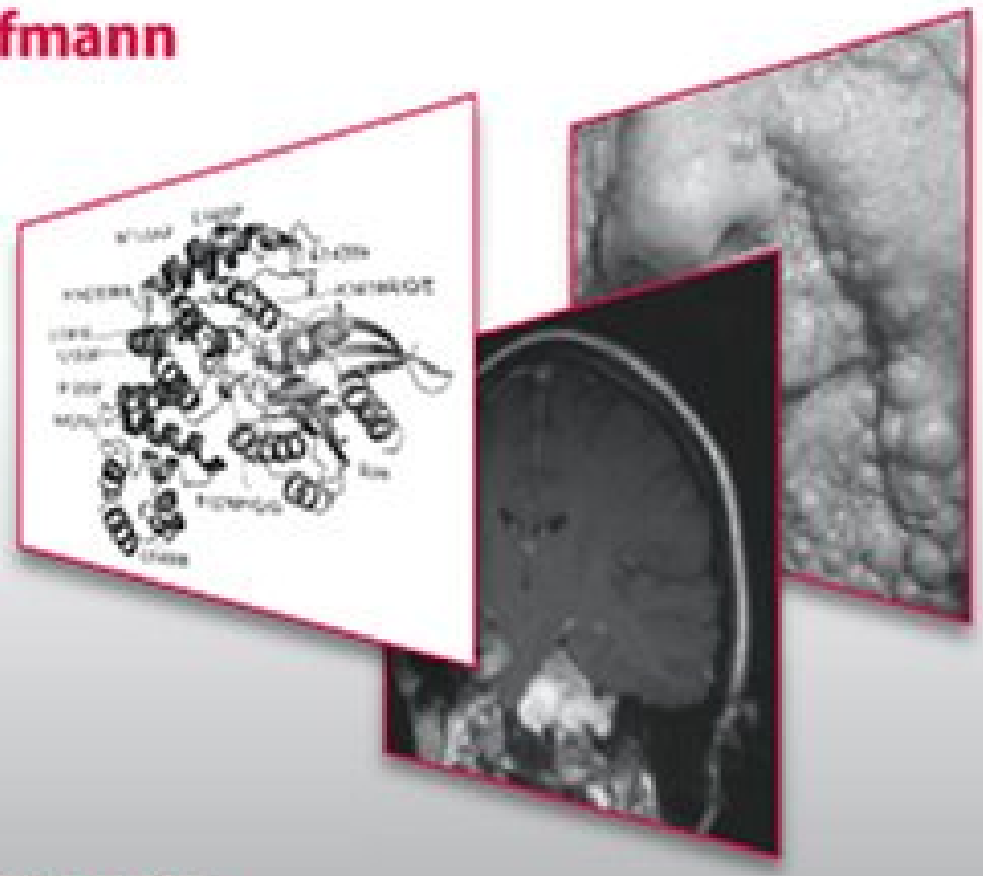
Monographs in Human Genetics
Editor: M. Schmid
Vol. 16

Neuro-fibromatoses

Editor
D. Kaufmann



KARGER

Neurofibromatoses

Monographs in Human Genetics

Vol. 16

Series Editor

Michael Schmid *Würzburg*

Neurofibromatoses

Volume Editor

Dieter Kaufmann *Ulm*

28 figures, 14 in color, and 15 tables, 2008

KARGER

Basel · Freiburg · Paris · London · New York · Bangalore · Bangkok · Shanghai · Singapore · Tokyo · Sydney

Dieter Kaufmann
Institute of Human Genetics
University of Ulm
Albert-Einstein-Allee 11
D–89070 Ulm

Library of Congress Cataloging-in-Publication Data

Neurofibromatoses / volume editor, Dieter Kaufmann.
p. ; cm.– (Monographs in human genetics, ISSN 0077–0876 ; v.16)
Includes bibliographical references and indexes.
ISBN 978–3–8055–8520–0 (hard cover : alk. paper)
1. Neurofibromatosis–Genetic aspects. 2. Neurofibromatosis. I. Kaufmann, Dieter, 1948– II. Series.
[DNLM: 1. Neurofibromatoses–genetics. 2. Neurofibromatoses–physiopathology. W1 MO567P v.16 2008 / QZ 380 N4934 2008]
RC280.N4N4864 2008
616.99'383042–dc22 2008009252

Bibliographic Indices. This publication is listed in bibliographic services, including Current Contents® and Index Medicus.

www.karger.com
Printed in Switzerland on acid-free and non-aging paper (ISO 9706) by Reinhardt Druck, Basel
ISSN 0077–0876
ISBN 978–3–8055–8520–0

Contents

VII Editorial
Schmid, M. (Würzburg)

IX Preface
Kaufmann, D. (Ulm)

1 The Neurofibromatoses: Classification, Clinical Features and Genetic Counselling
Huson, S.M. (Manchester)

21 Treatment and Management of Neurofibromatosis 1
Mautner, V.-F. (Hamburg); Boltshauser, E. (Zürich)

32 Neurofibromatosis Type 1 and Other Syndromes of the Ras Pathway
Stevenson, D.A.; Swensen, J.J.; Viskochil, D.H. (Salt Lake City, Utah)

46 *NF1* Gene Structure and NF1 Genotype/Phenotype Correlations
Upadhyaya, M. (Cardiff)

63 *NF1* Mutational Spectrum
Messiaen, L.M. (Birmingham, Ala.); Wimmer, K. (Vienna)

78 Clinical Phenotypes in Patients with *NF1* Microdeletions
Tinschert, S. (Dresden)

89 Structure of the *NF1* Gene Region and Mechanisms Underlying Gross *NF1* Deletions
Kehrer-Sawatzki, H. (Ulm)

103 *NF1* Gene Evolution in Mammals
Assum, G.; Schmegner, C. (Ulm)

113 Structure and Function of Neurofibromin
Welti, S.; D'Angelo, I.; Scheffzek, K. (Heidelberg)

129 Composition of Neurofibromas, *NF1* Expression, and Comparison of Normal and *NF1* Haploinsufficient Cells
Peltonen, J.; Peltonen, S. (Turku)

143 Somatic *NF1* Mutations in Tumors and Other Tissues
De Raedt, T. (Leuven); Maertens, O. (Ghent); Serra, E. (Llobregat); Legius, E. (Leuven)

154 NF2: Mutations and Management of Disease
Evans, D.G.R.; Wallace, A. (Manchester)

167 Function of Merlin in Genesis of Tumours and Other Symptoms of NF2
Hanemann, C.O. (Plymouth)

177 Molecular Studies on Schwannomatosis
Kluwe, L. (Hamburg)

189 Author Index

190 Subject Index

Editorial

The present volume of *Monographs in Human Genetics* focuses on 'Neurofibromatoses', important autosomal dominant genetic disorders of the nervous system that primarily affect the development and growth of neural cell tissues. They encompass a set of distinct disorders that cause tumors to grow on nerves and, in addition, can affect the development of non-nervous tissues such as bones and skin. The first precise clinical and pathological characterization of neurofibromatosis (type I) was published in 1882 by Friedrich Daniel von Recklinghausen, a German pathologist who then practiced and taught medicine at the University of Strasbourg.

The genes and mutations causing neurofibromatoses have been identified in recent years. Moreover, very much has been elucidated about the complex molecular mechanisms leading to these diseases. By no means is it easy for the non-specialist to get an overview of the many aspects and the current knowledge of neurofibromatoses. Therefore, this volume of *Monographs in Human Genetics* aims to contribute a timely update and compilation of the manifold data obtained for these disorders by clinical studies, genetics and molecular biology.

I thank all the authors for their interesting contributions, the editor Dieter Kaufmann for his invaluable and constant efforts in organizing this book and processing the manuscripts, and the publisher Thomas Karger for his engagement in this book series.

Michael Schmid
Würzburg, December 2007

Preface

'When a disease-causing gene is identified, a causal therapy is only a few steps away'. The neurofibromatoses, covered in this volume of the series 'Monographs in Human Genetics', illustrate that this is not so easy. A chapter called 'Causal Therapy of the Neurofibromatoses' is as yet missing. Still, the understanding of the molecular mechanisms underlying these diseases has increased enormously after the identification of the respective genes, which raises the patient's hopes for successful therapies. Patients with Neurofibromatosis type 1 (NF1), formerly known as Morbus Recklinghausen, have led a shadowy existence for centuries as demonstrated by the literary character of the 'Hunchback of Notre Dame'. This changed after foundation of patient support groups and raising large-scale funds for research. These funds were essential for advancing investigation of the neurofibromatoses and thereby caused great progress in their understanding. The data on these diseases have become very complex. In many places the present book can be seen only as an introduction to the neurofibromatoses.

The neurofibromatoses, predisposing to multiple tumours of the peripheral nervous system, are often considered classical tumour suppressor diseases. In NF1 multiple dermal neurofibromas dominate, in the much rarer Neurofibromatosis type 2 (NF2) and schwannomatosis schwannomas are the most frequent tumour type. This book centers on the genetic mechanisms underlying these three diseases. As NF1 is the most frequent of the three, the majority of chapters deal with this disease.

While the symptoms of NF2 and schwannomatosis are largely restricted to the various tumour types, NF1 presents with a distinctive pleiotropy for non

tumor-associated symptoms. These symptoms, e.g. learning disabilities or bone abnormalities, can have the same significance for the clinic as the multiple tumours. Since the symptoms do not adhere to academic boundaries interdisciplinary medical care is required. This topic and the necessary diagnostic and therapeutic measures are covered at the beginning of this book.

The *NF1* gene is very large. One report concerns its structure and a second its evolution in mammals. Identifying specific *NF1* mutations is laborious. The mutational spectrum and some genotype/phenotype correlations are addressed with special focus on *NF1* microdeletions and associated phenotypes. NF1 shows intrafamilial variability of symptoms which is very meaningful for genetic counselling. The causes for this variability are still not known, except for the stochastic of second hit mutations in tumour progenitor cells and the higher noise in neurofibromin-associated signal transduction pathways in *NF1* haploinsufficient cells.

Most tumours of NF1 and NF2 are slow growing, primarily benign and very rarely undergo malignant transformation. One chapter deals with somatic (second hit) mutations of the *NF1* gene in these tumours and also in other tissues and tumour types. Furthermore there is a description of how essential the interaction of *NF1* deficient cells with *NF1* haploinsufficient cells is. It turns out that the *NF1* gene product neurofibromin has various functions and is involved in the regulation of numerous signalling pathways. This may explain the observed pleiotropy. Some domains of neurofibromin could be characterised on a molecular level. This is shown in detail together with knowledge of the resulting neurofibromin functions.

The pathways regulated by neurofibromin overlap in part with those influenced by merlin, the protein product of the *NF2* gene. One chapter covers the function of merlin in tumours, the mutations found in NF2 and the necessary clinical management of disease. In the meantime, schwannomatosis can be reliably distinguished from NF2 and NF1 in the clinic. The concluding chapter reports on the status of the molecular investigations of this disease.

I wish to thank Karger publishers and Michael Schmid (series editor of *Monographs in Human Genetics*) for the opportunity to make study of neurofibromatoses more popular. Furthermore, I want to thank all authors for their contributions. I hope that this book will arouse greater interest in these very common inherited tumour diseases and thereby help to step forward to causal therapies.

Dieter Kaufmann
Ulm, December 2007

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 1–20

The Neurofibromatoses: Classification, Clinical Features and Genetic Counselling

S.M. Huson

Medical Genetic Research Group and Regional Genetic Service, University of Manchester and Central Manchester and Manchester Childrens Hospitals NHS Trust, Manchester, UK

Abstract

Neurofibromatosis type 1 (NF1) is one of the commonest dominant disorders in man. Recent molecular genetic studies have identified some clinically useful genotype-phenotype correlations. A non-allelic but phenotypically similar condition has also been reported. In this article, additions to the current NF1 diagnostic criteria and a new NF classification system are proposed. In the differential diagnosis the importance of identifying families with the mismatch repair deficient phenotype is highlighted. The clinical problems that can occur in NF1 are summarized and newly recognised associations included (nail bed glomus tumours and increased risk of breast cancer in women). The key factors of importance in genetic counselling for families with NF are discussed.

Historically the different kinds of neurofibromatosis were 'lumped' together under the umbrella terms neurofibromatosis (NF) or von Recklinghausen's disease. With the application of modern methods of genetic research during the last two decades of the last century the main types of NF, types 1 (NF1) and type 2 (NF2), have been shown to be distinct both clinically and genetically [1–3]. More recently subtypes of NF1, both allelic and non-allelic, have been defined [4–6] as has the NF2 related condition, schwannomatosis [7, 8].

These distinctions are more than just an academic exercise (and one geneticists like myself are fond of!); the various forms of NF have quite different implications for patients in terms of management and genetic counselling.

Neurofibromatosis: Definition and Classification

As we learn more about the distinct natural history and molecular pathogenesis of NF1 and NF2 it is tempting to agree with the recent suggestion that NF1 and its subtypes would be better defined as 'neuro-cardio-facial-cutaneous (NFNC) syndromes', along with Noonan, LEOPARD, cardio-facio-cutaneous (CFC) and Costello syndromes [9, 10]. These conditions all share a variable degree of learning difficulty, cardiac defects, overlapping facial dysmorphism, short stature, macrocephaly and skin abnormalities. Patients with all of the conditions, except CFC, have been shown to have an increased risk of specific, and overlapping, malignancies. They have also been shown to result from germline mutations in the evolutionarily conserved Ras-MAPKinase pathway. These conditions are discussed further in this volume by Stevenson, Swensen and Viskochil [11]. NF2 may be more appropriately part of a separate group along with schwannomatosis.

Such major changes of classification and terminology need to be discussed and agreed by an international consensus group. For the purposes of this article I will use the working definition of NF that I have developed in clinical practice and propose an update to the NIH consensus conference classification of NF [1, 12].

NF Definition

The neurofibromatoses are a group of conditions which predispose to tumours of the nervous system and abnormal skin pigmentation. Each type is defined by the presence/absence of café au lait (CAL) spots and skinfold freckling, what kind of peripheral nerve tumour develops (neurofibromas vs. schwannomas) and other features, particularly in the eye, specific to each form.

NF Classification

Riccardi first recognised the importance of clear distinction of the different types of NF1 and proposed a numerical classification system based upon the presence/absence of CAL spots, skinfold freckling, neurofibromas/schwannomas and Lisch nodules [13]. This classification formed the basis for subsequent discussions and developments and was replaced by the NIH consensus conference classification and diagnostic criteria [12]. Subsequent clinical studies recognized the need for revision of the NF2 diagnostic criteria [1, 14]. Viskochil and Carey [15] envisaged a classification based around molecular genetic classification; at the time, this proposal was not taken up but as I will discuss it would now seem timely to add genotypic data to the diagnostic criteria. Based upon a review of the current literature and clinical experience, an up to date NF classification is summarised in table 1 [16–20]. For completeness, previous terminology for the different types is included [12].

Table 1. Proposed classification system for the neurofibromatoses

NF type [references]	Previous names	Disease frequency	Gene/chromosome	CAL spots	Skinfold freckling	Peripheral nerve tumours	Eye findings	Other frequent clinical features
NF1 and related disorders								
NF1 [1–3]	Von Recklinghausen or peripheral NF	1/3,000 (birth incidence)	*NF1*/17	≥6	Yes	Dermal, nodular and plexiform neurofibromas	Lisch nodules (LN) Optic nerve gliomas (ONGs)	Learning and behaviour problems; predisposition to certain malignancies and numerous other complications: see table 2
NF1 subtypes – Allelic (A)								
A1 NF1 microdeletions [4]	Accounted for at least some designated NF-Noonan syndrome	1/60,000 (assuming accounts for 5% NF1 mutations)	*NF1*/17	Clinical features of NF1 with tendency to excessive numbers of dermal neurofibromas, more severe learning problems, increased frequency of certain NF1 complications including cardiovascular and malignant peripheral nerve sheath tumour. In addition microdeletion cases usually have specific craniofacial dysmorphism and a tendency to be taller rather than shorter than average for their age.				
A2 Del AAT exon 17 [5]	Autosomal dominant CAL spots only	?	*NF1*/17	≥6	Yes	None reported to date	LN less frequent than usual	Majority of reported families have pigmentary features only with lower frequency of all complications except pulmonary stenosis

Table 1. (continued)

NF type [references]	Previous names	Disease frequency	Gene/chromosome	CAL spots	Skinfold freckling	Peripheral nerve tumours	Eye findings	Other frequent clinical features
A3 Watson syndrome [16]	NA	Very rare	*NF1*/17	≥6	Yes	Adults have few if any dermal Nfs; other kinds of neurofibromas not reported	LN less frequent than normal; no ONGs reported	Mild learning problems more frequent than usual; as is pulmonary stenosis. Other complications not reported.
A4 Localised/ segmental NF1 [17, 18]	Segmental NF1	1/36,000–40,000 (prevalence)	Somatic *NF1* mutations	Cutaneous features of NF1 (pigmentation and/or neurofibromas) limited to one or more segments of body. Associated NF1 complications relatively uncommon-combined frequency of 5.6% in one large series.				
NF1 subtypes – Non-Allelic (NA)								
NA1 SPRED1 phenotype [6]	Autosomal dominant CAL spots only	?	*SPRED1*/15	≥6	Yes	None	None	Main other NF1 feature in most people was macrocephaly. A few had learning problems, two had ADHD. Several people had lipomas (see text).

NF2 and related disorders								
NF2 [1–3, 19]	Central or Wishart	1/25,000 (birth incidence)	*NF2*/22	<6 but occur at higher frequency than in general population	No	Predominantly schwannomas histologically but neurofibromas can occur. Clinically only NF2 plaques are distinguishable from dermal/ nodular/plexiform neurofibromas	Juvenile Posterior subcapsular lens opacity/ cortical cataract in 70–80%; retinal abnormalities in 22%	Most frequent feature are bilateral vestibular Schwannomas. Meningiomas, other cranial nerve and spinal root schwannomas, ependymomas.
NF2 subtypes – Allelic (A)								
A1 Localised/ Segmental NF2 [17]	NA	1/970,000 [17]	*NF2*/22	NF2 associated cranial tumours localised to one half of brain. When patients have spinal or peripheral nerve schwannomas localised to one part of body it is impossible to know clinically if this is form of NF2 or Schwannomatosis.				
NF2 subtypes – Non-Allelic (NA)								
NA1 Schwan-nomatosis [7, 8, 20]	NA	1/580,000 [17]	*INI1/ SMARCB1*/22	None	None	Peripheral nerve and spinal root schwannomas	None	None

Neurofibromatosis Type 1

NF1 is not only the commonest form of NF but also one of the commonest dominant disorders in man. As reviewed below and summarised in table 1 specific subtypes of NF1 are emerging where clinical features tend to be more uniform in families; in a general NF1 clinic they represent 5% of cases seen at most. In the majority of families NF1 is a highly variable and unpredictable condition. It is this aspect of the disease which is most difficult for families and physicians alike – for the former the uncertainty of what may happen in the future and for their Doctors how to inform people about NF1 without causing unnecessary anxiety and over-medicalising the condition, whilst still encouraging to report unusual symptoms at an early stage.

NF1 Clinical Features

These are summarised in table 2 [21–23]. Clinically I find it useful to divide them into three categories:

(1) *Major defining features*. These are CAL spots (fig. 1), skinfold freckling (fig. 2), Lisch nodules and the different kinds of neurofibromas (fig. 3). They are present in the majority of NF1 patients and are the key diagnostic features. Only neurofibromas are associated with significant medical problems.

(2) *Minor features*. These are features which occur in an excess of patients with NF1 but are not specific enough to be included in the diagnostic criteria. They include short stature, macrocephaly and pectus excavatum [3].

(3) *Complications*. Virtually all of these can occur as isolated events in the general population. However, people with NF1 have an increased relative risk of their occurrence although the actual incidence of a given problem in NF1 may still be very low as they are such rare events in the general population. The most frequent complications are discussed in detail in the next chapter. New associations with NF1 are still being recognised. The most recent are glomus tumours of the nail beds [24] – these are an important cause of digital pain in NF1 and several of the patients I have diagnosed had been extensively investigated for more proximal peripheral nerve tumours initially.

Another limitation to literature interpretation is the limited prospective natural history data. Many of the earlier studies of large patient cohorts were cross-sectional and therefore would be expected to under-estimate the life time frequency of some complications, particularly malignancy. More recent studies have shown the life time risk of malignant peripheral nerve sheath tumours to be in the range of 8–13% [25]. Also an increased risk of breast cancer in women with NF1 under 50 years of age has only emerged as a much larger cohort than previously has been followed prospectively [26].

Table 2. NF1 clinical features and age of onset [21–26]

Disease feature	Frequency (%)[a]	Age of presentation
Café au lait (CAL) spots	>99	0–2 years
Skinfold freckling	67	3–5 years
Dermal neurofibromas	>95 of adults	≥7 years, usually postpubertal
Nodular neurofibromas	48[b]	Occasionally <10 years, usually start developing from teens
Plexiform neurofibromas		
All lesions	30.0	0–18 years
Large lesions of head and neck	1.2	0–1 year
Limbs/trunk lesions associated with hypertrophy	5.8	0–5 years
Lisch nodules	90–95	≥3 years
Macrocephaly	45	Birth
Short stature	31.5	Birth
Intellectual handicap		
Severe	0.8	0–5 years
Moderate	2.4	0–5 years
Minimal/learning difficulties	29.8	0–5 years
Epilepsy		
No known cause	4.4	Lifelong
Secondary to disease complications	2.2	Lifelong
Hypsarrhythmia	1.5	0–5 years
CNS tumors		
Optic glioma	1.5[c]	0–20
Other CNS tumors	1.5	Lifelong
Spinal neurofibromas	1.5	Lifelong
Aqueductal stenosis	1.5	Lifelong, usually <30 years
Malignancy		
MPNST	1.5[d]	Lifelong
Rhabdomyosarcoma	1.5	0–5 years
Orthopedic		
Scoliosis, requiring surgery	4.4	0–18 years
Scoliosis, less severe	5.2	0–18 years
Long bone dysplasia	3.7	0–2 years
Vertebral scalloping	10.0[e]	Lifelong
Gastrointestinal stromal tumours	2.2	Lifelong
Renal artery stenosis	1.5	Lifelong
Pheochromocytoma	0.7	≥10 years
Duodenal carcinoid	1.5	≥10 years
Juvenile xanthogranuloma	0.7	0–1 year
Congenital glaucoma	0.7	0–1 year

Table 2. (continued)

Disease feature	Frequency (%)[a]	Age of presentation
Sphenoid wing dysplasia	<1	Congenital
Lateral meningocele	<1	Lifelong
Juvenile myelomonocytic leukemia	<1	0–18 years
Cerebrovascular disease	<1	Childhood
Glomus tumours of nailbed	<1	Usually in adults
Breast cancer in women	Relative risk ×3 for those <50 years	

[a]Data mainly from Huson et al. [21].
[b]Tucker et al. [56].
[c]Optic glioma seen in 15% of children if imaging study done [22].
[d]Life time risk may be as high as 8–13% [25].
[e]Data from Riccardi and Eichner [23].

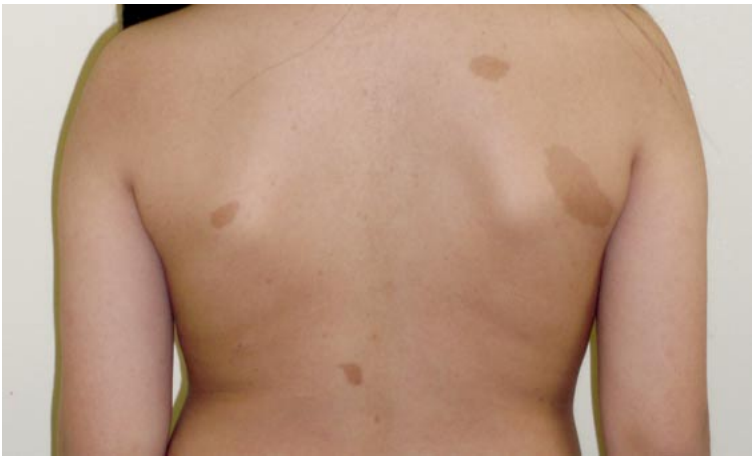


Fig. 1. Typical CAL spots in a child with NF1.

In clinical practise it is important to note the ages at which the different complications usually present. Parents find it helpful to know that a given complication can no longer develop; e.g. I have never seen tibial pseudarthrosis present after a child has begun walking or detected a serious facial plexiform after the age of three.

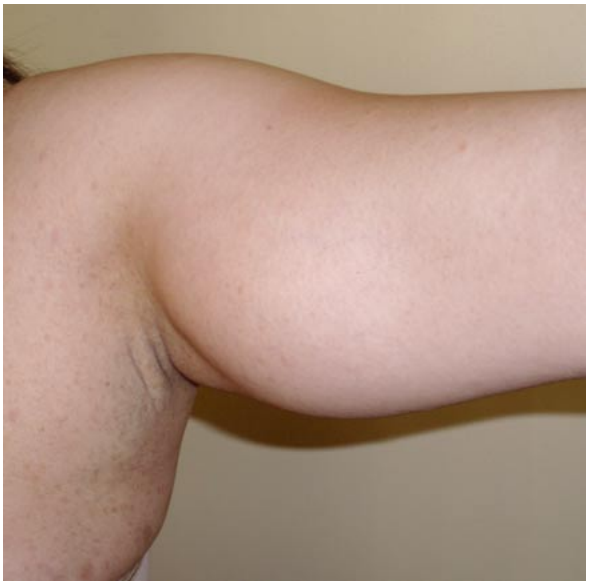


Fig. 2. Axillary freckling in NF1. The patient had only recently been diagnosed in her mid forties after presenting with a rapidly enlarging, painful swelling of the left arm (also shown); this was found to be a Malignant Peripheral Nerve Sheath Tumour (MPNST).

NF1 Diagnostic Criteria

The NF1 diagnostic criteria were agreed at the NIH 1987 NF consensus conference [12]. They have stood the test of time well until relatively recently. They are shown in table 3 along with a suggested update. Given the improved detection methods for *NF1* mutations [27], at least two examples of clinically important genotype-phenotype correlation and confirmation of non-allelic heterogeneity [6], it seems timely that 'a mutation in the *NF1* gene' is added.

The recently recognised phenotype of mismatch repair deficiency syndrome (MMR-D) [28, 29] also means that the term 'first degree relative' needs further definition. MMR-D patients have biallelic mutations in one of the mismatch repair genes associated with hereditary non-polyposis colon cancer (HNPCC) in the heterozygous state. Inheritance of MMR-D is therefore recessive but the heterozygous parents are at risk of HNPCC. The overlap with NF1 arises because many of the reported cases have multiple CAL spots; Bandipalliam has recently reported a systematic literature review [29]. The majority of affected individuals are reported as having CAL spots only although these usually have irregular edges and pigmentation which is unusual in NF1,

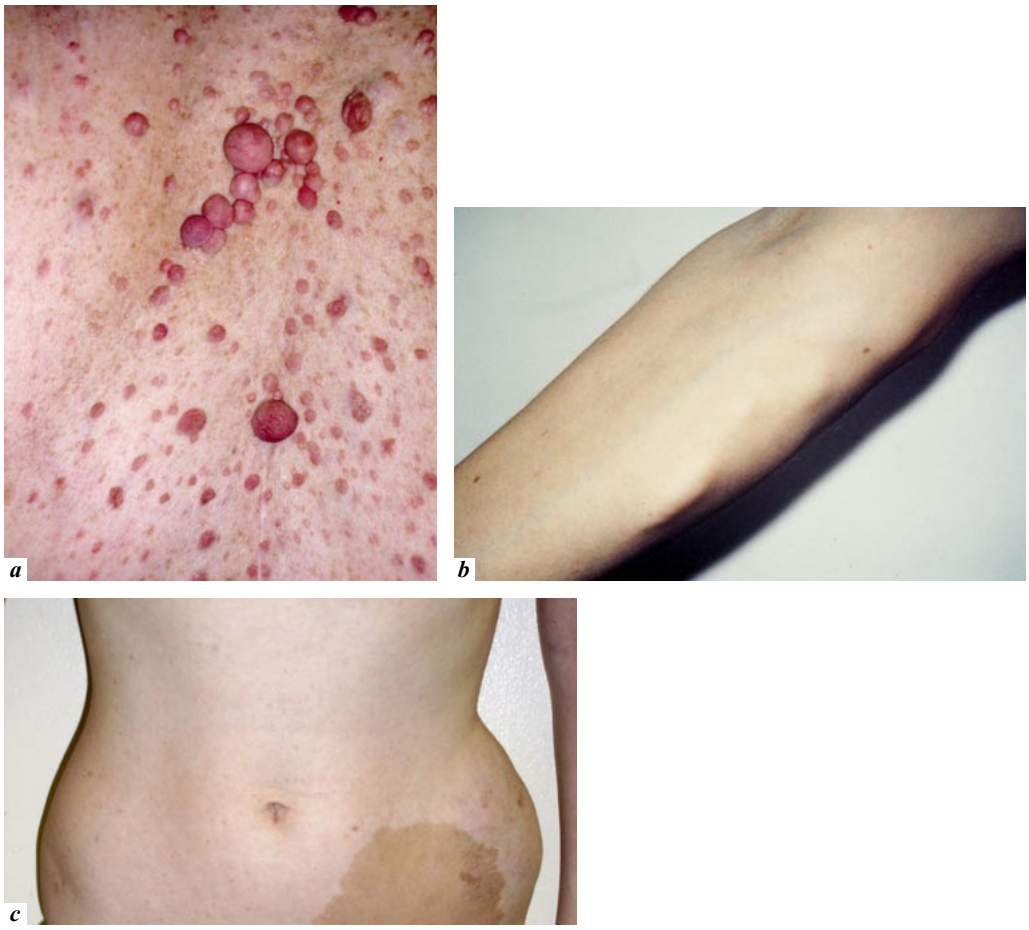


Fig. 3. The different kinds of neurofibroma. (***a***) Dermal neurofibromas. (***b***) Nodular neurofibromas. (***c***) Diffuse plexiform neurofibroma over lower left flank.

however as they may have an affected sibling the NIH criteria for NF1 can be met. The difficulty is that CAL spots are seen as a phenotypic marker in DNA repair syndromes and ring chromosome syndromes. It is difficult to know whether they are simply a reflection of faulty DNA repair mechanisms and unstable mitosis. Perhaps a comment on the other syndromes in which CAL spots can occur and their appearance needs also to be added to the criteria.

However, at least some MMR-D cases have other features of NF1. Of the 26 cases reviewed by Bandipalliam, 16 had CAL spots only; five had CAL spots plus

Table 3. Suggested changes to NIH NF1 diagnostic criteria

The NIH Consensus Development Conference Statement diagnostic criteria for NF1 [11] are met in an individual who has two or more of the following:

1. Six or more café-au-lait macules of over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals.
2. Two or more neurofibromas of any type or one plexiform neurofibroma.
3. Freckling in the axillary or inguinal regions.
4. Optic glioma.
5. Two or more Lisch nodules (iris hamartomas).
6. A distinctive osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudarthrosis.
7. A first-degree relative (parent, sibling, or offspring) with NF1 by the above criteria.

Suggestions for updating criteria:

1. Six or more café-au-lait macules of over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals.
2. Two or more neurofibromas of any type or one plexiform neurofibroma.
3. Freckling in the axillary or inguinal regions.
4. Optic glioma.
5. Two or more Lisch nodules (iris hamartomas).
6. A distinctive osseous lesion such as anterolateral bowing of the lower leg with or without pseudarthrosis.
7. A parent or offspring with NF1 by the above criteria.
8. A pathogenic mutation in the *NF1* gene
   - The CAL spots in NF1 usually have a smooth contour and uniform depth of pigmentation. Patients with DNA repair and ring chromosome syndromes can have ≥6 CAL coloured areas but with both an irregular contour and pigmentation depth.
   - Patients with segmental NF1 may have ≥2 of criteria 1–6 but limited to one (usually) or more distinct body segments.

another disease feature which would have led to an independent diagnosis of NF1, two of these had siblings with just CAL spots; one was said to have had a fibroma at age 15 years but no spots and in three the skin had not been commented on in the original report. Although mismatch repair analysis has been undertaken in all reports, *NF1* genetic analysis has not. Therefore whether an *NF1* mutation is present and if it is identical in the familial cases is not known. A further report [30] is of an affected child with NF1 features limited to half the body suggesting a somatic *NF1* mutation. One possible explanation for the association of MMR-D and NF1 is that the *NF1* gene may be a particular target for mismatch repair (MMR) mutagenesis which has been shown in two in vitro studies [31, 32].

This MMR deficient phenotype is important to recognise for two reasons, the much higher risk of early malignancy in the patient, and the risk of HNPCC related malignancy in the patient and the heterozygote parents. None of the

parents in the reports have shown any features of NF1. The majority of reported children have been born into known HNPCC families.

As a result of their studies of long bone dysplasias in NF1 Stevenson et al. [33] have recently suggested alteration to the wording of criterion 6 of the NIH criteria and I have included this in the revised criteria (table 3).

The final problem I have encountered is that cases with segmental NF1 [17] can meet the criteria unless the significance of the distribution of the skin changes is appreciated. Several segmental patients have sought specialist review in our clinic having been confidently diagnosed with NF1 elsewhere, on the basis of a segment of skin with ≥6 CAL spots and unilateral skinfold freckling. Therefore, a comment regarding distribution needs to be included in the criteria.

NF1 Genotype-Phenotype Correlation

To date only two correlations of clinical significance have been reported: the NF1 microdeletion phenotype [4] and the exon 17 del AAT associated with absence of neurofibromas [5]. These, along with the recent report of mutations in the *SPRED1* gene causing a NF1 like phenotype [6], mean there is an increasing role for NF1 genotyping in clinical practice. This is further discussed in this volume by Upadhyaya [34].

Family studies have suggested that the variation in expression seen in the majority of NF1 families is caused by the influence of modifying genes [35].

NF1 Subtypes

The most frequent of these is mosaic or segmental NF1. The other subtypes have been defined from the rare families in which the majority of affected individuals exhibit very similar NF1 features in two or more generations. In my experience such families represent <5% of an NF1 clinic population. The importance of their recognition is the similarity of phenotype in family members. Until recently, it was only possible to diagnose these subtypes in multigenerational families. However, molecular genetic diagnosis of some of them is now possible.

Segmental or Localized NF1

The term segmental or localized NF1 is used to describe the patients with disease features limited to one or more body segments. Ruggieri and Huson [17] estimated disease prevalence to be between 1 in 36,000 to 40,000 individuals in the general population. Most patients are asymptomatic and seek medical opinion because of the unusual appearance of the skin. In the majority of patients the

area involved is unilateral and varies in size from a narrow strip to one quadrant and occasionally one half of the body. Some patients have more than one segment involved on both sides of the midline, either in a symmetrical or asymmetrical arrangement. Within the affected area the patients either have NF1-related pigmentary changes, neurofibromas alone, or both. Ruggieri and Huson reported 124 cases, eight of the patients having a solitary plexiform neurofibroma as their only manifestation. Seven of the remaining 116 patients (6.9%) had specific NF1 complications, including learning difficulties, plexiform neurofibromas, optic pathway gliomas, and pseudarthrosis. Other NF1 complications were only identified in patients with segmental pigmentary changes. The importance of recognizing this group is for their different natural history and because they have much lower recurrence risks in offspring. There are, however, well recorded examples of parents with segmental NF1 diagnosed through children with full-blown NF1 [36]. These parents are gonosomal mosaics.

Since the first clinical descriptions it had been assumed segmental NF1 resulted from somatic mutation of the *NF1* gene. Proving this through mutation analysis turned out to be more difficult than anticipated [36–38]. Maertens et al. [18] have recently shown that it is necessary to perform *NF1* mutation analysis in Schwann cells derived from neurofibromas and melanocytes from the CAL spots of the affected segment to identify the causal mutation. Their work also demonstrates that the phenotype reflects the embryonic timing and, therefore, the neural crest-derived cell type involved in the somatic mutation. This work is important both clinically and for providing insights into the pathogenesis of different NF1 disease features. From a clinical viewpoint, patients with segmental NF1 sometimes find the small, but definite risk of a child with generalized NF1 too big a risk. We now have the methods by which the causative mutation can be found to enable prenatal diagnosis.

The CAL-only Phenotype

This term is used to describe multi-generational families with multiple CAL spots as their main disease feature sometimes associated with axillary freckling. Prior to mutation testing, one family had been shown to be linked to the NF1 region and two not [40–42]. Two recent studies have further elucidated the genetic basis of this phenotype:

a) *NF1 Exon 17 3-bp inframe deletion*. Upadhyaya et al. [5] recently reported 21 unrelated probands (14 familial and 7 sporadic) with the same c.2970–2972 del AAT (p.990delM) mutation but no cutaneous neurofibromas and no clinically obvious plexiform neurofibromas. Of the total cohort (n = 47), only one had had a symptomatic spinal neurofibroma removed. Thirty of the 47 individuals had axillary freckling. There was also a different frequency of complications, with a much lower frequency of learning problems,

macrocephaly and short stature; a similar frequency of scoliosis but with an increased frequency of pulmonary stenosis. The main importance of the phenotype was the lack of dermal neurofibromas in adult patients.

b) *SPRED1 gene and CAL spots only phenotype*. Brems et al. [6] recently reported mutations in the *SPRED1* gene on chromosome 15. In a large NF1 clinic they identified five families with CAL spots, axillary freckling, macrocephaly and Noonan like facies in some individuals. No neurofibromas or Lisch nodules were present. The only other features which overlap with NF1 were learning problems in some individuals, ADHD in two people and pectus excavatum in several. Several individuals had lipomas, but as these are relatively common in the general population, their significance is uncertain. As is the finding of individual cases with lung cancer (non-smoker age 42), childhood malignant tumour (possibly Wilms) and colon adenoma (age 45). When *NF1* mutations were not identified, they did linkage studies in the two largest families and mapped the locus to chromosome 15. In this region *SPRED1* was recognized as an ideal candidate, as it negatively regulates MAPK signaling like neurofibromin. Mutations were found in all five families. They then extended their studies to 86 unrelated patients who had negative *NF1* testing and CAL spots ± freckling only and found 7/86 (8%) had *SPRED1* mutations.

The clinical importance of these findings is that clinically useful natural history predictions can be made through finding one of these mutations. Prospective studies of their frequency in children presenting with multiple CAL but no family history are needed.

Watson Syndrome

Watson [42] described autosomal dominant inheritance of pulmonary stenosis, multiple café au lait spots, and intelligence at the lower end of the normal range. At that time pulmonary stenosis was not recognized as an NF1 complication and all family members had mild learning problems which is unusual in NF1. A few similar families have since been reported. Allanson et al. [43] followed up the original Watson patients and confirmed that their phenotype had remained distinct from NF1. Although a few individuals had Lisch nodules on slit lamp examination and some had developed neurofibromas, both of these features were present at a much lower frequency than is usually seen in NF1. They demonstrated linkage with markers for the *NF1* gene. Since then three different mutations in the *NF1* gene have been reported in Watson syndrome (an 80-kb deletion, an in-frame tandem duplication in exon 28 and the exon 17 3-bp deletion discussed above [44, 45] respectively). This suggests that the *NF1* mutation alone is not sufficient to explain this distinctive phenotype.

Neurofibromatosis-Noonan Syndrome (NFNS)

The present author has never been convinced that a specific NFNS phenotype exists, as suggested by Opitz and Weaver [46]. A systematic survey of the Noonan phenotype in a series of NF1 patients also concluded that this was not a unique syndrome [47]. However, a reasonable number of patients with NF1 do have facial features that overlap with mild Noonan syndrome, with mild ptosis and hypertelorism, down-slanting palpebral fissures, and posteriorly rotated ears [47, 48]. These patients have *NF1* gene mutations [49]. Pectus excavatum can also occur in both conditions; it was present in 31% of Riccardi's NF1 patients [23]. Given the recent findings of mutations in other components of the Ras-MAPK pathway in Noonan and other syndromes with features overlapping NF1 and Noonan's, one possibility to explain the variable NFNS phenotype is the interaction with functional polymorphisms in other genes in the pathway.

Spinal NF

Patients with NF1 can develop neurofibromas on the dorsal spinal roots, either as a single entity or at several consecutive levels as part of a plexiform neurofibroma. There is a subset of NF1 patients where the spinal root tumours are the principle feature. Families with this consistent phenotype have been reported [50–52]. Pulst et al. [52] reported one family which did not map to the NF1 locus. Messiaen et al. [53] have recently described 22 adult patients with the phenotype which they define as an entity where patients present with few NF1 pigmentary features and absence of dermal neurofibromas but with multiple spinal neurofibromas with or without involvement of peripheral nerves. They identified NF1 mutations in 18/22 of the cohort, suggesting there may be genetic heterogeneity. They found a very different spectrum of mutations compared with the general NF1 population with an over-representation of missense and splice mutations. These findings may point towards a different requirement for dermal vs. spinal root neurofibromas.

Genetic Counselling in NF1

The first step in the genetic counselling process is confirmation of diagnosis. In our NF clinic we occasionally see people referred where other causes of CAL like pigmentation or patchy skin pigmentation in general (e.g. ring chromosome syndromes, children of parents with different skin colouring) or cutaneous/subcutaneous tumours (e.g. lipomas) have been diagnosed as NF1. Once these diagnoses have been excluded then it is important to ensure the patient has typical NF1 rather than one of the much rarer subtypes described above. Patients with the severe form of NF2 can present in early childhood

with a couple of CAL spots, and tumours which clinically are plexiform neurofibromas (but are histologically usually schwannomas). The most useful skin sign in this form of NF2 is the NF2 plaque which present as areas of brown-orange skin which are slightly raised and roughened and may be hairy. Eye examination is also helpful as NF1 and NF2 have quite distinct features. In cases where diagnosis is uncertain evaluation in a specialist NF clinic can be helpful.

Once the diagnosis of NF1 is made counselling depends on the family situation as follows:

People with Typical NF1

Patients with NF1 have a 50/50 chance of an affected child. The problem is that apart from the rare subtypes discussed above, the phenotype does not 'breed true' even in families. Therefore we can not predict how a child will be affected. A person's perception of risk is influenced by his/her own NF1 experience and that seen in other family members. It is important to stress that, in most families the presence of an NF1 complication in a parent does not mean an increased risk of that problem in their children. Based on the Welsh population study [54], if one assumes that people will be most concerned about the risk of severe learning problems, the different complications that develop in childhood and cause lifelong morbidity and the risk of an NF1 related malignant tumour, then the risks of an offspring with one of these is around 1 in 12.

The majority of couples decide that they will have children without prenatal diagnosis, although if a test were available to predict severe NF1 the situation would probably be very different. With improved mutation detection rates [27] both prenatal and preimplantation genetic diagnosis is available for NF1. In familial cases DNA marker studies can also be used.

Often the risks to children are only one aspect of the counselling session. The consultant's main concerns are often the natural history of NF1 and the likely cosmetic burden of dermal neurofibromas.

Children at Risk of NF1

It is extremely rare for there to be any NF1 features picked up on ultrasound examination in pregnancy or at birth. There are a handful of reports of massive plexiform neurofibromas being detected in late pregnancy or at birth. Most NF1 children have no immediate problems, although sometimes one or more CAL spots will already be present. The majority of children with NF1 will have developed multiple CAL spots by their first birthday but some, particularly with pale colouring may not develop obvious spots until their second birthday and rarely later. In our clinic we therefore see the children at around

3 months, 15 months and around 2 years. If there are no CAL spots we then offer a final check around the fifth birthday. We have not routinely offered early mutation testing to determine status and few families have requested it.

There are rare families with <6 CAL spots, particularly with spinal NF, and in these mutation testing at an appropriate age is necessary and may be better delayed until the child can take an active part in the counselling process.

Counselling Parents of Sporadic Cases

As 50% of cases are the result of new mutations, this is a common genetic clinic scenario. In a follow-up study of children with ≥6 CAL spots Korf [55] found 95% of them develop typical NF1. Hitherto, it has been usual to counsel that NF1 is the most likely diagnosis and follow as appropriate. With the recent reports of the exon 17 deletion and SPRED1 phenotypes, with a much milder natural history, testing at least for these subtypes should now be considered in children presenting with only CAL spots +/– skinfold freckling. Obviously if there is an associated NF1 complication such as a plexiform neurofibroma or pseudarthrosis then a diagnosis of typical NF1 is secured. If the parents are considering more children then examination of their skin and irides is recommended because of the rare cases of segmental NF1 who are gonosomal mosaics. If examination is normal I counsel that the risk is little above the general population risk of a new mutation (around 1/6,000) as pure gonadal mosaicism in NF1 is an extremely rare event. Counselling parents with segmental NF1 is difficult and one can only use empiric risks. Animal studies suggest that it is proportional to the body area involved. Molecular genetic diagnosis through analysis of specific cell types from the affected area with the potential for a prenatal test is now possible [18].

References

1 Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, et al: The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. JAMA 1997;278:51–57.

2 Huson SM, Korf BR: The Phakomatoses; in Rimoin DL, Connor JM, Pyeritz RE, Korf BR (eds): Principles and Practice of Medical Genetics, ed 5. Edinburgh, Churchill Livingstone, 2007, pp 2817–2850.

3 Korf BR, Rubenstein AE: Neurofibromatosis: A Handbook for Patients, Families, and Health Care Professionals. New York, Thieme Medical Publishers, 2005.

4 Tinschert S: Clinical phenotypes in patients with *NF1* microdeletions; in Kaufmann D (ed): Neurofibromatoses. Monogr Hum Genet 2008;16:78–88.

5 Upadhyaya M, Huson SM, Davies M, Thomas N, Giovannini S, et al: A complete absence of cutaneous neurofibromas associated with a 3-bp in-frame deletion in exon 17 of the *NF1* gene (c.2970_2972 delAAT): A clinically significant genotype-phenotype correlation? Am J Hum Genet 2007;80:140–151.

6 Brems H, Chmara M, Sahbatou M, Denayer E, Taniguchi K, et al: Germline loss-of-function mutations in *SPRED1* cause a neurofibromatosis type 1-like phenotype. Nat Genet 2007;39: 1120–1126.
7 MacCollin M, Chiocca EA, Evans DG, Friedman JM, Horvitz R, et al: Diagnostic criteria for schwannomatosis. Neurology 2005;64:1838–1845.
8 Hulsebos TJM, Plomp AS, Wolterman RA, Robanus-Maandag Els C, Baas F, Wesseling P: Germline mutation of *INI1/SMARCB1* in familial schwannomatosis. Am J Hum Genet 2007;80:805–810.
9 Bentires-Alj M, Kontardis MI, Neel BG: Stops along the RAS pathway in human genetic disease. Nat Med 2006;12:283–285.
10 Denayer E, Legius E: What's new in the neuro-cardio-facial-cutaneous syndromes? Eur J Pediatr 2007;431:535–537.
11 Stevenson DA, Swensen JJ, Viskochil DH: Neurofibromatosis type 1 and other syndromes of the Ras pathway; in Kaufmann D (ed): Neurofibromatoses. Monogr Hum Genet 2008;16:32–45.
12 Stumpf D: Consensus development conference of neurofibromatosis. Arch Neurol 1988;45: 575–578.
13 Riccardi VM: Neurofibromatosis; clinical heterogeneity. Curr Prob Cancer 1982;VII:1–34.
14 Baser ME, Friedman JM, Wallace AJ, Ramsden RT, Joe H, Evans DGR: Evaluation of clinical diagnostic criteria for neurofibromatosis 2. Neurology 2002;59:1759–1765.
15 Viskochil DH, Carey JC: Nosological considerations of the neurofibromatoses. J Dermatol 1992;19:873–880.
16 Allanson JE, Upadhyaya M, Watson GH, Partington M, Lakey D, et al: Watson syndrome: Is it a sub-type of neurofibromatosis? J Med Genet 1991;28:752–756.
17 Ruggieri M, Huson SM: The clinical and diagnostic implications of mosaicism in the neurofibromatoses. Neurology 2001;56:1433–1443.
18 Maertens O, De Schepper S, Vandesompele J, Brems H, Heyns I, et al: Molecular dissection of isolated disease features in mosaic neurofibromatosis type 1. Am J Hum Genet 2007;81:243–251.
19 Evans DGR, Wallace A: NF2: mutations and management of disease; in Kaufmann D (ed): Neurofibromatoses. Monogr Hum Genet 2008;16:154–166.
20 Kluwe L: Molecular studies on Schwannomatosis; in Kaufmann D (ed): Neurofibromatoses. Monogr Hum Genet 2008;16:177–188.
21 Huson SM, Harper PS, Compston DAS: Von Recklinghausen neurofibromatosis. A clinical and population study in south-east Wales. Brain 1988;111:1355–1381.
22 Listernick R, Louis DN, Packer RJ, Gutmann DH: Optic pathway gliomas in children with neurofibromatosis 1: consensus statement from the NF1 Optic Pathway Glioma Task Force. Ann Neurol 1997;41:143–149.
23 Riccardi VM, Eichner JE: Neurofibromatosis: Phenotype, Natural History, and Pathogenesis. Baltimore, Johns Hopkins University Press, 1992.
24 De Smet L, Sciot R, Legius E: Multifocal glomus tumours in the fingers of two patients with neurofibromatosis type 1. J Med Genet 2002;39:e45.
25 Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A: Malignant peripheral nerve sheath tumours in neurofibromatosis 1. J Med Genet 2002;39:311–314.
26 Sharif S, Moran A, Huson SM, Iddenden R, Shenton A, et al: Women with neurofibromatosis 1 are at moderately increased risk of developing breast cancer and should be considered for early screening. J Med Genet 2007;44:481–484.
27 Messiaen LM, Callens T, Mortier G, Beysen D, Vandenbroucke I, et al: Exhaustive mutation analysis of the NF1 gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. Hum Mutat 2000;15:541–555.
28 Scott RH, Mansour S, Pritchard-Jones K, Kumar D, MacSweeney F, Rahman N: Medulloblastoma, acute myelocytic leukemia and colonic carcinomas in a child with biallelic *MSH6* mutations. Nat Clin Pract Oncol 2007;4:130–134.

29 Bandipalliam P: Syndrome of early onset colon cancers, hematologic malignancies and features of neurofibromatosis in HNPCC families with homozygous mismatch repair mutations. Fam Cancers 2005;4:323–333.
30 Pratt CB, Parham DM, Rao BN, Fleming ID, Dilawari R: Multiple colorectal carcinomas, polyposis coli, and neurofibromatosis. J Natl Cancer Inst 1988;80:1170–1172.
31 Wang Q, Montmain G, Ruano E, Upadhyaya M, Dudley S, et al: Neurofibromatosis type 1 gene as a mutational target in a mismatch repair-deficient cell type. Hum Genet 2003;112:117–123.
32 Gutmann DH, Winkeler E, Kabbarah O, Hedrick N, Dudley S, et al: *Mlh1* deficiency accelerates myeloid leukemogenesis in neurofibromatosis 1 (Nf1) heterozygous mice. Oncogene 2003;22: 4581–4585.
33 Stevenson DA, Viskochil DH, Schorry EK, Crawford AH, D'Astous J, et al: The use of anterolateral bowing of the lower leg in the diagnostic criteria for neurofibromatosis type 1. Genet Med 2007;9:409–412.
34 Upadhyaya M: *NF1* gene structure and NF1 genotype/phenotype correlations; in Kaufmann D (ed): Neurofibromatoses. Monogr Hum Genet 2008;16:46–62.
35 Easton DF, Ponder MA, Huson SM, Ponder BAJ: An analysis of variation in expression of NF1:evidence for modifying genes. Am J Hum Genet 1993;53:305–315.
36 Consoli C, Moss C, Green S, Balderson D, Cooper DN, Upadhyaya M: Gonosomal mosaicism for a nonsense mutation (R1947X) in the *NF1* gene in segmental neurofibromatosis type 1. J Invest Dermatol 2005;125:463–466.
37 Tinschert S, Naumann I, Stegmann E, Buske A, Kaufmann D, et al: Segmental neurofibromatosis is caused by somatic mutation of the neurofibromatosis type 1 (NF1) gene. Eur J Hum Genet 2000;8:455–459.
38 Schultz ES, Kaufmann D, Tinschert S, Schell H, von den Driesch P, Schuler G: Segmental neurofibromatosis. Dermatology 2002;204:296–297.
39 Charrow J, Listernick R, Ward K: Autosomal dominant multiple café-au-lait spots and neurofibromatosis 1: evidence of non-linkage. Am J Med Genet 1993;45:606–608.
40 Brunner HG, Hulsebos T, Steijlen PM, der Kinderen DJ, vd Steen A, Hamel BC: Exclusion of the neurofibromatosis 1 locus in a family with inherited café-au-lait spots. Am J Med Genet 1993;46:472–474.
41 Abeliovich D, Gelman-Kohan Z, Silverstein S, Lerer I, Chemke J, et al: Familial café-au-lait spots: a variant of neurofibromatosis type 1. J Med Genet 1995;32:985–986.
42 Watson GH: Pulmonary stenosis, cafe-au-lait spots, and dull intelligence. Arch Dis Child 1967;42:303–307.
43 Allanson JE, Upadhyaya M, Watson GH, Partington M, MacKenzie A, et al: Watson syndrome: is it a subtype of type 1 neurofibromatosis? J Med Genet 1991;2811:752–756.
44 Tassabehji M, Strachan T, Sharland M, Colley A, Donnai D, et al: Tandem duplication within a neurofibromatosis type 1 (NF1) gene exon in a family with features of Watson syndrome and Noonan syndrome. Am J Hum Genet 1993;53:90–95.
45 Upadhyaya M, Shen M, Cherryson A, Farnham J, Maynard J, et al: Analysis of mutations at the neurofibromatosis 1 (NF1) locus. Hum Mol Genet 1992;1:735–740.
46 Opitz JM, Weaver DD: The neurofibromatosis-Noonan syndrome. Am J Med Genet 1985;21: 477–490.
47 Colley A, Donnai D, Evans DG: Neurofibromatosis/Noonan phenotype: a variable feature of type 1 neurofibromatosis. Clin Genet 1996;49:59–64.
48 Tullu MS, Muranjan MN, Kantharia VC, Parmar RC, Sahu DR, et al: Neurofibromatosis-Noonan syndrome or LEOPARD syndrome? A clinical dilemma. J Postgrad Med 2000;46:98–100.
49 Baralle D, Mattocks C, Kalidas K, Elmslie F, Whittaker J, et al: Different mutations in the *NF1* gene are associated with Neurofibromatosis-Noonan syndrome (NFNS). Am J Med Genet 2003;119:1–8.
50 Ars E, Kruyer H, Gaona A, Casquero P, Rosell J, et al: A clinical variant of neurofibromatosis type one: familial spinal neurofibromatosis. Am J Hum Genet 1998;62:834–841.

51 Poyhonen M, Leisti E-L, Kytolo S, Leist J: Hereditary spinal neurofibromatosis: a rare form of NF1? J Med Genet 1997;34:184–187.
52 Pulst SM, Riccardi VM, Fain P, Korenberg JR: Familial spinal neurofibromatosis: clinical and DNA linkage analysis. Neurology 1991;41:1923–1927.
53 Messiaen L, Callens T, Williams JB, Babovic-Vuksanovic D, Huson S, et al: Genotype-phenotype correlations in spinal NF. Poster presentation, Am Soc Hum Genet 2007.
54 Huson SM, Compston DA, Harper PS: A genetic study of von Recklinghausen neurofibromatosis in south east Wales. II. Guidelines for genetic counselling. J Med Genet 1989;26:712–721.
55 Korf BR: Diagnostic outcome in children with multiple café au lait spots. Pediatrics 1992;90: 924–927.
56 Tucker T, Wolkenstein P, Reviz J, Zeller J, Friedman JM: Association between benign and malignant nerve sheath tumors in NF1. Neurology 2005;65:205–211.

Susan M. Huson
St Mary's Hospital
Hathersage Road
Manchester, M13 0JH (England)
Tel. +44 161 276 5152, Fax +44 161 276 6145, E-Mail Susan.huson@cmmc.nhs.uk

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 21–31

Treatment and Management of Neurofibromatosis 1

V.-F. Mautner[a], *E. Boltshauser*[b]

[a]Clinic for Maxillofacial Surgery, Section of Phakomatoses, University Hospital Hamburg-Eppendorf, Hamburg, Germany; [b]Division of Paediatric Neurology, University Children's Hospital Zürich, Zürich, Switzerland

Abstract

Neurofibromatosis 1 is a common condition with an autosomal dominant pattern of inheritance. Clinical management of patients with NF1 is complex due to the diversity of symptoms within patients. An interdisciplinary approach focusing different therapeutic interventions in each discipline is necessary to achieve optimal clinical care. Clinical diagnosis is based on NIH NF1 criteria. Therapeutic interventions in children with NF1 should focus the following aspects: general development (motor/cognitive function), visual symptoms, pubertal development (delayed/precocious puberty), blood pressure (renal artery stenosis), cardiovascular examination (congenital heart disease), evaluation of bones and spine (scoliosis associated with plexiform neurofibromas, pseudarthrosis), and examination of skin (cutaneous/subcutaneous and plexiform neurofibromas). Most of adult NF1 patients do not develop life-threatening complications. Disfiguring skin tumors are main burdens and can be removed successfully by surgery. It is essential to identify at risk patients for developing vascular complications, spinal tumors, progressive plexiform tumors, malignant peripheral nerve sheath tumors and/or gastrointestinal stromal tumors. As of yet, there are no reliable patterns to sort out at risk patients. Patients should present on a regular annual basis to a specialist in collaboration with their family practitioner. In view of the complex burden of NF1, psychological support has to be considered.

Neurofibromatosis 1 (NF1) is a common condition with an autosomal dominant pattern of inheritance. Within the last decade there has been significant progress in the therapeutic interventions based on molecular and clinical studies (such as neuropsychological and neuroimaging studies). Clinical management of patients with NF1 is still complex due to the diversity of symptoms. An interdisciplinary approach focusing on different therapeutic interventions in each discipline is necessary to achieve optimal clinical care.

NF1 in Children

Clinical diagnosis is based on clinical investigation to meeting NF1 diagnostic criteria [1]. Once the diagnosis is considered, patients should be referred to physicians skilled in the diagnosis of NF1. The clinical evaluation of children with NF1 should include the following aspects: general development (motor/cognitive function), visual symptoms, pubertal development (delayed/precocious puberty), blood pressure (renal artery stenosis), cardiovascular examination (congenital heart disease, pulmonary stenosis), evaluation of bones and spine (especially scoliosis associated with plexiform neurofibromas, pseudarthrosis), and examination of skin (neurofibromas, cutaneous and subcutaneous and plexiform neurofibromas).

Cardinal Features in the Pediatric Age Group

General Delay of Development

In the pediatric age group, there are few main cardinal features. The first one is a developmental delay (in ~40% of children – personal observation). The NF1-child frequently fails to reach normal developmental milestones in the expected time range. Impaired language or motor functions (such as clumsiness) and muscle hypotonia are typical difficulties in the first years of life. Due to hypotonia of abdominal muscles NF1-infants often have a protuberant belly and a tendency to funnel chest. It is important to stress that these deficits should be defined clearly. Other organic reasons have to be excluded and physiotherapeutic interventions are recommended – in our experience – similar to children with other disabilities.

Deficits in Cognitive Function

Cognitive deficits in NF1-children are a main burden (30–60%). While café-au-lait spots and plexiform neurofibromas have aesthetic significance, specific learning disabilities in NF1-children may result in failing to achieve their full academic and social potential, and thus cause serious problems. Depressed performance in verbal and nonverbal tasks and impaired global language belong to the characteristic features. Visual spatial problems and working memory impairment are part of specific learning problems in NF1-children [2]. A detailed neuropsychological developmental assessment should be performed in children with any cognitive deficits in order to determine which areas of cognitive function need support. It is necessary to perform a neuropsychological assessment prior to school entrance and to determine early if a child has low (IQ <70 = rare in NF1) or mild intellectual deficit (average range IQ <85 = more frequent in NF1) in order to place in appropriate educational classes. All of this information helps to establish a coordinated liason between parents, teachers, pediatricians and psychologists in order to provide optimal support of an NF1

child. In a recent study Hyman et al. found problems with academic achievements in 52% of 81 children, 30% had a more general learning problem, 'only' 20% a specific learning disability. There was a significant comorbidity of learning problems and attention-deficit-hyperactivity disorder [3].

Attention Deficit Hyperactivity Disorder (ADHD)

Another aspect affecting academic achievement of children with NF1 are problems with attention or attention and impulse control. These problems frequently meet the DSM-IV criteria for ADHD at a higher incidence rate than expected, as noted in clinical experience and several studies. The deficits of attention and noted behavioral problems in NF1 children are the main reasons for the problems in school and lead to lower level of vocational training. Consequently, children with NF1 can experience lack of self-confidence and social acceptance. In our experience, children with NF1 respond satisfactorily to systematic use of low doses ($<$15 mg) of methylphenidate. Therefore, those children whose history and presenting symptoms suggest ADHD should be given an evaluation for sustained attention and impulse control. This evaluation should be performed independent of any intellectual deficits, as children with ADHD and lower IQ ($<$80) have also demonstrated improved performance after medication. It is important to follow children closely who are under medication to monitor if the stimulant drug is effective or should be discontinued, as attentional problems may decrease with age [4]. Recent studies have confirmed our clinical impression: children with NF1 have impaired social skills [5], and a high prevalence of psychological disturbances and sleeping disorders [6]. It is thus not surprising that children and adolescents with NF1 have impaired quality of life and psychological adjustment [7]. It is very important to pay attention to these limitations in the follow-up.

Cutaneous Neurofibromas and Plexiform Neurofibromas

Cutaneous neurofibromas rarely occur prior to the age of ten years; they rather tend to develop in the late teen aged years. The late teen aged years are a vulnerable age of life. The adolescent needs sensitive education about his/her NF1 and – in some instances – psychological support.

In the first years of life, the child with NF1 can present with a congenital plexiform neurofibroma (PNF) at any location of the body. Frequently these tumors are misdiagnosed as lymphangioma. These congenital tumors can cause disfigurement, pain and functional deficits. PNF tend to show net-like growth patterns along nerve roots extending from a main nerve root to a small distal branch. PNF can be divided in different growth types: superficial, invasive and displacing. Superficial tumors are frequently palpable and associated with hyperpigmentation, thickening and hypertrophy of the skin or hair excess. Each of these symptoms

may be the only clinical sign of a superficial PNF that shows only skin involvement. However, a PNF can be located superficially and show internal growth at the same time. It is important to investigate PNF by an ultrasound or MRI in order to explore their growth characteristics. Imaging studies have revealed that asymptomatic internal PNF can be detected in childhood showing up with a displacing or invasive growth pattern [8]. It is important to be aware of the longitudinal studies, which have demonstrated that PNF show progression mainly in childhood and adolescence and progression slows down with increasing age [9].

In general, surgical options are limited due to the tendency of PNF to infiltrate surrounding tissues. However, it is worthwhile to analyze each tumor with regard to location and growth, because tumor resection can be performed best in small superficial PNF at an early stage. Follow up periods of more than five years have shown that a complete resection can be achieved successfully. Complete removal is frequently impossible, but even partial and/or repeated resection of tumors can be very beneficial for the patients (for example, developing PNF of the ankle joint does not allow a patient to wear a normal shoe) [10]. PNF located in the region of bones (especially limbs) can be associated with overgrowth of extremities and require the interaction between surgeons and orthopedic surgeons to carry out the best surgical strategy.

Over the last few years, several agents as farnesyl transferase inhibitors, anti-angiogenesis drugs or fibroblast inhibitors have been tested in clinical trials to inhibit or decrease PNF growth. There is no evidence that such drugs stabilize or decrease PNF growth. Malignant peripheral nerve sheath tumors (MPNST) develop from PNF. In children, MPNST are extremely rare and complications may occur – in our experience – in patients with internal plexiform tumors [11].

Bone Manifestations

Pseudarthrosis

About 2% of patients with NF1 develop bowing of the long bones, particularly the tibia, and/or pseudarthrosis. In most of these children, an incident of minimal trauma can lead to a fracture of the bone [12].Therefore prophylactic splinting has been advocated in infants at risk to prevent a fracture. The surgical management of pseudarthrosis is frequently not satisfactory. Currently, there are two main options: the Iliazarov procedure as one treatment approach or alternatively, treatment by transfer of a vascularized fibular graft from the contralateral extremity. Recently, successful treatment was reported by applicating recombinant human bone morphogenetic protein [13].

Scoliosis

Monitoring of the spine is an important task of the follow-up examinations. Patients with clinical evidence of incipient scoliosis need close intervals

for monitoring the progression of scoliosis (months). Patients should be referred to an orthopedic surgeon and receive radiologic imaging including MRI of the spine. The early onset of a rapid progressive scoliosis, which is characteristic for NF1, is often referred to as a dystrophic scoliosis. Dystrophic scoliosis commonly affects the lower cervical and upper thoracic spine, it may involve several segments and causes distortion of the vertebral bodies and ribs and requires early surgical intervention [14].

Short Stature – Megalencephaly

Short stature is a common finding in children with NF1, as is megalencephaly. Specific growth charts for young NF1 patients are available [15]. Growth hormone deficiency is exceptional. In such patients, hormonal replacement treatment is considered safe, not contributing to accelerated growth of neurofibromas.

Vascular Complications

Regular monitoring of blood pressure is already required in children, as hypertension may result from renal artery stenosis or other complex hypodiaphragmatic vascular dysplasia. Children with NF1 have an increased prevalence of moyamoya syndrome (progressive arterial occlusion of the circle of Willis), presenting as transient-ischaemic attacks or stroke.

Astrocytomas

Optic Pathway Gliomas

Optic pathway gliomas (OPG) are pilocytic astrocytomas (grade I), that occur in 7–15% of children with NF1. But only 5% of these tumors become symptomatic and only children under seven years of age have a high risk of developing a symptomatic tumor [16]. Since screening for asymptomatic optic tumors in children with NF1 typically does not produce any clinical consequences to the patient, an MRI of the brain is not recommended as a routine examination. However, as these young children do not complain of visual problems, children should have visual assessment performed annually to assess for any unreported problems. Asymptomatic children should have one baseline assessment of color vision and visual field at appropriate developmental age. A visual assessment in (very) young children who may have cognitive deficits is very difficult. Parents should be made aware that visual problems of children such as failing to pick up small toys, or frequent dropping of toys, may be first indicators of optic gliomas. A complete ophthalmological examination should be done on an annual basis for any child with NF1. The annual check up should include assessment of visual acuity, color vision, visual fields, ophthalmoscope and slit-lamp examination. Ocular alignment and rotations, pupillary light responses, and refractive status

with cycloplegia examinations are recommended. Ophthalmoscopy should include indirect and, when possible, direct examination.

Accelerated linear growth in children may be the first manifestation of a chiasmatic glioma, even in the presence of a normal ophthalmological investigation. A precocious puberty is frequently related to hypothalamic involvement of the tumor.

The vast majority of intraorbital optic nerve gliomas in NF1 patients never progress once they have come to medical attention. However, during regular follow up examinations progressive visual loss, decrease of visual fields or proptosis indicates a tumor progression. Close follow up by MRI and ophthalmological examination in short intervals may confirm this finding and indicate whether therapeutic interventions are needed. The treatment of children with OPG depends on location and extension of the tumor, while biopsy is rarely necessary. Chemotherapy is usually administered using vincristine and carboplatin [17, 18]. Neurosurgeons are mostly involved when debulking of extensive gliomas is necessary. Even though there is evidence that radiotherapy will at least temporarily stop tumor growth, it is not suitable in young children because of the potential of secondary malignancy, vascular change, endocrine consequence and neuropsychological deficits [19].

Astrocytomas Outside Optic Pathways

Astrocytomas may occur in all parts of the central nervous systems in addition to the optic pathway. Cerebellar low grade gliomas have a good prognosis after neurosurgery. Brainstem tumors are low grade gliomas as well. In the context of neurofibromatosis these also tend to have a rather benign course. Detection may be 'incidental' by neuroimaging. Treatment should only be considered if tumor progression and clinical signs are obvious. Treatment modality (chemotherapy, radiotherapy) should be tailored to the individual situation.

Neurofibromatosis Type 1 in Adults

Clinical care of adults with NF1 can be a challenge. Most of the NF1 patients do not develop life-threatening complications and disfiguring skin tumors are the main burden. It is essential to identify those patients who are at risk to develop vascular complications, spinal tumors, progressive plexiform tumors, MPNST and/or gastrointestinal stromal tumors (GIST). There are no known reliable patterns to sort out the at risk patients. Therefore, patients should be followed by an NF1 specialist on a regular annual basis in collaboration with the family practitioner. Patients with mild form of the disease should at least be made aware of unusual complications as described above [20].

Implications and Treatment of Neurofibromas

Neurofibromas are benign peripheral nerve sheath tumors showing focal cutaneous or subcutaneous location. Neurofibromas can lead to transient stinging and itching. Sometime these tumors induce pain and cause problems by wearing of clothing. Multiplicity of these tumors may cause a disfigurement which may have a psychological impact on the individual's self-image, partnerships and social relations.

Therefore referral to surgeons skilled in the removal of neurofibromas is indicated and plastic surgeons should be consulted whenever disfigurement is present. This is especially true for neurofibromas of the face and neck. The result of surgery is dependent on dimension of tumor, its localization and its structure (diffuse, nodular, or pedunculated). Pedunculated neurofibromas can be excised with very satisfactory results. Neurofibromas can be removed by different techniques: by scalpel, laser or electrocauterization. In our experience the scalpel is useful for the larger, exophytic tumors. The use of laser and electrocauterization is helpful for tumors with intracutaneous localization containing a great amount of blood vessels. Completely resected tumors typically do not relapse. There is no proven benefit of carbon dioxide laser treatment in comparison to the removal of neurofibromas by a scalpel. There is also no evidence that neurofibromas tend to have a malignant change. The itching of neurofibromas can be very troublesome to patients and the effect of antihistamines is not always satisfactory in resolving the itching.

Hormones and Neurofibromas

It is obvious that hormonal factors contribute to the growth of neurofibromas, as neurofibroma growth is stimulated by puberty and pregnancy. A recent study has shown that 75% of neurofibromas carry progesterone receptors. However, there is no evidence that the combined oral contraceptive pill or progesterone only pill may contribute to neurofibroma growth [21]. During pregnancy obstetrician and NF clinicians should be aware that spinal and pelvic neurofibromas may progress rapidly and these neurofibromas should be monitored closely.

Plexiform Neurofibromas

Plexiform neurofibromas (PNF) are comprised of the same cell type as dermal ones, but have an expanded extracellular matrix. Congenital PNF can be present in the first years of life, but may also become apparent later in life. Deeply located tumors may lead to pain and neurological deficits; thus, these patients need special care and close follow up intervals. Patients developing deficits or pain should undergo surgery whenever a positive outcome is suggested. Peripheral malignant nerve sheath tumors are associated with deeply

located PNF (displacing/invasive growth type). Superficial PNF mainly cause esthetic problems, especially when located in the face. Across patients of different ages, this type of tumor does not show infiltration into muscles. This fact supports the hypothesis that these tumors do not change growth type throughout life [8].

Neurofibromas and plexiform neurofibromas can lead to sensory and motor deficits. An annual examination has to include careful neurological status to detect first symptoms indicating possible spinal cord compression which needs a neurosurgical intervention.

MPNST

NF1 patients have an approximate lifetime risk of 10% for the transformation of a PNF into an MPNST. Predominantly the transformation occurs in individuals aged 20–35 years [22]. Clinical indicators of malignancy are a persistent or increasing pain, swelling and increase in size, and/or neurological deficits. Survival rates in NF1 patients with MPNST are low. A main reason may be a delay in the diagnosis itself. The most effective intervention in MPNST is the complete removal of the tumor with wide margins. However, options for surgical treatment may be limited due to size and location of the sarcoma. There is a high local recurrence rate after surgery and early onset of metastases [23].

Positron emission tomography (PET) with [^{18}F] Fluorodeoxyglucose (FDG), currently performed in combination with CT on a combined PET/CT scanner, can be a useful diagnostic tool in differentiating benign PNF from MPNST [24]. The final preoperative diagnosis of MPNST is usually achieved by histopathology and immunohistochemistry on fine needle aspiration samples or biopsy. The most important therapeutic intervention in MPNST remains complete removal of the tumor with margins as wide as possible ($\geq$10 cm). Radiotherapy may help to provide local control and delay the onset of local recurrence. But so far, it has little effect on long-term survival which is determined by metastases. Chemotherapy is usually restricted to patients who have metastatic disease, to date only very few drugs or drug combinations have been shown to be effective. The result is from a treatment regimen that applies doxorubicin in combination with ifosfamide. These drugs are administered either as palliative therapy to reduce size of tumor and/or to enable resection of tumors which were initially unresectable. Recently, the detection of receptor tyrosine kinases PDGFRα and c-kit oncogene in MPNST of NF1 patients has led to potentially new forms of treatment. This new treatment form is based on receptor tyrosine kinase inhibitors such as STI 571 (Imatinib). The first clinical trials investigating the therapeutic benefit of receptor tyrosine kinase inhibitors in MPNST have been initiated [25].

Vascular Problems

Hypertension occurs in association with NF1 and may have several different causes, including pheochromocytoma (2%) and renal artery stenosis (2%). The renal artery stenosis is one manifestation of a vasculopathy that can occur localized or generalized [26]. Other clinical manifestations of the NF1 induced vasculopathy include carotid artery stenosis/occlusion, haemorrhage and aneurysm. The diagnosis should be considered in hypertensive children and adults. Tests for clarification of diagnosis are renal duplex ultrasound examination and magnetic resonance angiography. Renovascular hypertension can usually be treated by surgical repair of abnormal renal arteries, revascularization of kidney or unilateral nephrectomy.

Patients with NF1 who present with sustained or paroxysmal hypertension, unexplained agitation and anxiety, tachycardia, palpitation, headache, perspiration and flushing are likely to have pheochromocytoma. Diagnostic evaluation should include a 24-hour urine collection to quantify the excretion of vanillin-mandelic acid and specific catecholamines (metanephrine, epinephrine, and norepinephrine). MRI is very sensitive for the detection of the tumor.

Decreased Mineral Density

Surgical treatment of patients with scoliosis reveals that bony structures are frequently malacic. These observations led to mineral density studies, which have shown that bone mineral density is frequently decreased in NF1 patients [27]. In recent observations, serum vitamin D concentrations among NF1 patients are much lower than in controls and this might be pathogenically related. The fact that patients with NF1 and osteomalacia respond well to treatment with pharmacological doses of vitamin D and calcium raises the possibility that similar treatment may help to prevent the development of osteomalacia and osteoporosis in people with NF1 [28].

Gastrointestinal Stromal Tumors

Gastrointestinal stromal tumors (GIST) most commonly occur sporadically, but an increased tendency for these tumors is suggested in NF1 patients. A great majority of tumors occur in the jejunum or ileum, with a multiplicity of tumors within each patient. Other locations include duodenal and gastric tumor involvement. Clinically, the most common presentations are gastrointestinal bleeding and anemia over several years. Recent studies have demonstrated that most NF1 patients who underwent surgery have a good long-term prognosis in opposite to their sporadic counterparts [29].

Psychological Aspects – Burden of Neurofibromatosis

The psychological burdens of NF1 are due to many different factors, in particular esthetic problems, anxiety of potential complications, social isolation,

impaired professional performance resulting from learning disabilities. Anxiety and depression are common and psychological support and antidepressive medication may be necessary. There is little knowledge of the lifelong performance of NF1 affected adults who have suffered cognitive difficulties in their childhood. The interaction between the disease and cognitive impairment is unknown. Recently, a study was carried out to investigate the psychological impact of ADHD in adults with NF1. The authors suggested that attention deficits in a subgroup of NF1 patients persisted lifelong. The lifelong ADHD produced interpersonal distress, and poorer psychological or relationship functioning. The ADHD had a significant effect independent of the NF1 itself on reduced life quality [30]. As with NF1 children who have ADHD, stimulant medication is frequently effective in adults.

NF1 Clinics

Without doubt there is a need for an interdisciplinary approach to assure an optimal treatment of patients with NF1. A specialized center should provide the expertise that covers the differing aspects of the disease. The role of these experts is to monitor complex cases, to give advice to general practitioners and to educate patients and their families [20].

References

1 National Institutes of Health Consensus Development Conference Statement. Arch Neurol 1988;45:578–579.

▶2 Cutting LE, Koth CW, Denkla MB: How children with neurofibromatosis type 1 differ from 'typical' learning disabled clinical attenders: non-verbal learning disabilities revisited. Dev Neuropsychol 2000;17:29–24.

▶3 Hyman SL, Shores EA, North KN: Learning disabilities in children with neurofibromatosis type 1: subtypes, cognitive profile, and attention-deficit-hyperactivity disorder. Dev Med Child Neurol 2006;48:973–977.

▶4 Mautner VF, Kluwe L, Thakker SD, Leark RA: Treatment of ADHD in neurofibromatosis type 1. Dev Med Child Neurol 2002;44:164–170.

▶5 Barton B, North KN: Social skills of children with neurofibromatosis type 1. Dev Med Child Neurol 2004;46:553–563.

▶6 Johnson H, Wiggs L, Stores G, Huson S: Psychological disturbance and sleep disorders in children with neurofibromatosis type 1. Dev Med Child Neurol 2005;47:237–242.

▶7 Graf A, Landolt M, Capone Mori A, Boltshauser E: Quality of life and psychological adjustment in children and adolescents with neurofibromatosis type 1. J Pediatr 2006;149:348–353.

▶8 Mautner VF, Hartmann M, Kluwe L, Friedrich RE, Fünsterer C: MRI growth patterns of plexiform neurofibromas in patients with neurofibromatosis type 1. Neuroradiology 2006;48:160–165.

9 Mautner VF, Schooler T, Fünsterer C, Hartmann M, Friedrich RE, Friedman J: Longitudinal study of plexiform neurofibroma growth in people with Neurofibromatosis. Poster at NNFF Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis 2005 Aspen, Colorado.

▶10 Friedrich RE, Schmelzle R, Hartmann M, Fünsterer C: Resection of small plexiform neurofibromas in neurofibromatosis type 1 children. World J Surg Oncol 2005;3:6.

▶11 Friedrich RE, Hartmann M, Mautner VF: Malignant peripheral nerve sheath tumors (MPNST) in NF1-affected children. Anticancer Res 2007;27:1957–1960.

12 Korf BR: Diagnostic outcome in children with multiple café-au-lait spots. Pediatrics 1992;90:924–927.
13 Young-In-Lee F, Sinicropi STM, Lee FS, Vitale MG, Roye DP, Choi IH: Treatment of congenital pseudarthrosis of the tibia with recombinant human bone morphogenetic protein-7 (rhBMP-7). J Bone Joint Surg 2006;88:627–633.
14 Riccardi VM: Skeletal system; in Friedman JM, Gutman DH, MacCollin M, Riccardi VM (eds): Neurofibromatosis, Phenotype, Natural History and Pathogenesis, ed 3. Johns Hopkins University Press, Baltimore and London, 1999, pp 250–271.
15 Szudek J, Birch P, Friedman JM: Growth in North American white children with neurofibromatosis 1 (NF1). J Med Genet 2000;37:933–938.
16 Listernick R, Ferner RE, Liu GT, Gutmann DH: Optic pathway gliomas in neurofibromatosis-1: controversies and recommendations. Ann Neurol 2007;61:189–198.
17 Packer RJ, Alter J, Allen J, Phillips P, Geyer R, et al: Carboplatin and vincristine chemotherapy for children with newly diagnosed progressive low-grade gliomas. J Neurosurg 1997;86:747–754.
18 Gnekow AK, Kortmann RD, Pietsch T, Emser A: Low grade chiasmatic-hypothalamic glioma-carboplatin and vincristin chemotherapy effectively defers radiotherapy within a comprehensive treatment strategy – report from the multicenter treatment study for children and adolescents with a low grade glioma – HIT-LGG 1996 – of the Society of Pediatric Oncology and Hematology (GPOH). Klin Padiatr 2004;216:331–342.
19 Sharif S, Ferner R, Birch JM, Gillespie JE, Gattamaneni HR, Baser ME, Evans DG: Second primary tumors in neurofibromatosis 1 patients treated for optic glioma: substantial risks after radiotherapy. J Clin Oncol 2006;24:2570–2575.
20 Ferner RE, Huson SM, Thomas N, Moss C, Willshaw H, et al: Guidelines for the diagnosis and management of individuals with neurofibromatosis 1 (NF1). J Med Genet 2006;44:81–88.
21 Lammert M, Mautner VF, Kluwe L: Do hormonal contraceptives stimulate growth of neurofibromas? A survey of 59 patients. BMC Cancer 2005;5:16.
22 Evans DG, Baser ME, Mc Gaughran J, Sharif S, Howard E, Moran A: Malignant peripheral nerve sheath tumors in neurofibromatosis 1. J Med Genet 2002;39:311–314.
23 Ferner RE, Gutmann DH: International consensus statement on malignant peripheral nerve sheath tumors in neurofibromatosis. Cancer Res 2002;62:1573–1537.
24 Brenner W, Friedrich RE, Gawad KA, Hagel C, von Deimling A, et al: Prognostic relevance of PDG PET in patients with neurofibromatosis type-1 and malignant peripheral nerve sheath tumors. Eur J Nucl Med Mol Imaging 2006;33:428–432.
25 De Wit M, Mautner V, Bokemeyer C: An open-labeled phase II study of imantinib treatment of patients with malignant peripheral nerve sheath tumors and investigation of molecular targets. [Abstract] Anticancer Res 2005;25:4844.
26 Friedman JM, Arbiser J, Epstein JA, Gutmann DH, Huot SJ, et al: Cardiovascular disease in neurofibromatosis 1: a report of the NF1 Cardiovascular Task Force. Genet Med 2003;4:105–111.
27 Lammert M, Kappler M, Mautner VF, Lammert K, Storkel S, Friedman JM, Atkins D: Decreased bone mineral density in patients with neurofibromatosis 1. Osteoporos Int 2005;16:1161–1166.
28 Lammert M, Friedman JM, Roth HJ, Friedrich RE, Kluwe L, et al: Vitamin D deficiency associated with number of neurofibromas in neurofibromatosis 1. J Med Genet 2006;43:810–813.
29 Miettinen M, Fetsch JF, Sobin LH, Lasota J: Gastrointestinal stromal tumors in patients with neurofibromatosis 1: a clinicopathologic and molecular genetic study of 45 cases. Am J Surg Pathol 2006;30:90–96.
30 Wiesner D, Mautner VF, Leark RA: Cognitive and psychosocial functioning of NF adults with/without history of ADHD. J Int Neuropsychol Soc 2003;9:564.

Prof. Victor-F. Mautner
University Hospital Hamburg-Eppendorf
Clinic for Maxillofacial Surgery, Section of Phakomatoses
Martinistr. 52, DE–20246 Hamburg (Germany)
Tel. +49 40 42803 4068, Fax +49 40 42803 5966, E-Mail v.mautner@uke.uni-hamburg.de

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 32–45

Neurofibromatosis Type 1 and Other Syndromes of the Ras Pathway

D.A. Stevenson[a,b], *J.J. Swensen*[c], *D.H. Viskochil*[a,b]

[a]Division of Medical Genetics, Department of Pediatrics, University of Utah, Salt Lake City, [b]Shriners Hospital for Children Intermountain, Salt Lake City and [c]ARUP Laboratories and Department of Pathology, University of Utah, Salt Lake City, Utah, USA

Abstract

Neurofibromin, the *NF1* protein product, functions as a tumor suppressor through negative regulation of the Ras signal transduction pathway. Neurofibromatosis type 1 is an autosomal dominant condition due to *NF1* haploinsufficiency in conjunction with double inactivation in tissue. Several other clinical syndromes have been delineated with overlapping phenotypes of NF1. The overlapping clinical phenotype in many of these syndromes has led to the identification of disease-causing mutations in genes within the Ras signal transduction pathway.

Neurofibromatosis type 1 (NF1) is diagnosed clinically, in which an individual must fulfill 2 of 7 clinical criteria [1, 2]. Individuals with NF1 usually harbor inactivating mutations in the *NF1* gene, which encodes the protein product neurofibromin, a Ras-GAP protein (also reviewed by Peltonen and Peltronen, this book) [3]. The Ras GTPases interact with multiple pathways including the Raf/Mek/ Erk MAPK (mitogen-activated protein kinase) cascade, which regulates cellular growth and differentiation. Although NF1 is a distinct condition based on phenotype, several other syndromes have overlapping clinical features leading to the identification of disease-causing mutations in genes within the Ras signal transduction pathway. Understanding the genotype-phenotype relations of those other syndromes may lead to effective therapies for some manifestations of NF1. Fine-tuned quantification of the down-stream readout of *NF1* haploinsufficiency (i.e. mTOR, Akt) may identify appropriate therapies for various manifestations.

The Ras Pathway

The *KRAS, HRAS* and *NRAS* genes encode the four related p21 proteins, Kras-4A, Kras-4B, Hras and Nras, that lie at the core of the Ras pathway [4, 5]. Kras-4A and Kras-4B are both expressed from the *KRAS* gene and differ only at their C-termini as a result of alternative splicing. The p21 proteins belong to the Ras GTPase superfamily, whose members function as switches that regulate numerous intracellular pathways [6]. Ras GTPase activity results in the hydrolysis of bound GTP to GDP, which is accompanied by a conformational change in the protein that alters interactions with other cellular components. Ras possesses weak intrinsic GTPase activity that is regulated by accessory proteins. Guanine nucleotide exchange factors (GEFs) activate Ras by catalyzing the exchange of bound GDP for GTP, while GTPase activating proteins (GAPs) downregulate Ras by enhancing the hydrolysis of bound GTP to GDP. The Ras p21 proteins share common GEFs and GAPs as well as downstream effectors. They regulate signals passed from receptors at the plasma membrane to intracellular pathways that control processes such as cellular proliferation and differentiation. While GTP-bound, they transduce a signal received from upstream pathway components. Upon hydrolysis of GTP to GDP, signaling to downstream targets is terminated.

With the exception of their C-termini, the four p21 proteins are highly related. The unique C-terminus of each Ras is the site of a combination of post-translational modifications that influence the intracellular and plasma membrane microlocalization of that isoform [7, 8]. Such differences in cellular microlocalization can modify the interactions available to a particular Ras. Tissue-specific expression differences between the *RAS* genes also likely contribute unique characteristics to each isoform [9]. There is, however, evidence that considerable functional redundancy exists between these proteins. Transgenic mice with homozygous disruption of the exon specific to *Kras-4A* have no apparent phenotype [10], and mice with homozygous disruption of *Hras* and *Nras*, individually or in combination, have phenotypes that are subtle [11, 12]. In contrast, homozygous disruption of the entire *Kras* gene (both splice variants) results in embryonic lethality in mice, indicating a non-redundant role for at least Kras-4B, although some combined requirement for both isoforms cannot be excluded [13].

In a canonical Ras pathway at the plasma membrane, a receptor tyrosine kinase (RTK) dimerizes and autophosphorylates tyrosines in its cytoplasmic domain in response to binding ligand, an extracellular growth factor. The intracellular phosphotyrosines on the RTK are able to bind select proteins with SH2 domains. One SH2 protein, Grb2, acts as an adaptor for the binding of 1 of 2 related GEFs, Sos1 or Sos2, to the RTK. The bound GEF interacts with Ras to

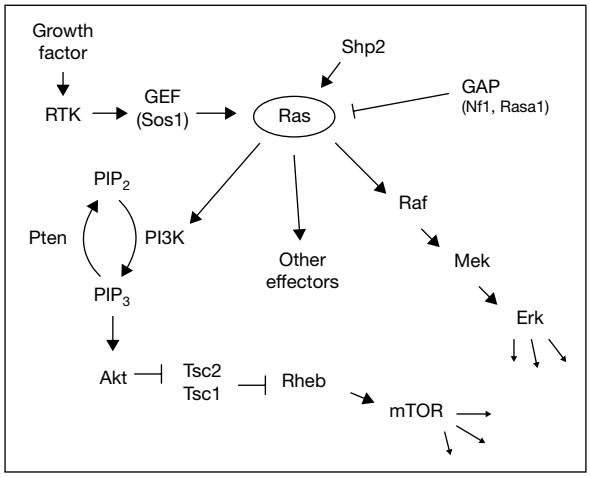


Fig. 1. Diagram of the Ras pathway.

'activate it', which initiates signaling to downstream effectors. GAPs, the best known of which are neurofibromin (encoded by the *NF1* gene) and Rasa1 (also known as p120-RasGAP), act to downregulate the signal.

Activated GTP-bound Ras regulates signaling through a number of downstream effector pathways. The best known of these are the Raf/Mek/Erk MAPK (mitogen activated protein kinase) cascade and the PI3 kinase (PI3K)/Akt pathway (fig. 1). The PI3K/Akt pathway is discussed in a later section. The MAPK cascade is an evolutionarily conserved pathway in which a series of 3 protein kinases are sequentially activated through phosphorylation as a means of transducing a signal to intracellular targets [reviewed in 14, 15]. The Raf/Mek/Erk MAPK cascade is involved in the regulation of cell growth and differentiation. In this pathway, Ras-GTP recruits 1 of 3 Raf serine/threonine kinases (Braf, Araf or Raf1/Craf) to the plasma membrane where it is activated. Activated Raf phosphorylates 1 of 2 Mek dual-specificity kinases (Mek1 or Mek2), which, in turn, phosphorylates 1 of 2 Erk serine/threonine kinases (Erk1 or Erk2). Activated Erk phosphorylates targets such as transcription factors and other signaling proteins.

Germline and somatic mutations can lead to hyperactive signaling of the Ras pathway through several mechanisms. At the core of the pathway, they may reduce the intrinsic GTPase activity of Ras, increase the activity of a GEF, or reduce the function of a GAP. Activating somatic mutations of *RAS* and other Ras-pathway genes occur in a variety of cancers. Historically, work with acute transforming retroviruses in the murine model identified *ras* oncogenes, and

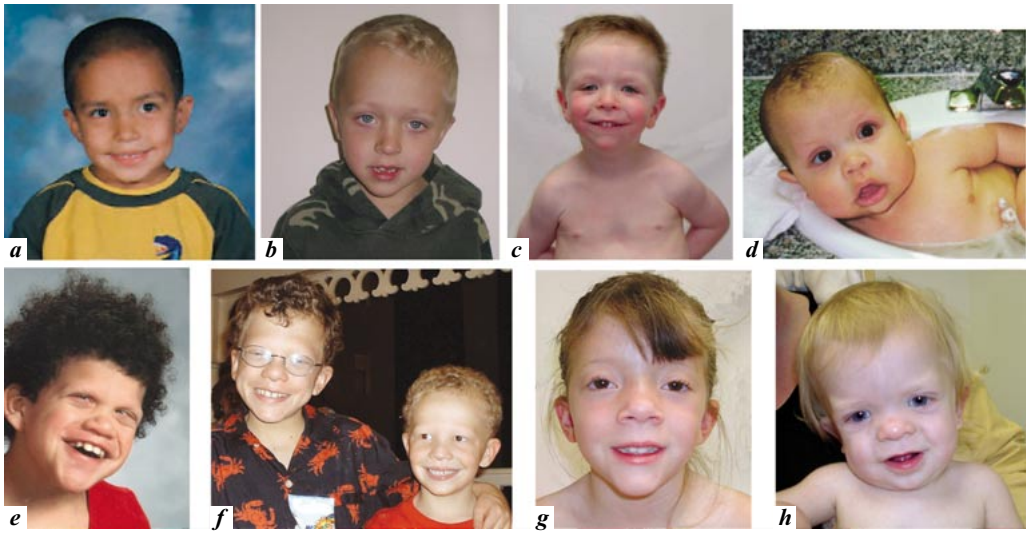


Fig. 2. Photographs of selected individuals with syndromes of the Ras pathway. ***a*** Individual with neurofibromatosis type1 (*NF1* intragenic mutation). ***b*** Individual with clinical diagnosis of NF1. ***c*** Individual with NF1 (*NF1* whole-gene deletion). ***d*** Individual with Costello syndrome (*HRAS* mutation). ***e*** Individual with cardio-facio-cutaneous (CFC) syndrome (*BRAF* mutation). ***f*** Two unrelated individuals with CFC syndrome (*MEK2* mutations). ***g*** Individual with clinical diagnosis of Noonan syndrome. ***h*** Individual with Noonan syndrome (*PTPN11* mutation).

subsequently transforming *RAS* genes were discovered in bladder carcinoma cell lines, the first human oncogenes [16]. The distinct tumor spectrum associated with each *RAS* gene [17] is consistent with the proteins having functional specificity conferred by some combination of their expression patterns and their cellular microlocalizations. In the germline, activating mutations in the *RAS* genes and genes encoding components of Ras pathways, both upstream and downstream, result in various clinical syndromes that have overlapping phenotypes with NF1. Several of these syndromes feature an increased risk of malignancy.

Although NF1 is a distinct condition based on phenotype, several other syndromes have overlapping clinical features. These include Noonan syndrome, NF-Noonan syndrome (NFNS), LEOPARD syndrome, Watson syndrome, cardio-facio-cutaneous (CFC) syndrome, and Costello syndrome (fig. 2). Given the overlapping phenotypes, longstanding controversy has existed in the delineation of these syndromes, and whether or not they are distinct syndromes or simply variable expressivity of the same condition. However, the overlapping phenotypes in each of these syndromes provided clues for the identification of

disease-causing genes encoding proteins within the Ras pathway, emphasizing the importance of Ras in human embryology, in addition to cell proliferation.

Watson Syndrome

Watson syndrome was first described in 1967 [18], in which 3 families were reported to have variable expression of pulmonary stenosis, café-au-lait macules, freckling and low normal or dull intelligence. Other individuals with a similar phenotype were subsequently reported [19]. Neurofibromas were not originally reported in the families described by Watson [18], but further evaluation by Allanson et al. revealed Lisch nodules and neurofibromas in some of the individuals [20]. Given the overlap of these manifestations with NF1, examination of the *NF1* gene in individuals with Watson syndrome was logical. Different mutations in the *NF1* gene (an 80-kb deletion, and an in-frame tandem duplication of 42 bases in exon 28) were subsequently reported in individuals with Watson syndrome [21, 22]. Thus, Watson syndrome falls within the spectrum of variable expressivity of NF1 or, if truly a discrete entity, is allelic to NF1.

LEOPARD Syndrome

LEOPARD syndrome is characterized by multiple lentigines, electrocardiographic-conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. The majority of individuals with LEOPARD syndrome have *PTPN11* mutations, allelic to Noonan syndrome [23–26]. Interestingly, the *PTPN11* mutations observed in LEOPARD syndrome have a dominant negative effect, unlike Noonan syndrome. There has been one case, however, of a de novo missense mutation in exon 18 of the *NF1* gene in a patient reported to have features of LEOPARD syndrome [27], suggesting the possibility that LEOPARD syndrome could be genetically heterogeneous and allelic to NF1.

Noonan Syndrome

Noonan syndrome is an autosomal dominant syndrome with the clinical findings of short stature, pectus deformities, congenital heart defects (most commonly pulmonic stenosis and hypertrophic cardiomyopathy), learning disorders, and a characteristic facial gestalt (ptosis, posteriorly rotated ears, and hypertelorism) [28–30]. NF1 and Noonan syndrome have overlapping clinical

findings and one study reported that approximately 10% of individuals with NF1 had some evidence of Noonan syndrome [31]. In 2001, missense mutations in the *PTPN11* gene were found in approximately 50% of cases with Noonan syndrome [32]. Given the clinical overlap of NF1 and Noonan syndrome, it is not surprising that mutations in *PTPN11* cause Noonan syndrome as the *PTPN11* encoded protein (SHP-2) relays signals to the Ras proteins including Kras and Hras [reviewed in 33]. Subsequently, heterozygous germline mutations in *KRAS* and *SOS1* were found in a small number of individuals with Noonan syndrome [34–37].

The non-receptor-type protein tyrosine phosphatase SHP-2 is composed of 2 amino-terminal Src-homology 2 (SH2) domains (N-SH2 and C-SH2) arranged in tandem, a phosphotyrosine phosphatase domain, and a carboxy-terminal tail [30, 32, 38]. The *PTPN11* mutations in Noonan syndrome affect the amino N-SH2 and phosphotyrosine phosphatase domains, resulting in a gain of function with increased SHP-2 activity [38] and hyperactivation of Erk [39].

Although Noonan syndrome has phenotypic overlap with NF1 and NFNS, the phenotypic overlap of Noonan syndrome with CFC and Costello syndrome has been more commonly discussed [33], which has subsequently led to a better understanding of the molecular etiology of these overlapping syndromes.

NF-Noonan Syndrome

NF-Noonan syndrome (NFNS) is probably the most debated of the herein described syndromes in terms of its definition as a distinct syndrome. NF1 has strict clinical diagnostic criteria, but the phenotypic findings for Noonan syndrome are more subjective. Potential explanations for NFNS have included variable expressivity of NF1 or Noonan syndrome, co-occurrence of two distinct conditions, allelic heterogeneity, or a separate genetically distinct disorder [40, 41].

One group identified mutations in the *NF1* gene with lack of mutations in *PTPN11* in 16/17 individuals described as having NFNS [40]. Another study evaluated 7 individuals with variable phenotypes of the NFNS spectrum, and all had mutations of *NF1* without *PTPN11* mutations [42]. Stevenson et al. described a family with the pigmentary findings of NF1, in which all affected family members had variable phenotypic findings of Noonan syndrome [41]. These individuals had a 3-bp in-frame deletion of the *NF1* gene (c.2970_2972 delAAT). Subsequently, other individuals with the same mutation were described, in which there was an absence of neurofibromas providing evidence of a clinically significant NF1 genotype-phenotype correlation, although a Noonan phenotype was not frequently reported in other individuals [43].

Many NF1 individuals have clinical features similar to Noonan syndrome (i.e. pectus abnormalities, pulmonic stenosis, similar facial gestalt), and it is likely that the NFNS phenotype represents variable expressivity of NF1. Colley et al. [31] examined 94 individuals with NF1 and reported that 9.5% had evidence of Noonan syndrome. Although NF1 is classically described as having intrafamilial variable expressivity, familial clustering of Noonan syndrome features in individuals with NF1 has been reported [31, 41, 44], which suggests that NFNS is a discrete entity, although individually the array of clinical findings are still part of the phenotypic spectrum of NF1. *NF1* genotype-phenotype relations may exist for the Noonan features within the context of NF1, but the NFNS phenotype may also be the result of modifier genes.

It is clear that the Noonan features observed in NF1 are within its broad variable phenotype, which is now better understood given the converging pathways of the downstream targets of the *NF1* and *PTPN11* protein products. Still, the discussion of NFNS vs. NF1 has potential clinical utility. The presence of familial clustering of the NFNS phenotype suggests that echocardiography would be warranted in offspring of individuals with NFNS compared to those with NF1. In addition, future studies may show that individuals with a Noonan facial gestalt in the context of NF1 are more likely to present with pulmonary stenosis, necessitating early echocardiographic evaluation and potential surgical intervention.

Costello Syndrome

The clinical features associated with Costello syndrome include mental retardation, papillomata, cardiac defects including hypertrophic cardiomyopathy and valvar pulmonic stenosis, curly and sparse hair, characteristic coarse facial features with full cheeks and lips, ulnar deviation of the hands, and thick skin with deep palmar creases [45]. The facial features of Costello syndrome are similar to the facial features observed in Noonan syndrome and cardio-facio-cutaneous (CFC) syndrome. Due to the phenotypic overlap with Noonan syndrome, downstream targets of *PTPN11* were potential candidate genes for Costello syndrome. Aoki et al. originally identified *HRAS* mutations in individuals with Costello syndrome, and subsequently 86–100% of individuals with Costello syndrome have been shown to have constitutional *HRAS* activating mutations [46–49]. It is not surprising that solid tumors such as rhabdomyosarcomas, bladder carcinomas, and neuroblastomas are associated with Costello syndrome (approximately 15%), as similar *HRAS* mutations are observed somatically in neoplasms of other individuals [46, 50]. The p.Gly12Ser amino acid substitution is the most frequent mutation in Costello syndrome and has been observed in a low frequency in human tumors, while the p.Gly12Val

mutant is more common in somatic tumors [49]. The occurrence of neoplasms is in contrast to CFC syndrome where malignant tumors are not common.

Cardio-Facio-Cutaneous (CFC) Syndrome

CFC syndrome and Costello syndrome can be difficult to distinguish clinically. Individuals with CFC syndrome can have sparse and wooly hair with sparse or absent eyebrows, pulmonic stenosis, cardiac rhythm abnormalities, characteristic facial features (hypertelorism, down-slanting palpebral fissures, ptosis, short nose, posteriorly rotated ears, and a broad face), keratosis pilaris, hyperkeratosis of the limbs and face, feeding problems, and mental retardation. Germline activating mutations in *BRAF*, *MEK1* and *MEK2* have been found in individuals with CFC syndrome [52]. The study by Rodriguez-Viciana et al. reported that 78% of patients with CFC syndrome in their cohort had *BRAF* mutations [52]. Interestingly, oncogenic somatic mutations in *BRAF* (differing from those in CFC syndrome) are found in ~7% of human cancers [53].

Germline mutations in *KRAS* have also been found in patients with CFC syndrome [35, 50, 51], which have also been observed in Noonan syndrome [35, 50]. Zenker et al. suggested that gain-of-function mechanisms with *KRAS* mutations lead to accumulation of mutant Ras proteins in the active GTP-bound state [50].

Capillary Malformation Syndromes

Capillary malformation-arteriovenous malformation (CMAVM) lies within the spectrum of vascular and vascular/overgrowth syndromes. CMAVM and Parkes Weber syndromes are often described synonymously, and are similar to Klippel-Trenauney syndrome. The primary differentiating features of Parkes Weber syndrome from Klippel-Trenauney syndrome consist of fast flow arterial malformations with a rarity of lymphatic malformations [54]. CMAVM is the result of inactivating *RASA1* mutations [55]. Vasculopathies are associated with NF1 including cerebrovascular abnormalities, renal artery dysplasia, and aortic coarctation [56, 57], but their frequency is not high [58, 59]. Of note, other common features of NF1 are not observed in CMAVM. This is surprising given that *RASA1* encodes the p120-RasGAP protein, a smaller peptide than neurofibromin, that also down-regulates the Ras-MAPK signaling transduction pathway [54, 55, 60] via activation of the intrinsic GTPase of Ras.

Along this spectrum of vascular manifestations, loss-of-function mutations in *CCM1*, encoding Krit1 (Krev interaction trapped-1 protein), have been

reported in individuals with type 1 cerebral capillary malformations [54, 61, 62]. Krit1 interacts with Rap1a, which is a member of the Ras family of GTPases, and is an antagonist of Ras transformation [54, 61, 63]. Cerebrovascular abnormalities are associated with NF1, although not frequent. One study reported that 2.5% of a cohort of 316 children with NF1 who underwent a brain MRI had an abnormality of the cerebrovascular system, which included narrowed or ectatic vessels, vascular stenoses, aneurysms and moyamoya [59]. The impact of abnormal angiogenesis in individuals with NF1 may help to explain some of the clinical findings of NF1. For example, there is evidence of narrowing of the periosteal vessels around the pseudarthrosis site in individuals with NF1 who have tibial pseudarthrosis [64, 65]. Arterial malformations, particularly cerebral vascular malformations, are possibly underappreciated in the NF1 population [66]. However, somatic events may be required for the development of vascular abnormalities in NF1, explaining the low frequency of the vascular defects.

Syndromes of the PI3K/Akt Pathway

The PI3K/Akt pathway functions in the regulation of cellular growth, proliferation, survival and metabolism [67, 68]. At the apex of the pathway (see fig. 1), PI3K can be activated in response to growth factors both through Ras-dependent and (or) Ras-independent mechanisms. Ras can bind and activate the catalytic subunit of PI3K, which then phosphorylates the lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), to produce the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3) [67, 68]. PIP_3 promotes activation of the Akt serine/threonine kinase, which phosphorylates substrates that include tuberin, the product of the *TSC2* gene. Unphosphorylated tuberin forms a complex with hamartin (product of the *TSC1* gene), that functions as a GAP for the Ras-family GTPase, Rheb. The inactivation of the tuberin/hamartin complex resulting from phosphorylation by Akt allows Rheb-GTP to accumulate, and this activates the mTOR serine/threonine kinase. The best known substrates of mTOR, S6K (p70 ribosomal protein S6 kinase) and 4EBP1 (eIF4E binding protein), function in the regulation of intracellular protein translation. Activation of mTOR is associated with cell proliferation. Pten, a lipid phosphatase, dephosphorylates PIP_3 to downregulate the pathway.

Several genes within the PI3K/Akt pathway are mutated in syndromes that have clinical overlap with NF1, and they include a predisposition to hamartomas, lipomas, pigmentary anomalies, and malignancy [67, 69]. These syndromes include tuberous sclerosis complex (resulting from germline mutations in *TSC1* and *TSC2*) and a set of overlapping conditions collectively known as

the PTEN hamartoma tumor syndrome (PHTS) that result from germline mutations in the *PTEN* gene. PHTS associated conditions include Bannayan-Riley-Ruvalcaba syndrome, Cowden syndrome, and Proteus syndrome.

Based on where neurofibromin acts in the Ras pathway, the phenotype of NF1 likely expresses contributions from multiple Ras effector pathways. The overlap of neurocutaneous tumors in the PI3K/Akt pathway syndromes with those in NF1 suggests a potential role for the PI3K/Akt pathway in the phenotypic features of NF1. Yet, there is little other overlap of clinical features except for macrocephaly, and the types of neurocutaneous tumors differ in NF1 compared to those in PHTS. This is surprising, given the convergence of Ras pathway readout into the PI3K/mTOR pathway, and the observation that mutation of the *NF1* gene has been demonstrated to result in dysregulation of the PI3K/Akt pathway [70]. One example of the involvement of the mTOR pathway in NF1 is the observation of hyperactivation of the mTOR pathway in tissue from optic gliomas of *Nf1* mutant mice and in pilocytic astrocytomas in humans with NF1 [71]. There is also evidence that Erk regulates mTOR [72], and therefore investigation of other pathways besides the PI3K/Akt pathway may be important when selecting therapeutic targets based on overlapping phenotypes.

Conclusion

The phenotypic overlap between the herein described syndromes highlights the potential for modifier genes in variable clinical expression of NF1. This is further complicated by the associated age-related manifestations and the evolution of the phenotype over time. In some instances, the incorporation of genotype may be an important variable to integrate into the diagnostic evaluation and discussion of anticipatory guidance with families of individuals with an atypical clinical presentation of the spectrum of findings seen in NF1. Presently, management decisions rarely depend on the *NF1* gene mutation. In the future, genotype-phenotype relations could become clearer and may eventually help in clinical management. Still, individuals with similar genotypes (such as individuals with CFC, Noonan, and Costello syndrome who have *KRAS* mutations) have a broad spectrum of clinical findings [50], giving credence to other modifiers that may influence the phenotype.

Medical therapies are still less than optimal for many of the complications associated with NF1 (i.e. malignant peripheral nerve sheath tumors, tibial pseudarthrosis, plexiform neurofibromas, dystrophic scoliosis). A better understanding of the Ras-MAPK pathway and its interaction with other converging signal transduction pathways will provide potential targets for therapeutic interventions for specific manifestations of NF1. Therapeutic agents successful in

treating complications of other syndromes within the Ras pathway will be natural candidates for therapy of some medical complications of NF1.

Acknowledgements

We thank the patients and their families, CFC International, the Costello Syndrome Family Network, Rick Guidotti, Positive Exposure, Heather Hanson, and Pilar Magoulas for their help in obtaining and providing photographs. Dr. Stevenson and Dr. Viskochil are supported by research grants #1 K23 NS052500 and #R01 NS050509 from the National Institute of Neurological Disorders and Stroke. We thank the Clinical Genetics Research Program, the Children's Health Research Center, and the Clinical Research Center at the University of Utah through a Public Health Services research grant #M01-RR00064 from the National Center for Research Resources for support in phenotype analysis.

References

1 Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, et al: The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. JAMA 1997;278:51–57.
2 Neurofibromatosis. Conference statement. National Institutes of Health Consensus Development Conference. Arch Neurol 1988;45:575–578.
3 Viskochil D, White R, Cawthon R: The neurofibromatosis type 1 gene. Ann Rev Neurosci 1993;16:183–205.
4 Bar-Sagi D: A Ras by any other name. Mol Cell Biol 2001;21:1441–1443.
5 Hingorani SR, Tuveson DA: Ras redux: rethinking how and where Ras acts. Curr Opin Genet Dev 2003;13:6–13.
6 Wennerberg K, Rossman KL, Der CJ: The Ras superfamily at a glance. J Cell Sci 2005;118:843–846.
7 Rocks O, Peyker A, Kahms M, Verveer PJ, Koerner C, et al: An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. Science 2005;307:1746–1752.
8 Hancock JF: Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 2003;4:373–384.
9 Leon J, Guerrero I, Pellicer A: Differential expression of the ras gene family in mice. Mol Cell Biol 1987;7:1535–1540.
10 Plowman SJ, Williamson DJ, O'Sullivan MJ, Doig J, Ritchie AM, et al: While K-ras is essential for mouse development, expression of the K-ras 4A splice variant is dispensable. Mol Cell Biol 2003;23:9245–9250.
11 Esteban LM, Vicario-Abejon C, Fernandez-Salguero P, Fernandez-Medarde A, Swaminathan N, et al: Targeted genomic disruption of H-ras and N-ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development. Mol Cell Biol 2001;21:1444–1452.
12 Umanoff H, Edelmann W, Pellicer A, Kucherlapati R: The murine N-ras gene is not essential for growth and development. Proc Natl Acad Sci USA 1995;92:1709–1713.
13 Johnson L, Greenbaum D, Cichowski K, Mercer K, Murphy E, et al: K-ras is an essential gene in the mouse with partial functional overlap with N-ras. Genes Dev 1997;11:2468–2481.
14 Johnson GL, Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002;298:1911–1912.
15 Schaeffer HJ, Weber MJ: Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 1999;19:2435–2444.

16 Malumbres M, Barbacid M: RAS oncogenes: the first 30 years. Nat Rev Cancer 2003;3:459–465.
17 Forbes S, Clements J, Dawson E, Bamford S, Webb T, et al: Cosmic 2005. Br J Cancer 2006;94: 318–322.
18 Watson GH: Pulmonary stenosis, café-au-lait spots, and dull intelligence. Arch Dis Child 1967;42:303–307.
19 Partington MW, Burggraf GW, Fay JE, Frontini E: Pulmonary stenosis, café au lait spots and dull intelligence: the Watson syndrome revisited. Proc Greenwood Genet Center 1985;4:105 (abstract).
20 Allanson JE, Upadhyaya M, Watson GH, Partington M, MacKenzie A, et al: Watson syndrome: is it a subtype of type 1 neurofibromatosis? J Med Genet 1991;28:752–756.
21 Upadhyaya M, Shen M, Cherryson A, Farnham J, Maynard J, Huson SM, Harper PS: Analysis of mutations at the neurofibromatosis 1 (*NF1*) locus. Hum Mol Genet 1992;1:735–740.
22 Tassabehji M, Strachan T, Sharland M, Colley A, Donnai D, Harris R, Thakker N: Tandem duplication within a neurofibromatosis type I (*NF1*) gene exon in a family with features of Watson syndrome and Noonan syndrome. Am J Hum Genet 1993;53:90–95.
23 Digilio MC, Conti E, Sarkozy A, Mingarelli R, Dottorini T, et al: Grouping of multiple-lentigines/LEOPARD and Noonan syndromes on the *PTPN11* gene. Am J Hum Genet 2002;71: 389–394.
24 Legius E, Schrander-Stumpel C, Schollen E, Pulles-Heintzberger C, Gewillig M, Fryns JP: *PTPN11* mutations in LEOPARD syndrome. J Med Genet 2002;39:571–574.
25 Nyström AM, Annerén G, Bondeson ML: Noonan syndrome with café-au-lait spots and LEOPARD syndrome: evidence for genetic heterogeneity. Am J Hum Genet 2003;73(suppl):725.
26 Sarkozy A, Conti E, Digilio MC, Marino B, Morini E, et al: Clinical and molecular analysis of 30 patients with multiple lentigines LEOPARD syndrome. J Med Genet 2004;41:e68.
27 Wu R, Legius E, Robberecht W, Dumoulin M, Cassiman JJ, Fryns JP: Neurofibromatosis type 1 gene mutation in a patient with features of LEOPARD syndrome. Hum Mut 1996;8:51–56.
28 Noonan JA: Hypertelorism with Turner phenotype: A new syndrome with associated congenital heart disease. Am J Dis Child 1968;116:373–380.
29 Allanson JE: Noonan syndrome; in Cassidy SB, Allanson JE (eds): Management of Genetic Syndromes. Wiley, New York, 2001, pp 253–268.
30 Allanson J: The first Noonan syndrome gene: *PTPN11*, which encodes the protein tyrosine phosphatase SHP-2. Pediatr Res 2002;52:471.
31 Colley A, Donnai D, Evans DGR: Neurofibromatosis/Noonan phenotype: a variable feature of type 1 neurofibromatosis. Clin Genet 1996;49:59–64.
32 Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, et al: Mutations in *PTPN11*, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet 2001;29: 465–468.
33 Lopez-Rangel E: Overlapping clinical phenotypes: the road to identifying dysmorphology signaling pathways and their associated risks. Clin Genet 2007;71:43–44.
34 Carta C, Pantaleoni F, Bocchinfuso G, Stella L, Vasta I, et al: Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. Am J Hum Genet 2006;79:129–135.
35 Schubbert S, Zenker M, Rowe SL, Boll S, Klein C, et al: Germline *KRAS* mutations cause Noonan syndrome. Nat Genet 2006;38:331–336.
36 Roberts AE, Araki T, Swanson KD, Montgomery KT, Schiripo TA, et al: Germline gain-of-function mutations in *SOS1* cause Noonan syndrome. Nat Genet 2007;39:70–74.
37 Tartaglia M, Pennacchio LA, Zhao C, Yadav KK, Fodale V, et al: Gain-of-function *SOS1* mutations cause a distinctive form of Noonan syndrome. Nat Genet 2007;39:75–79.
38 Tartaglia M, Kalidas K, Shaw A, Song X, Musat DL, et al: *PTPN11* mutations in Noonan syndrome: Molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. Am J Hum Genet 2002;70:1555–1563.
39 Mohi MG, Williams IR, Dearolf CR, Chan G, Kutok JL, et al: Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by *Shp2* (*PTPN11*) mutations. Cancer Cell 2005;7:179–191.

40 De Luca A, Bottillo I, Sarkozy A, Carta C, Neri C, et al: *NF1* gene mutations represent the major molecular event underlying neurofibromatosis-Noonan syndrome. Am J Hum Genet 2005;77: 1092–1101.
41 Stevenson DA, Viskochil DH, Rope AF, Carey JC: Clinical and molecular aspects of an informative family with neurofibromatosis type 1 and Noonan phenotype. Clin Genet 2006;69:246–253.
42 Hüffmeier U, Zenker M, Hoyer J, Fahsold R, Rauch A: A variable combination of features of Noonan syndrome and neurofibromatosis type 1 are caused by mutations in the *NF1* gene. Am J Med Genet 2006;140A:2749–2756.
43 Upadhyaya M, Huson SM, Davies M, Thomas N, Chuzhanova N, et al: An absence of cutaneous neurofibroma associated with a 3-bp in-frame deletion in exon 17 of the *NF1* gene (c.2970_2972 delAAT): Evidence of a clinically significant NF1 genotype-phenotype correlation. Am J Hum Genet 2007;80:140–151.
44 Abuelo DN, Meryash DL: Neurofibromatosis with fully expressed Noonan syndrome. Am J Med Genet 1988;29:937–941.
45 Johnson JP, Golabi M, Norton ME, Rosenblatt RM, Feldman GM, et al: Costello syndrome: phenotype, natural history, differential diagnosis, and possible cause. J Pediatr 1998;133:441–448.
46 Aoki Y, Niihori T, Kawame H, Kurosawa K, Ohashi H, et al: Germline mutations in *HRAS* proto-oncogene cause Costello syndrome. Nat Genet 2005;37:1038–1040.
47 Estep AL, Tidyman WE, Teitell MA, Cotter PD, Rauen KA: *HRAS* mutations in Costello syndrome: detection of constitutional activating mutations in codon 12 and 13 and loss of wild-type allele in malignancy. Am J Med Genet A 2006;140:8–16.
48 Kerr B, Delrue MA, Sigaudy S, Perveen R, Marche M, et al: Genotype-phenotype correlation in Costello syndrome: *HRAS* mutation analysis in 43 cases. J Med Genet 2006;43:410–405.
49 Zampino G, Pantaleoni F, Carta C, Cobellis G, Vasta I, et al: Diversity, parental germline origin, and phenotypic spectrum of de novo *HRAS* missense changes in Costello syndrome. Hum Mutat 2006;28:265–272.
50 Zenker M, Lehmann K, Schulz AL, Barth H, Hansmann D, et al: Expansion of the genotypic and phenotypic spectrum in patients with *KRAS* germline mutations. J Med Genet 2007;44:131–135.
51 Niihori T, Aoki Y, Narumi Y, Neri G, Cave H, et al: Germline *KRAS* and *BRAF* mutations in cardio-facio-cutaneous syndrome. Nat Genet 2006;38:294–296.
52 Rodriguez-Viciana P, Tetsu O, Tidyman WE, Estep AL, Conger BA, et al: Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. Science 2006;311:1287–1290.
53 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, et al: Mutations of the *BRAF* gene in human cancer. Nature 2002;417:949–954.
54 Timur AA, Driscoll DJ, Wang Q: Biomedicine and disease: the Klippel-Trenaunay syndrome, vascular anomalies and vascular morphogenesis. Cell Mol Life Sci 2005;62:1434–1447.
55 Eerola I, Boon LM, Mulliken JB, Burrows PE, Dompmartin A, et al: Capillary malformation-arteriovenous malformation, a new clinical genetic disorder caused by *RASA1* mutations. Am J Hum Genet 2003;73:1240–2149.
56 Sobata E, Ohkuma H, Suzuki S: Cerebrovascular disorders associated with von Recklinghausen's neurofibromatosis: a case report. Neurosurgery 1988;22:544–549.
57 Friedman JM, Arbiser J, Epstein JA, Gutmann DH, Huot SJ, et al: Cardiovascular disease in neurofibromatosis 1: report of the NF1 Cardiovascular Task Force. Genet Med 2002;4:105–110.
58 Finley JL, Dabbs DJ: Renal vascular smooth muscle proliferation in neurofibromatosis. Hum Pathol 1988;19:107–110.
59 Rosser TL, Vezina G, Packer RJ: Cerebrovascular abnormalities in a population of children with neurofibromatosis type 1. Neurology 2005;64:553–555.
60 Gibbs JB, Marshall MS, Scolnick EM, Dixon RA, Vogel US: Modulation of guanine nucleotides bound to Ras in NIH3T3 cells by oncogenes, growth factors and the GTPase activating protein (GAP). J Biol Chem 1990;265:20347–20442.
61 Laberge-le Couteulx S, Jung HH, Labauge P, Houtteville JP, Lescoat C, et al: Truncating mutations in *CCM1* encoding KRIT1, cause hereditary cavernous angiomas. Nat Genet 1999;23:198–193.
62 Sahoo T, Johnson EW, Thomas JW, Kuehl PM, Jones TL, et al: Mutations in the gene encoding KRIT1, a Krev-1/rap 1a binding protein, cause cerebral cavernous malformations (CCM1). Hum Mol Genet 1999;8:2324–2333.

63 Zawistowski JS, Serebriiskii IG, Lee MF, Golemis EA, Marchuk DA: KRIT1 association with the integrin-binding protein ICAP-1:a new direction in the elucidation of cerebral cavernous malformations (CCM1) pathogenesis. Hum Mol Genet 2002;11:389–396.
64 Hermanns-Sachweh B, Senderek J, Alfer J, Klosterhalfen B, Büttner R, Füzesi L, Weber M: Vascular changes in the periosteum of congenital pseudarthrosis of the tibia. Pathol Res Pract 2005;201:305–312.
65 Kuorilehto T, Kinnunen P, Nissinen M, Alanne M, Leskela HV, Lehenkari P, Peltonen J: Vasculopathy in two cases of NF1-related congenital pseudarthrosis. Pathol Res Pract 2006;202:687–690.
66 Lin AE, Birch PH, Korf BR, Tenconi R, Niimura M, et al: Cardiovascular malformations and other cardiovascular abnormalities in neurofibromatosis 1. Am J Med Genet 2000;95:108–117.
67 Inoki K, Corradetti MN, Guan KL: Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 2005;37:19–24.
68 Wullschleger S, Loewith R, Hall MN: TOR signaling in growth and metabolism. Cell 2006;124:471–484.
69 Pilarski R, Eng C: Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome. J Med Genet 2004;41:323–326.
70 Johannessen CM, Reczek EE, James MF, Brems H, Legius E, Cichowski K: The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc Natl Acad Sci USA 2005;102:8573–8578.
71 Dasgupta B, Yi Y, Chen DY, Weber JD, Gutmann DH: Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumors. Cancer Res 2005;65:2755–2760.
72 Ma L, Chen Z, Erdjument-Bromage H, Tempst P, Pandolfi PP: Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 2005;121:179–193.

David A. Stevenson, MD
University of Utah Health Sciences Center
Department of Pediatrics, Division of Medical Genetics, 2C412 SOM
Salt Lake City, Utah 84132 (USA)
Tel. +1 801 581 8943, Fax +1 801 585 7252, E-Mail david.stevenson@hsc.utah.edu

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 46–62

NF1 Gene Structure and NF1 Genotype/Phenotype Correlations

M. Upadhyaya

Institute of Medical Genetics, School of Medicine, Cardiff University, Heath Park, Cardiff, UK

Abstract

Neurofibromatosis type 1 (NF1, MIM 162200) is a complex disorder that affects many cell types and involves multiple body systems. This inherited disorder is due to germline mutations of the *NF1* gene; this 17q11.2-located gene spans 280 kb of genomic DNA, and has 61 exons, of which 4 are alternatively spliced, that code for an ~9-kb open reading frame. A large 60-kb intron of the *NF1* gene contains three unrelated genes, *EVI2A*, *EVI2B* and *OMG*, that are transcribed from the opposite strand. A number of highly homologous *NF1* pseudogene-like sequences are present on several chromosomes. Neurofibromin, the *NF1* gene product, is a large (2818 amino acids) ubiquitously expressed protein that is present at low concentration in most tissues, with brain showing the highest protein concentrations. Neurofibromin is a recognised tumour suppressor gene relating to its main functional role in inactivating cellular Ras protein, although several additional roles for the protein are now recognised.

Establishing a close correlation between specific disease gene mutations and an associated clinical phenotype in affected patients can be a challenging task in molecular clinical genetics. For NF1, only two such genotype-phenotype correlations have been reported for the *NF1* gene: the first relates to patients with large germline gene deletions who often present with large numbers of neurofibromas for their age, dysmorphic features and learning disabilities, and may have a higher risk of developing malignancy; the second association involves a small number of NF1 patients whose germline *NF1* mutation is a specific 3-bp deletion in exon 17 of the gene and who show a complete absence of neurofibromas, including dermal, subcutaneous and superficial plexiform neurofibromas, and often present with a milder form of the disease, with a significant reduction of many NF1 features.

Neurofibromatosis type 1 (NF1) [MIM 162200] is a common autosomal dominant disorder, with a prevalence of 1 in 4,000, that exhibits complete

penetrance of the mutant gene by age 5 [1]. This is a complex disorder that affects many cell types and multiple body systems.

The *NF1* Gene

In most NF1 patients, their inherited disorder results from an inactivating germline mutation of the 17q11.2-located *NF1* gene. This large gene spans 280 kb of genomic DNA, contains 61 exons, and encodes a 12-kb mRNA transcript, with an ~9 kb open reading frame (fig. 1) [2].

Genes within Intron 27b

The *NF1* gene has two large (>60 kb) introns, 1 and 27b, of which intron 27b contains three small unrelated genes, *EVI2A*, *EVI2B* and *OMG*, each of which has two exons. *EVI2A* encodes a 232 amino acid polypeptide that is expressed in the brain and bone marrow. The *OMG* gene codes for a 416 amino acid cell adhesion protein expressed primarily in oligodendrocytes. Each of these genes is transcribed in the reverse orientation to the *NF1* gene (fig. 1). The possible role of these three genes, whether individually or together, in regulating *NF1* gene expression is at present unknown.

Alternative Splicing of the NF1 Gene

The *NF1* gene has four alternatively spliced exons, 9a, 10a-2, 23a and 48a, and in each case the inclusion of any one of these exons does not disrupt the overall reading frame. A number of different *NF1* transcripts have been found to be differentially expressed in various tissues in normal individuals. For example, the ubiquitously expressed type II *NF1* transcript includes the alternatively spliced exon 23a, that results in a 63-bp in-frame insertion in the GAP-related domain (GRD) of neurofibromin [3], and this transcript is conserved in several species [4]. The resultant type II neurofibromin, with an additional 21 amino acids, exhibits a significantly reduced GAP activity but conversely demonstrates an increased affinity for Ras in comparison to type I neurofibromin that lacks exon 23a. Equivalent levels of type I and type II *NF1* transcripts are expressed in many normal tissues. Interestingly, a mouse NF1 model constructed to produce only type I neurofibromin (−23a) was found to exhibit learning disabilities [5].

Another alternatively spliced transcript that includes exon 48a, resulting in a 54-bp in-frame insertion, is abundantly expressed in muscle [6], while another transcript containing exon 9a, that inserts an additional 30 bp, is highly expressed in the central nervous system [7]. The alternatively spliced product containing exon 10a-2, and adding 45 bp to the transcript, was reported to be

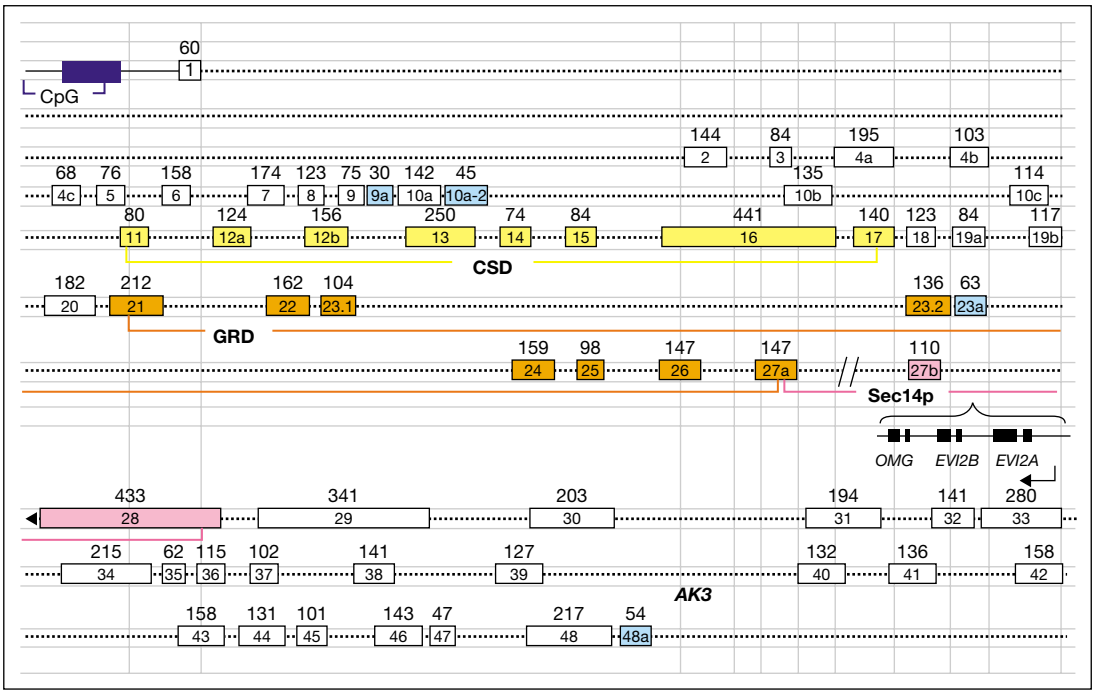


Fig. 1. Structure of the *NF1* gene. Each of the 61 exons is shown (rectangles) and numbered according to accepted *NF1* gene nomenclature, and its size (in base pairs) indicated. The four alternatively spliced exons (9a, 10a-2, 23a and 48a) are identified (blue boxes). The intervening introns are shown by dotted lines and each is drawn to scale, with the exception of introns 1 and 27b, both of which are >60 kb in length. The core promoter of the gene (−341 bp to −261 bp) is shown (dark blue box), and 'CpG' denotes the CpG island of the gene (−731 to +261 bp; [79]). Exons that encode for three of the recognised domains in neurofibromin are indicated: 'CSD', is the cysteine/serine-rich domain [80] (yellow boxes); 'GRD', is the GTPase activating protein (GAP) related domain (orange boxes); and 'Sec14p', indicates the gene region that has homology to a lipid-binding domain of the *Saccharomyces cerevisiae* phosphatidylinositol transfer protein Sec 14p [81] (pink boxes). The three embedded genes in intron 27b (*OMG*, *EVI2A*, and *EVI2B*) are indicated (black boxes), an arrow indicates the opposite direction of transcription, and 'AK3', denotes an adenylate kinase 3 pseudogene located in intron 39.

expressed at a much lower level than was the normal type I RNA in all human primary and tumour cells examined [8]. A large number of different sized *NF1* transcripts have been described, many of which involve exon-specific deletions (exons 4b, 29, 30, 33, 37, 43 and 45), as well as sequence insertions (31-bp

from intron 4a) [9–12]. Indeed, it has been suggested that quantitative differences between these different transcripts in patients may contribute to the clinical phenotypic variability often seen in patients [12].

NF1 Gene Promoter Region

The *NF1* gene promoter is located within a CpG-island-containing genomic region that exhibits a high degree of sequence conservation with *NF1* genes found in many other organisms [13]. To date, no pathogenic mutations of the *NF1* promoter have yet been reported [14, 15]. Hypermethylation of the *NF1* promoter regions does not appear to be a common mechanism that inactivates the normal allele in any NF1-related tumours [16, 17].

3′ Untranslated Region (3′ UTR)

The 3′ untranslated region (UTR) of the human *NF1* gene is 3.5 kb in length and it also exhibits a high level of sequence conservation, indicating its possible functional importance either for regulating mRNA stability, or for controlling translational efficiency of the gene.

NF1 Pseudogenes

Fluorescence in situ hybridisation (FISH) analysis, using a labelled *NF1* cDNA, was used to identify a number of partial pseudogene sequences located on various human chromosomes (2q12–q13, 12q11, 14p11–q11, 15q11.2, 18p11.2, 21p11–q11 and 22p11–q11 [18–27]), with at least two separate regions of sequence homology on both chromosome 15 and 22 [19]. Many of these pseudogenic sequences display significant homology (>90%) to the 17q11.2-located *NF1* sequences, however they all have various inactivating nucleotide substitutions, insertions, or deletions. All of these pseudogene sequences are thought to be the result of multiple independent partial duplications of the 17q11.2-located *NF1* gene followed by sequential inter-chromosomal transposition events [28].

While sequence evidence indicates that none of these pseudogene sequences are likely to encode a functional protein, it has been suggested that these highly homologous sequences might act as a potential reservoir of *NF1* mutations, and as such are likely to increase the *NF1* mutation rate by inter-chromosomal gene conversion events [29]. However, as very few disease-causing *NF1* mutations appear to have an equivalent in any of the identified pseudogenic sequences, then such inter-chromosomal gene conversion events are likely to make a very limited contribution to the overall *NF1* mutation rate [28].

The use of patient-derived mRNA for *NF1* mutation screening has been suggested as a way to circumvent any potential problems created by these

pseudogenes. The recent demonstration of limited transcription of the *NF1* pseudogenes on chromosome 2, 15 and 21 [30] may however complicate functional *NF1* mutational analysis at the mRNA level and also the molecular diagnosis for NF1.

Neurofibromin: the NF1 Gene Product

Neurofibromin is a large (2818 amino acids) ubiquitously expressed protein that is present at low concentration in many tissues [31] and with its highest levels of expression found in cells of the central nervous system, where it is usually associated with tubulin [32]. Neurofibromin is a recognised tumour suppressor protein related to its main functional role in down-regulating functionally active cellular Ras proteins. The protein is a member of a large family of evolutionarily conserved proteins, the mammalian GTPase activating protein-related proteins (GAP-related proteins) [33]. Neurofibromin functions by greatly promoting the basal intrinsic levels of GTPase activity of the Ras protein itself, this catalyses the conversion of the active GTP-bound form of Ras in the cell to the inactive GDP-bound form of the protein, and this significantly decreases cell proliferation and tissue growth.

Clinical Features

Neurofibromatosis type 1 can involve multiple tissues and its cardinal clinical features are pigmentary changes of the skin, such as multiple café-au-lait (CAL) spots, patches of skin that contain giant pigment macules that may be present in much smaller numbers in normal skin [34] and axillary and inguinal freckling, benign peripheral nerve sheath tumours (neurofibromas), and pigmented Lisch nodules in the iris. The development of many of these features is strongly age dependent, with six or more CAL spots, (>0.5 cm in children) usually present by the 2nd year of life. The numbers of CAL spots often increase during childhood but they may fade with age. Cutaneous neurofibromas usually only develop during adolescence or in early adulthood, and are present in most 20-year-old patients. Neurofibromas are rarely observed prior to 7 years and then often increase in size and number as the patients age [1, 35, 36]. While most dermal neurofibromas are readily visible some are only evident on palpation of the skin. Plexiform neurofibromas (PNF) are the other main type, these are usually much larger and often involve multiple nerves. All neurofibromas develop as outgrowths from cells of the peripheral nerve sheath and are therefore formed mainly from Schwann cells, along with fibroblasts, mast cells, and perineurial cells all contained within an extracellular matrix. Unlike dermal neurofibromas, PNF normally develop in early infancy and then continue to

grow throughout the patients' lifetime. PNF are important as a small number of their constituent Schwann cells have the propensity for transformation into peripheral nerve sheath tumours (MPNST). Computer tomography and magnetic resonance imaging now permit rapid assessment of the presence of internal PNF and MPNST in the NF1 patient population.

Variants of NF1

A number of rare families are increasingly being recognised in which the affected individuals in at least two generations exhibit similar NF1 clinical features that may be linked, or unlinked, to the *NF1* gene [37–41], and such families may represent as much as 15% of the NF1 population [37]. Many of these families often exhibit considerable intra-familial variation, both in the number of major features and in the occurrence of complications. One such type of family are those who present with CAL spots alone, either with or without skinfold freckling [37–39]. Other examples include families with Watson syndrome, characterized by pulmonary stenosis, learning problems, and a paucity of cutaneous neurofibromas [41–45], and families who only have multiple spinal neurofibromas [40, 41]. Families have also been reported with both CAL spots and spinal neurofibromas.

Genotype/Phenotype Correlations

Attempts to try and establish a close association between a specific disease gene mutation and a characteristic clinical phenotype can be a challenging task in clinical genetics. For many inherited diseases the series of events and processes that separate a change in a DNA sequence and the expression of an associated disease feature is usually too complex to permit accurate correlation. In Huntington's disease (HD), for example, CAG repeat >36 results not only in HD but also exhibits a statistical correlation between repeat size and age of onset [46]. Similarly, fibroblast growth factor receptor (*FGFR*) mutations also show marked specific genotype/phenotype correlations [47].

In any attempt to interpret potential genotype/phenotype relationships, the combinatorial effects of possibly many different mutations and polymorphisms, both allelic and non-allelic, need to be considered. There are many examples in which different sequence variants involving the same gene, lead to very different effects on both the structure and function of the mutant protein, often producing considerable inter-individual phenotypic variation, or even resulting in apparently completely different clinical disorders. Perhaps, the best example of

such extreme variability is related to the different mutations that affect the human Lamin A/C gene (*LMNA*), in which at least 12 apparently different inherited disorders, including several muscular dystrophies, two progeria-like aging syndromes, and several variant lipodystrophies are all associated with specific *LMNA* mutations [48, 49]. However, close examination of the spectrum of *LMNA* mutations present in individuals affected with these various disorders has so far failed to find any obvious association between the particular clinical phenotype and either the mutation type, or its location within the gene [48, 49].

The identification of consistent genotype/phenotype correlations in NF1 is still in its infancy, due mainly to the considerable mutational heterogeneity of the *NF1* gene, and also because the size and complexity of the gene makes complete mutation screening both cost- and labour-intensive. The variable nature, location and developmental timing of *NF1* somatic mutations, that possibly determine the rate of progression and the severity of the disease expressed in different tissues may also obscure obvious genotype/phenotype relationships in NF1. It is also possible that the variable expression of different alternative *NF1* transcripts in various tissue types [50], or even abnormal *NF1* RNA editing, may also account for the wide range of clinical features observed [50]. In addition, the strong age-effects on clinical feature development, coupled with the lack of independence of many of these clinical features [51, 52], may also confound such studies. Researchers have tried to quantitatively analyze the familial variation in *NF1* and have found evidence for the possible involvement of modifying loci, and perhaps also the normal *NF1* allele, in the development of particular disease features [53, 54].

Modifying Genes in NF1

A possible role for modifier genes in NF1 disease phenotype expression was first proposed following the observation that monozygotic twins shared certain NF1 features [51, 53, 55]. By contrast, more distant affected relatives often exhibited more variable clinical phenotypes [53]. However, no germline modifying genes have yet been identified. Study of NF1 patients carrying *NF1* microdeletions has however provided evidence for an additional gene or genes that modify neurofibromagenesis. Genes that influence mitotic recombination, and thus the rate of loss of heterozygosity (LOH) as a second hit, also have been proposed as potential modifier genes that may account for the observed variation in neurofibroma number [56]. Wiest et al. [57] suggested that functional variants of genes involved in mismatch repair might also modulate the *NF1* mutation rate in a given patient. A number of alternative mechanisms have also been proposed to explain the clinical variability in NF1, including modifying

genes, allelic heterogeneity, a mutation in the second allele, somatic mosaicism and deletion of contiguous genes. The influences of environmental and stochastic factors have also been proposed to explain the marked inter-individual clinical phenotypic variation [58].

In mouse models of NF1, complex genetic and epigenetic interactions have been shown to control susceptibility to different NF1 tumours [59, 60]. These authors have identified a modifier of malignant peripheral nerve sheath tumours (MPNSTs) linked to mouse chromosome 9 that under variable genetic conditions either increases or decreases the incidence of MPNSTs.

In a previous NF1 study [61], two unrelated NF1 patients with the 4071delC mutation were identified, however, one patient was severely affected, developing CAL spots at 3 months, neurofibromas at 9 months, and a malignant schwannoma at 37 years, the second patient exhibited classical NF1 characterised by CAL spots, subcutaneous neurofibromas, axillary freckling, a large plexiform neurofibroma, a thoracic spinal deformity and mild cerebral atrophy. Similarly, a nonsense mutation in exon 31 has been reported in more than 14 unrelated NF1 patients, all of whom exhibit variable phenotypes [62]. Another more recent study found that NF1 individuals with missense germline mutations had a relatively lower risk of developing Lisch nodules compared to NF1 patients with either nonsense or frameshift mutations [63]. This preliminary finding requires confirmation in much larger NF1 patient populations.

Genotype/Phenotype Studies

To date in NF1, only two established genotype/phenotype correlations have been reported. The first involves NF1 patients whose germline *NF1* mutation is a large genomic deletion, such patients often present with a more severe disease status. They have many more dermal neurofibromas than would be expected for their age, may have dysmorphic features, and often develop learning disabilities [64]. Perhaps more importantly, such deleted NF1 patients may also exhibit an increased risk of developing MPNSTs [65, 66], although this particular genotype/phenotype association has recently been challenged [67]. Some 5% of all NF1 patients have microdeletions, a 1.4-Mb deletion (type I deletion) in about half of the cases and a 1.2-Mb deletion (type II deletion) in about 38% of the remaining deletion patients [68]. It should be noted that many of these deletion mutations are often found in a mosaic state in patients, with seven of eight type II deletions being mosaic deletions in a recent study [68]. As might be expected, none of the patients with mosaic deletions exhibited either facial dysmorphism or mental retardation, and they generally presented with a milder disease phenotype.

The second genotype/phenotype association relates to a recently reported correlation between the presence of a 3-bp deletion of the *NF1* gene and the complete absence of neurofibroma developments [42]. This specific AAT deletion in exon 17 of the *NF1* gene is also associated with a much milder NF1 phenotype in many patients. The striking finding is the complete absence of any cutaneous, subcutaneous, or superficial plexiform neurofibromas in these patients, and given that cutaneous neurofibromas represent a hallmark clinical feature of NF1, then a better understanding of why they fail to develop in almost all such adult patients may help to identify the underlying pathobiological mechanisms involved in NF1.

This 3-bp deletion mutation was originally identified in three NF1 families referred for *NF1* molecular diagnosis in Cardiff (fig. 2), all the affected individuals had the identical 3-bp deletion in exon 17 of the *NF1* gene and none had cutaneous neurofibromas.

In a subsequent international collaborative study [42], 22 unrelated NF1 probands (14 familial and 8 sporadic cases) were identified, all of whom have the same 3-bp deletion (c.2970–2972 del AAT – p.990delM) and none of whom had developed either cutaneous neurofibromas or any clinically-obvious plexiform neurofibromas. This AAT deletion in exon 17 of the *NF1* gene is predicted to remove one of two adjacent methionines (codons 991 and 992) (DMet991), these two methionine residues are located in a highly conserved region of neurofibromin that is likely to have an important functional role. It is the physical three-dimensional configuration and structure of a disease-associated protein, in conjunction with its functional interactions with other proteins, that is either responsible for, or contributes to, the observed variation in the clinical phenotype of a particular disorder. Thus, determination of the consequences of this specific methionine deletion on the overall configuration of the mutant protein could well reveal information on the potential functional role of this region of neurofibromin. The current lack of a suitable soluble polypeptide for this region of the protein however, precludes any immediate study of the effects of the mutation on the structure and configuration of the mutant protein (K. Scheffzek, personal communication).

The RT-PCR-based mutational analysis of mRNA from patients with the AAT deletion does indicate that the mutation is unlikely to directly effect the splicing of the *NF1* transcript.

This AAT-deficient mutation is located in the cysteine/serine rich domain (CSRD) of the protein [69], a domain that contains a number of potential cyclic adenosine monophosphate (cAMP)-dependent protein kinase A binding sites, indicating a possible role in cAMP signaling. No suitable functional assay measuring the overall activity of this CSRD domain is currently available. Indeed, to date the only biochemical test applicable to monitoring of neurofibromin

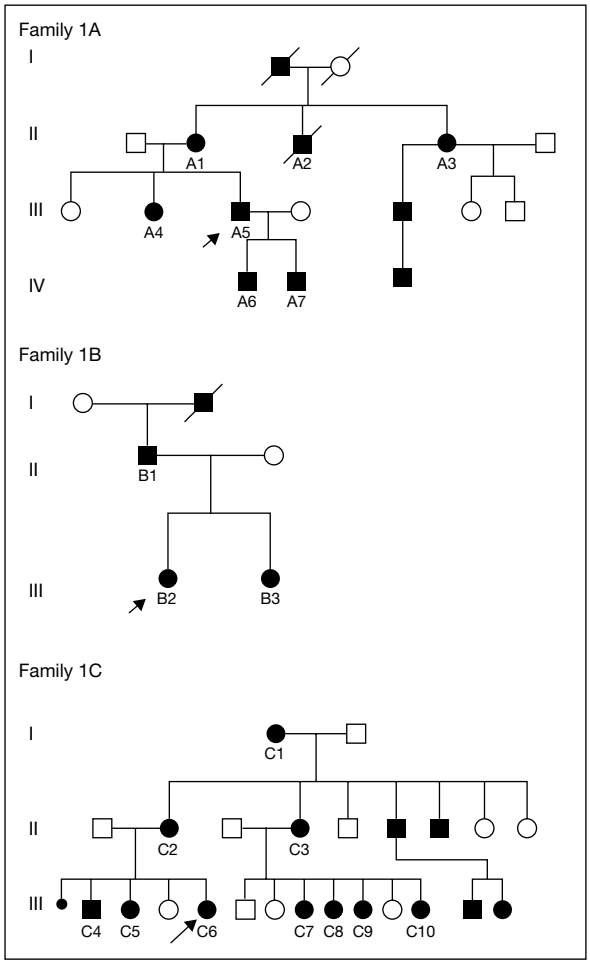


Fig. 2. Affected individuals (identified by family numbers) from three unrelated multigenerational NF1 families were clinically assessed and all had the same 3-bp AAT deletion and none have developed any cutaneous neurofibromas.

activity involves the assay that measures the intrinsic activity of the GRD [61, 69–71]. The increased activation of Ras observed in neurofibromin-deficient cells, often manifests itself by an increased activation of various downstream Ras effectors, including RAF, ERK1/2, PI3K, and p21-activation kinase [71]. It is the activation of these various signalling pathways that is likely to contribute to the increased proliferation observed in $Nf1^{-/-}$ Schwann cells and astrocytes,

as well as to the increased proliferation and motility of the heterozygous $Nf1^{+/-}$ mast cells present in neurofibromas.

It is possible that the methionine-deficient mutant neurofibromin functions as a hypomorphic allele, in which the loss of the single methionine results in a partial reduction in the overall level of neurofibromin in cells, but not to a level that results in neurofibroma development. Assessment of the effect of this mutation at the protein level is, therefore, required. Since this mutation is located on the border of the CSRD domain of neurofibromin, it is possible that the loss of methionine within this region may induce the aberrant phosphorylation of the threonine residue located immediately adjacent to the deleted methionine. In an attempt to address some of these questions, we propose to undertake a microarray analysis to assess changes both in gene expression analysis and in genomic copy number variation in order to identify potential modifying loci.

Neurofibromas are heterogeneous at the cellular level, mainly being composed of Schwann cells – along with fibroblasts, mast cells, and perineurial cells – although it is only the Schwann cells that undergo somatic mutation [72]. In a murine Nf1 model system, it was shown that it is the interaction of haploinsufficient mast cells ($Nf1^{+/-}$) with Schwann cells, which are completely deficient of neurofibromin that induces the development of neurofibromas [73]. It is, therefore possible that, in patients with the AAT deletion, this cellular interaction between mast cells and Schwann cells is disrupted, resulting in the failure of neurofibroma formation.

This recent study provides the first confirmed molecular evidence implicating the role of a specific 3-bp inframe deletion of the *NF1* gene in the determination of a particular clinical phenotype – namely, the almost complete lack of development of cutaneous, subcutaneous, and superficial plexiform neurofibromas. This finding is of clinical importance because plexiform neurofibromas have been shown to be associated with an increased risk of MPNSTs. It is not yet known whether this mutation is associated with the absence of asymptomatic internal neurofibromas, as no body or spinal imaging was undertaken in these patients. The absence of any form of neurofibroma has only previously been observed in families with familial CAL spots alone and also in some individuals with Watson syndrome, who often have few, if any, cutaneous neurofibromas.

This 3-bp deletion has been suggested to be the causative mutation in those rare families with chromosome 17-linked autosomal dominant CAL, it is proposed therefore that any patients presenting with CAL spots alone should be initially tested for the AAT deletion. The clinical data derived from this study also show a significant reduction of many NF1 features [43].

Molecular studies of individuals with NF1-Noonan (NFNS) have identified a variety of *NF1* mutations, several of which are also found in classical

NF1 patients who show no obvious NS-like features [74, 75]. This AAT deletion has so far only been identified in one family with NFNS, perhaps indicating that AAT-deletion mutation alone is insufficient to produce the NFNS phenotype, although this mutation may confer an increased predisposition to this phenotype. Phenotypic overlap has been demonstrated between NF1, Noonan syndrome, LEOPARD syndrome, cardio-facio-cutaneous (CFC) syndrome and Costello syndrome although these conditions are usually clinically distinguished. It was uncertain whether their common clinical features resulted from similarities in the underlying pathogenesis. A series of studies have demonstrated that each of these disorders is caused by germline mutations in a number of genes involved in the RAS-RAF-ERK-MAP-kinase cascade. Germline mutations in the *PTPN11* are found in almost half of all Noonan syndrome and all of LEOPARD syndrome cases [76]. Germline mutations in both *KRAS* and *SOS1* have also been found in Noonan syndrome, while mutations in the *BRAF*, *MAP2K* and *KRAS* gene are found in CFC patients. On the basis of these findings, it has been proposed that NF1, Noonan syndrome, LEOPARD syndrome, Costello syndrome and CFC syndrome should collectively be termed neuro-cardio-facial-cutaneous syndrome because of their overlapping clinical features and the central role that hyperactive Ras plays in their pathogenesis [76, 77].

In summary, the *NF1* gene displays several intriguing characteristics including the presence of three embedded genes, the presence of four alternatively spliced transcripts and their differential expression, multiple partial pseudogenes and a very high mutation rate which still remains enigmatic. *NF1* gene is a tumour suppressor gene [78] and is associated with many tumour types. It has been exciting few years for NF1, both in terms of clinical research and basic science. Our knowledge of the natural history, pathology, and pathogenesis of NF1 has greatly increased over the past decade, although this is still far from complete. Identification of additional genotype/phenotype correlations in this disorder would prove useful in both genetic counseling as well as expanding our knowledge of the aetiology of the disease. Studies based on genotype/phenotype correlation are however challenging, with molecular events between identification of a DNA genetic variant and an observable phenotype that may involve factors including genetic, environment and stochastic. Progress in molecular biology and neuroimaging and the development of mouse models have enhanced our understanding of the pathophysiology of NF1 and its clinical manifestations.

Neurofibromin is increasingly recognized to play a complex biochemical role within the cell, being involved in many critical signaling pathways, as reflected in the high evolutionary conservation of the protein. It has importance extending far beyond its role in the specific disorder neurofibromatosis and it

plays a fundamental role in key cellular processes. The development of targeted therapies for NF1 based on our improved understanding of the clinical features, molecular biology, proteomics and metabolic role of neurofibromin in different cell types including Schwann cells, neurons, astrocytes and mast cell, is anticipated in the near future.

Acknowledgements

I thank all my collaborators who have contributed to the genotype/phenotype studies. I am grateful to NF1 patients and their families for their support and to the Cancer Res UK and UK NF Association for the financial support.

References

1 Huson SM, Harper PS, Compston DAS: Von Recklinghausen neurofibromatosis: clinical and population study in south-east Wales. Brain 1988;111:1355–1381.

2 Viskochil DH: Gene structure and function; in Upadhyaya M, Cooper DN (eds): Neurofibromatosis Type 1: From Genotype to Phenotype. BIOS Publishers, Oxford, 1998, pp 39–56.

3 Suzuki V, Suzuki H, Kayama T, Yoshimoto T, Shibahara S: Brain tumors predominantly express the neurofibromatosis type 1 gene transcripts containing the 63 base insert in the region coding for GTPase activating protein-related domain. Biochem Biophys Res Commun 1991;181:955–961.

4 Andersen LB, Ballester R, Marchuk DA, Chang E, Gutmann DH, et al: A conserved alternative splice in the von Recklinghausen neurofibromatosis (*NF1*) gene produces two neurofibromin isoforms, both of which have GTPase-activating protein activity. Mol Cell Biol 1993;13:487–495.

5 Costa RM, Yang T, Huynh DP, Pulst SM, Viskochil DH, Silva AJ, Brannan CI: Learning deficits, but normal development and tumor predisposition, in mice lacking exon 23a of *Nf1*. Nat Genet 2001;27:399–405.

6 Gutmann D, Andersen L, Cole J: An alternatively spliced mRNA in the carboxy terminus of the neurofibromatosis type 1 (*NF1*) gene is expressed in muscle. Hum Mol Genet 1993;2:989–992.

7 Danglot G, Regnier V, Fauvet D, Vassal G, Kujas M, Bernheim A: Neurofibromatosis 1 (*NF1*) mRNAs expressed in the central nervous system are differentially spliced in the 5′ part of the gene. Hum Mol Genet 1995;4:915–920.

8 Kaufmann D, Müller R, Kenner O, Leistner W, Hein C, Vogel W, Bartelt B: The N-terminal splice product *NF1–10a-2* of the *NF1* codes for a transmembrane segment. Biochem Biophys Res Commun 2002;294:496–503.

9 Park VM, Kenwright KA, Sturtevant DB, Pivnick EK: Alternative splicing of exons 29 and 30 in the neurofibromatosis type 1 gene. Hum Genet 1998;103:382–385.

10 Ars E, Serra E, De la Luna S, Estivill X, Lázaro C: Cold shock induces the insertion of a cryptic exon in the neurofibromatosis type 1 (NF1) mRNA. Nucleic Acids Res 2000;28:1307–1312.

11 Thomson SA, Wallace MR: RT-PCR splicing analysis of the NF1 open reading frame. Hum Genet 2002;110:495–502.

12 Vandenbroucke I, Callens T, De Paepe A, Messiaen L: Complex splicing pattern generates great diversity in human NF1 transcripts. BMC Genomics 2002;3:13.

13 Hajra A, Martin-Gallardo A, Tarle SA, Freedman M, Wilson-Gunn S, Bernards A, Collins FS: DNA sequences in the promoter region of the *NF1* gene are highly conserved between human and mouse. Genomics 1994;21:649–652.

14 Horan MP, Osborn M, Cooper DN, Upadhyaya M: Functional analysis of polymorphic variation within the promoter and 5′ untranslated region of the neurofibromatosis type 1 (*NF1*) gene. Am J Med Genet 2004;131:227–231.

15 Osborn M, Cooper DN, Upadhyaya M: Molecular analysis of the 5′-flanking region of the neurofibromatosis type 1 (*NF1*) gene: identification of five sequence variants in NF1 patients. Clin Genet 2001;57:221–224.
16 Luijten M, Redeker S, van Noesel MM, Troost D, Westveld A, Hulsebos TJ: Microsatellite instability and promoter methylation as possible causes of *NF1* gene inactivation in neurofibromas. Eur J Hum Genet 2000;8:939–949.
17 Horan MP, Cooper DN, Upadhyaya M: Hypermethylation of the neurofibromatosis type 1 (*NF1*) gene promoter is not a common event in the inactivation of the *NF1* gene in NF1-specific tumours. Hum Genet 2000;107:33–39.
18 Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR, et al: cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of NF1 gene product. Genomics 1991;11: 931–940.
19 Gasparini P, Grifa A, Origone P, Coviello D, Antonacci R, Rocchi M: Detection of a neurofibromatosis type 1 homologous sequence by PCR: implications for the diagnosis and screening of genetic disease. Mol Cell Probes 1993;7:415–418.
20 Suzuki H, Ozawa N, Taga C, Kano T, Hattori M, Sakati Y: Genomic analysis of a NF1-related pseudogene on human chromosome 21. Gene 1994;147:247–250.
21 Purandare SM, Huntsman Breidenbach H, Li Y, Zhu XL, Sawada S, et al: Identification of neurofibromatosis 1 (NF1) homologous loci by direct sequencing, fluorescence in situ hybridization, and PCR amplification of somatic cell hybrids. Genomics 1995;30:476–485.
22 Hulsebos TJ, Bijleveld EH, Riegman PH, Smink LJ, Dunham I: Identification and characterization of NF1-related loci on human chromosomes 22, 14 and 2. Hum Genet 1996;98:7–11.
23 Kehrer-Sawatzki H, Schwickardt T, Assum G, Rocchi M, Krone W: A third neurofibromatosis type 1 (NF1) pseudogene at chromosome 15q11.2. Hum Genet 1997;100:595–600.
24 Barber JC, Cross IE, Douglas F, Nicholson JC, Moore KJ, Browne CE: Neurofibromatosis pseudogene amplification underlies euchromatic cytogenetic duplications and triplications of proximal 15q. Hum Genet 1998;103:600–607.
25 Luijten M, Wang Y, Smith BT, Westerveld A, Smink LJ, et al: Mechanism of spreading of highly related neurofibromatosis type 1 (*NF1*) pseudogenes on chromosome 2, 14 and 22. Eur J Hum Genet 2000;8:209–214.
26 Luijten M, Redeker S, Minoshima S, Shimizu N, Westerveld A, Hulsebos T: Duplication and transposition of the *NF1* pseudogene regions on chromosomes 2, 14, and 22. Hum Gen 2000;109: 109–116.
27 Fantes JA, Mewborn SK, Lese CM, Hedrick J, Brown RL, et al: Organisation of the pericentric region of chromosome 15: at least four partial gene copies are amplified in patients with a proximal duplication of 15q. J Med Genet 2002;39:170–177.
28 Luijten M, Fahsold R, Mischung C, Westerveld A, Nürnberg P, Hulsebos TJM: Limited contribution of interchromosomal gene conversion to *NF1* gene mutation. J Med Genet 2001;38:481–485.
29 Marchuk DA, Tavakkol R, Wallace MR, Brownstein BH, Taillon-Miller P, et al: A yeast artificial chromosome contig encompassing the type 1 neurofibromatosis gene. Genomics 1992;13: 672–680.
30 Yu H, Zhao X, Su B, Li D, Xu Y, et al: Expression of *NF1* pseudogenes. Hum Mutat 2005;26: 487–488.
31 Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, et al: Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. Science 1990;249: 181–186.
32 Gregory PE, Gutmann DH, Mitchell A, Park S, Boguski M, et al: Neurofibromatosis type 1 gene product (Neurofibromin) associates with microtubules. Somat Cell Mol Genet 1993;19:265–274.
33 Xu GF, O'Connell P, Viskochil DH, Cawthon R, Robertson M, et al: The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 1990a;62:599–608.
34 Benedict PH, Szabo G, Fitzpatrick TB, Sinesi SJ: Melanotic macules in Albright's syndrome and in neurofibromatosis. JAMA 1968;205:618–626.
35 Riccardi VM: Neurofibromatosis; Phenotype, Natural History, and Pathogenesis, ed 2. The John Hopkins University Press, Baltimore and London, 1992.

36 Ferner RE, Huson SM, Thomas N, Moss C, Willshaw H, et al: Guidelines for the diagnosis and management of individuals with neurofibromatosis 1. J Med Genet 2007;44:81–88.

37 Charrow J, Listernick R, Ward K: Autosomal dominant multiple cafe-au-lait spots and neurofibromatosis-1: evidence of non-linkage. Am J Med Genet 1993;45:606–608.

38 Brunner HG, Hulsebos T, Steijlen PM, der Kinderen DJ, vd Steen A, Hamel BC: Exclusion of the neurofibromatosis 1 locus in a family with inherited cafe-au-lait spots. Am J Med Genet 1993;46:472–474.

39 Abeliovich D, Gelman-Kohan Z, Silverstein S, Lerer I, Chemke J, Merin S, Zlotogora J: Familial cafe au lait spots: a variant of neurofibromatosis type 1. J Med Genet 1995;32:985–986.

40 Pulst SM, Riccardi VM, Fain P, Korenberg JR: Familial spinal neurofibromatosis: clinical and DNA linkage analysis. Neurology 1991;41:1923–1927.

41 Poyhonen M, Leisti EL, Kytölä S, Leisti J: Hereditary spinal neurofibromatosis: a rare form of NF1? J Med Genet 1997;34:184–187.

42 Upadhyaya M, Huson SM, Davies M, Thomas N, Chuzhanova N, et al: An absence of cutaneous neurofibromas associated with a 3-bp inframe deletion in exon 17 of the *NF1* gene (c.2970–2972 delAAT): evidence of a clinically significant NF1 genotype-phenotype correlation. Am J Hum Genet 2007;80:140–151.

43 Allanson JE, Upadhyaya M, Watson GH, Partington M, Mackenzie A, et al: Watson syndrome: is it a subtype of type 1 neurofibromatosis? J Med Genet 1991;28:752–756.

44 Upadhyaya M, Shen M, Cherryson A, Farnham J, Maynard J, Huson SM, Harper PS: Analysis of mutations in neurofibromatosis type 1. Hum Mol Genet 1992;1:735–740.

45 Tassabehji M, Strachan T, Sharland M, Colley A, Donnai D, Harris R, Thakker N: Tandem duplication within a neurofibromatosis type 1 (*NF1*) gene exon in a family with features of Watson syndrome and Noonan syndrome. Am J Hum Genet 1993;53:90–95.

46 Bates G, Harper P, Jones L: Huntington's Disease. Oxford Uni Press, New York, 2002, p 558.

47 Vajo Z, Francomano CA, Wilkin DJ: The molecular and genetic basis of fibroblast growth factor receptor 3 disorders: The Achondroplasia family of skeletal dysplasias, Muenke craniosynostosis, and Crouzon syndrome with acanthosis nigricans. Endocr Rev 2000;21:23–39.

48 Mattout A, Dechat T, Adam SA, Goldman RD, Gruenbaum Y: Nuclear lamins, disease and aging. Curr Opin Cell Biol 2006;18:335–341.

49 Burke B, Stewart CL: The laminopathies: the functional architecture of the nucleus and its contribution to disease. Annu Rev Genomics Hum Genet 2006;7:369–405.

50 Skuse GR, Cappione AJ: RNA processing and clinical variability in neurofibromatosis type I (NF1). Hum Mol Genet 1997;6:1707–1712.

51 Szudek J, Birch SJ, Friedman JM: The National Neurofibromatosis Foundation International Database Participants. Growth in North American white children with neurofibromatosis 1 (NF1). J Med Genet 2000;37:933–938.

52 Szudek J, Joe H, Friedman JM: Analysis of intrafamilial phenotypic variation in neurofibromatosis type 1 (NF1). Genet Epidemiol 2002;23:150–164.

53 Easton DF, Ponder MA, Huson SM, Ponder BA: An analysis of variation in expression of neurofibromatosis type 1 (NF1): evidence for modifying genes. Am J Hum Genet 1993;53:305–313.

54 Rasmussen SJ, Friedman JM: *NF1* gene and neurofibromatosis 1. Am J Epidemiol 2000;151:33–34.

55 Bahuau M, Pelet A, Vidaud D, Lamireau T, Bail B, et al: GDNF as a candidate modifier for neurofibromatosis enteric phenotype. J Med Genet 2001;38:638–643.

56 Serra E, Ars E, Ravella A, Sanchez A, Puig S, et al: Somatic *NF1* mutational spectrum in benign neurofibromas: mRNA splice defects are common among point mutations. Hum Genet 2001;108:416–429.

57 Wiest V, Eisenbarth I, Schmegner C, Krone W, Assum G: Somatic *NF1* mutation spectra in a family with neurofibromatosis type 1 toward a theory of genetic modifiers. Hum Mutat 2003;22:423–427.

58 Riccardi VM: Genotype, malleotype, phenotype, and randomness: lessons from neurofibromatosis-1 (NF-1). Am J Hum Genet 1993;53:301–304.

59 Reilly KM, Tuskan RG, Christy E, Loisel DA, Ledger J, et al: Susceptibility to astrocytoma in mice mutant for *Nf1* and *Trp53* is linked to chromosome 11 and subject to epigenetic effects. Proc Natl Acad Sci USA 2004;101:13008–13013.
60 Reilly KM, Broman KW, Bronson RT, Tsang S, Loisel DA, et al: An imprinted locus epistatically influences Nstr1 and Nstr2 to control resistance to nerve sheath tumors in a neurofibromatosis type 1 mouse model. Cancer Res 2006;66:62–68.
61 Upadhyaya M, Maynard J, Osborn M, Harper PS: Six novel mutations in the neurofibromatosis type 1 (NF1) gene. Hum Mutat 1997;10:248–250.
62 Upadhyaya M, Cooper DN: The mutational spectrum in neurofibromatosis type 1 and its underlying mechanisms; in Upadhyaya M, Cooper DN (eds): NF1: From Genotype To Phenotype. BIOS Publishers, Oxford, 1998.
63 Castle B, Baser ME, Huson SM, Cooper DN, Upadhyaya M: Evaluation of genotype-phenotype correlations in neurofibromatosis type 1. J Med Genet 2003;40:e109.
64 Kayes LM, Burke W, Riccardi VM, Bennet R, Ehrlich P, Rubenstein A, Stephens K: Deletions spanning the neurofibromatosis 1 gene: identification and phenotype of five patients. Am J Hum Genet 1994;54:424–436.
65 De Raedt T, Brems H, Wolkenstein P, Vidaud D, Pilotti S, et al: Elevated risk for MPNST in NF1 microdeletion patients. Am J Hum Genet 2003;72:1288–1292.
66 Kluwe L, Friedrich RE, Peiper M, Friedman J, Mautner VF: Constitutional *NF1* mutations in neurofibromatosis 1 patients with malignant peripheral nerve sheath tumors. Hum Mutat 2003;22:420.
67 Upadhyaya M, Spurlock G, Majounie E, Griffiths S, Forrester N, et al: The heterogeneous nature of germline mutations in NF1 patients with malignant peripheral nerve sheath tumours (MPNSTs). Hum Mutat 2006;27:716.
68 Kehrer-Sawatzki H, Kluwe L, Sandig C, Kohn M, Wimmer K, et al: High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the *JJAZ1* gene. Am J Hum Genet 2004;75:410–423.
69 Izawa I, Tamaki N, Saya H: Phosphorylation of neurofibromatosis type 1 gene product (neurofibromin) cAMP-dependent protein kinase. FEBS Lett 1996;382:53–59.
70 Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS, Downward J: Aberrant regulation of ras proteins in malignant tumor cells from type 1 neurofibromatosis patients. Nature 1992;356:713–715.
71 Arun D, Gutmann DH: Recent advances in neurofibromatosis type 1. Curr Opin Neurol 2004;17:101–105.
72 Serra E, Rosenbaum T, Winner U, Aldeo R, Ars E, et al: Schwann cells harbor the somatic *NF1* mutation in neurofibromas: evidence of two Schwann cells subpopulations. Hum Mol Genet 2000;9:3055–3084.
73 Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF: Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 2002;296:920–922.
74 Baralle D, Mattocks C, Kalidas K, Elmslie F, Whittaker J, et al: Different mutations in the *NF1* gene are associated with neurofibromatosis-Noonan syndrome (NFNS). Am J Med Genet A 2003;19:1–8.
75 De Luca A, Bottillo I, Sarkozy A, Carta C, Neri C, et al: *NF1* gene mutations represent the major molecular event underlying neurofibromatosis-Noonan syndrome. Am J Hum Genet 2005;77:1092–1101.
76 Bentires-Alj M, Kontardis MI, Neel BG: Stops along the RAS pathway in human genetic disease. Nat Med 2006;12:283–285.
77 Tartaglia M, Pennacchio LA, Zhao C, Yadav KK, Fodale V, et al: Gain-of-function *SOS1* mutations cause a distinctive form of Noonan syndrome. Nat Genet 2007;39:75–79.
78 Cichowski K, Jacks T: NF1 tumor suppressor gene function. Narrowing the GAP. Cell 2000;104:593–604.
79 Lee TK, Friedman JM: Analysis of NF1 transcriptional regulatory elements. Am J Med Genet A 2005;137:130–135.

80 Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, et al: Minor lesion mutational spectrum of the entire NF1 gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 2000;66:790–818.
81 Aravind L, Neuwald AF, Ponting CP: Sec14p-like domains in NF1 and Dbl-like proteins indicate lipid regulation of Ras and Rho signaling. Curr Biol 1999;9:R195–R197.

Meena Upadhyaya
Institute of Medical Genetics
School of Medicine, Cardiff University
Heath Park, Cardiff CF14 4XN (UK)
Tel. +44 2920 744 081, Fax +44 2920 747 603, E-Mail upadhyaya@cardiff.ac.uk

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 63–77

NF1 Mutational Spectrum

L.M. Messiaen[a], *K. Wimmer*[b]

[a]Department of Genetics at UAB, Medical Genomics Laboratory, Birmingham, Ala., USA; [b]Department of Medical Genetics, Medical University of Vienna, Vienna, Austria

Abstract

The *NF1* gene is a large and complex gene spread over 280 kb of genomic DNA on chromosome 17q11.2 and comprising 57 constitutive and at least 3 alternatively spliced exons. The *NF1* gene encodes a transcript with an open reading frame of 8,454 nucleotides. Mutation detection is a challenge due to the large size of the gene, the lack of mutational hotspots, the occurrence of a very diverse spectrum of mutation types and the presence of more than 30 unprocessed pseudogenes [1]. Several different techniques have been applied to look for mutations in this complex gene [2–4], which included already early on attempts to apply RNA-based assays, i.e. protein truncation testing (PTT) [5]. We initially started out using this PTT assay [6, 7], but soon realized its limitations which are (i) the necessity for complementary assays capable to detect non-truncating mutations and (ii) the need to overcome the problems inherent to all RNA-based approaches, i.e. illegitimate splicing and nonsense mediated RNA decay. However, we realized the enormous advantages of an RNA-based mutation analysis approach for a large and complex gene like *NF1* that is expressed in the lymphocytes, that has no hotspots for mutations, where mutations of all types exist and where multiple highly homologous unprocessed pseudogene sequences that are spread over the genome hamper specific DNA amplification. Therefore, we further refined and improved the assays so that they now represent the core of a comprehensive mutation analysis cascade that allows highly sensitive and effective NF1 testing. In the following, we will present this approach and the mutational spectrum deduced from its application to a large cohort of patients.

Comprehensive *NF1* Mutation Analysis Using a Cascade of Complementary Techniques – General Overview of the Approach

We applied a multi-step approach to analyze the entire *NF1* coding region, using an RNA-based center assay, complemented with additional methods allowing (i) to fully characterize the cDNA alterations at the gDNA level and (ii) to

identify microdeletions and intragenic copy number alterations. We identified the pathogenic *NF1* mutation in 1,770 unrelated patients submitted for clinical genetic testing to the Medical Genomics Laboratory at UAB. For all patients, an EDTA-blood sample was obtained and split into three aliquots (i) for genomic DNA extraction, (ii) for preparation of cell suspensions for interphase fluorescence in situ hybridization (FISH) and (iii) for short term lymphocyte cultures for RNA extraction. Short-term phytohemagglutinin (PHA)-stimulated lymphocyte cultures were set up to avoid illegitimate splicing, known to lead to multiple aberrant splice variants that impede the detection of mutations in an RNA-based approach [6, 7]. Before RNA extraction, cultures were treated with 200 μg/ml puromycin for 4 h to prevent the nonsense-mediated mRNA decay [6]. Total RNA was extracted and cDNA prepared as described [8]. The entire coding region was amplified in three overlapping fragments of ~4 kb (spanning the *NF1* exons 1–27b, 12a–35 and 22–49). All fragments were analyzed by gel electrophoresis allowing immediate identification of shortened fragments indicative for the presence of splice mutations or deletions [8]. Hereafter, the fragments were used as the starting material for direct cDNA sequencing. To cover the entire coding region 18 sequencing primers were used. Cycle sequencing reactions were performed with commercially available sequencing kits. The sequencing reactions were subsequently run on an automated capillary sequencer and analyzed using the sequence analysis program SeqScape V2.5 (ABI). In addition, exon 1 was analyzed by bidirectional direct sequencing of this exon from genomic DNA.

All alterations/mutations detected at the cDNA level were entirely characterized at the genomic level using exon/intron-specific or breakpoint-specific primers. As prescreen for total gene deletion (with or without deletion of additional flanking genes), microsatellite analysis using four intragenic markers was performed on an ABI3730 capillary sequencer. Patients showing only one allele for all microsatellite markers were further analyzed by multiplex ligation dependent probe amplification (MLPA) using the *NF1* microdeletion MLPA-kit (P122 MRC Holland) [9] and interphase FISH analysis (100 nuclei) using probes flanking the *NF1* gene, i.e. RP5–1002G3 and RP5–926B9, to confirm or rule out the presence of the total *NF1* gene deletion [6]. Finally, MLPA analysis (kits P081 and P082 MRC-Holland) was also performed for all patients in whom no mutation was identified after sequencing the entire coding region at the cDNA level in order to identify intragenic copy number alterations that escaped detection by the RT-PCR fragment analysis and direct sequencing approach [9]. The outline of the cascade of tests used in this comprehensive approach is depicted in figure 1. The 5′ and 3′UTR have not been systematically implemented in this approach, except for analysis of dosage alterations at positions c.1–717 and c.1–680 of the 5′UTR. Furthermore, no cytogenetic

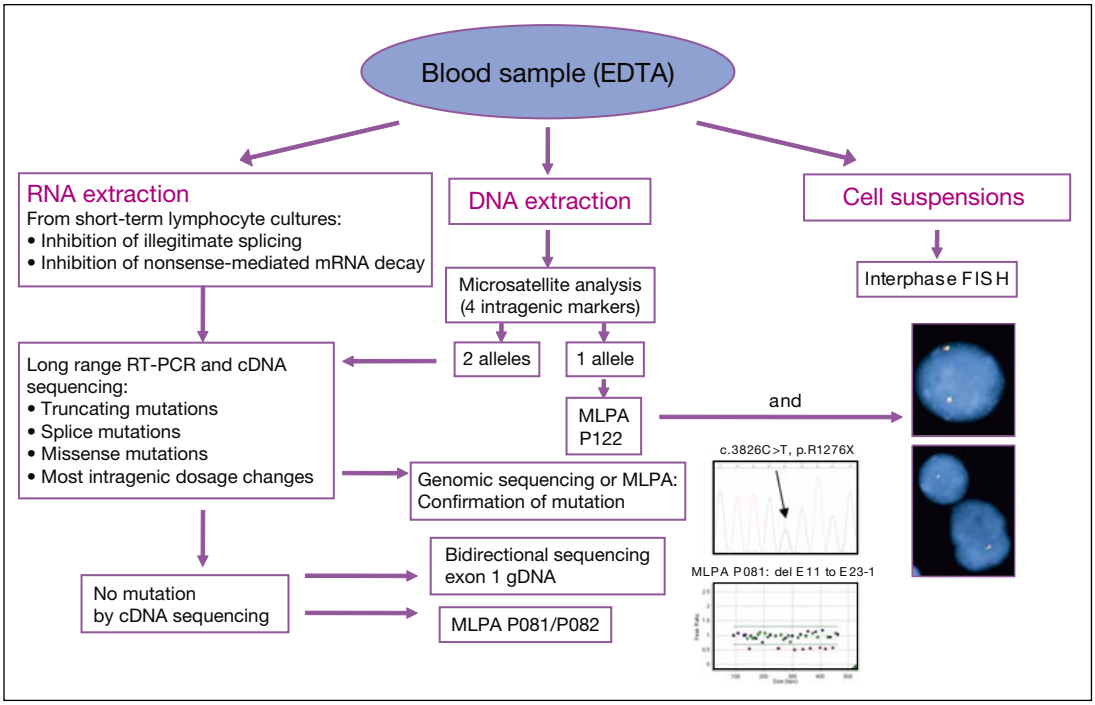


Fig. 1. Comprehensive *NF1* mutation analysis using a cascade of complementary techniques – general overview of the approach.

analysis was performed that would identify a (balanced) translocation or other cytogenetic abnormality resulting in NF1 or a NF1-like phenotype.

Spectrum of Mutations in 1,770 Unrelated Patients as Identified by A Comprehensive Multi-Step Approach

Comprehensive *NF1* mutation analysis using this RNA-based core assay supplemented with methods to identify *NF1* microdeletions as well as smaller copy-number changes identifies a mutation in 95% of non-founder NF1 patients fulfilling NIH criteria [6]. The sensitivity of a comprehensive RNA-based approach was confirmed in an independent cohort of well characterized patients [10]. The efficiency and reliability of this approach is based on the application of a set of complementary techniques that allow the detection of different mutation types, including unusual splicing defects occurring outside the

canonical AG/GT splice site sequences of the *NF1* gene. The higher detection rate compared to other methods is largely explained by the frequent occurrence of unusual splicing defects in the *NF1* gene.

Mutations in the *NF1* gene are spread over the entire coding region and include *NF1* microdeletions, intragenic copy number changes, i.e. deletions/duplications involving one to several exons, frameshift, nonsense, splice, missense mutations and in frame deletions or duplications involving one to several codons.

In total, 1,017 different *NF1* mutations were identified in this cohort of 1,770 unrelated patients in whom a mutation was found. 813 out of these 1,017 mutations were singular mutations. Hence, 46% of the patients (813/1,770) carry a 'private mutation' and 204 different mutations were observed more than once in the remainder of this cohort, i.e. 957 patients. This number includes four different types of *NF1* microdeletions (all encompassing the entire *NF1* gene with or without additional flanking genes) found in 89 unrelated patients as well as 200 minor-lesion mutations and intragenic copy number changes found twice to 30 times in the remaining 868 patients. Twenty-nine *different nonmicrodeletion* mutations residing in 23 different exons each occurred in >0.5% of the patients (ranging from 9/1,770 to 30/1,770) and together account for 413/1,770 of the mutations (23.3%). These mutations will be further discussed under the heading 'recurrent mutations'.

The *NF1* microdeletions found in 89 patients (5%) were of variable size. Seventy-six were 1.4 Mb in size (referred to as type I deletion [11, 12]) or larger; 9 were 1.2 Mb in size (referred to as type II deletions [13]), and 4 deletions were smaller than 1.2 Mb but still encompassed the entire *NF1* gene. 367 patients carried a nonsense mutation (20.7%). 478 patients had splicing mutations (27%). Of these, 186 affected the canonical AG/GT splice dinucleotides (10.5%), 46 affected intronic sequences at position +3 to +30 from the adjacent exon border (2.6%) and 38 affected intronic sequences at the position –3 to –30 from the adjacent exon border (2.1%), 172 patients carried a mutation within one of the *NF1* exons affecting splicing (9.7%), although at the gDNA level they presented as a nonsense, missense or silent alteration. Thirty-six patients carried a deep intronic alteration (2%) resulting in the creation of an intronic 5′ or 3′ splice site that is used in conjunction with a cryptic 3′ or 5′ splice site present in the wild type intronic sequence. 497 patients carried small (one to several nucleotides) deletions or duplications/insertions (28%). Forty-four of them were non-frameshifting mutations, i.e. a deletion (40/44) or duplication (4/44) leading to loss/gain of 1 (24 patients), 2 (16 patients), 3 (2 patients), 5 (1 patient) or 8 (1 patient) codons. The remaining 453 deletions/insertions caused a frameshift with 21 patients carrying a frameshift mutation due to insertion of 1 to 10 nucleotides (1.2%), 136 due to duplication of 1 to 13 nucleotides (7.7%) and 296 patients carried a frameshift mutation due to deletion of 1 to 20 nucleotides (16.7%). In

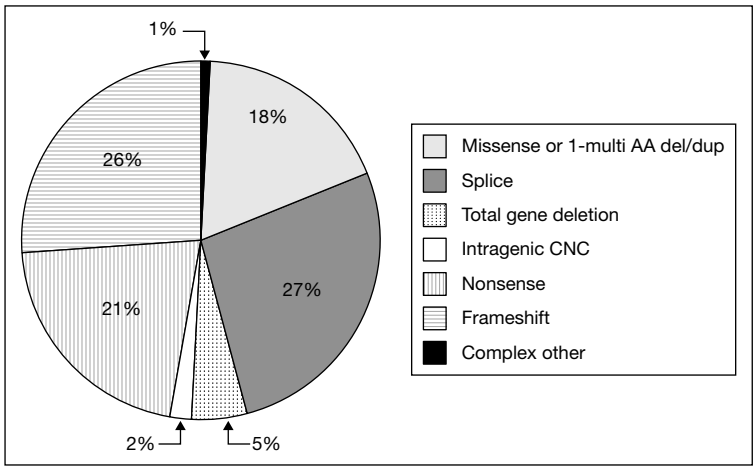


Fig. 2. Spectrum of mutations in 1,770 unrelated patients as identified by a comprehensive multi-step approach.

13 patients (0.7%), a unique complex mutation was identified, such as indels, multiple substitutions in nearby but non-adjacent nucleotides or a substitution plus deletion in nearby but non-adjacent nucleotides. 282 patients carried a missense mutation (15.9%). In 39 patients (2.2%), an intragenic copy number change was found to delete (35/39) or duplicate (4/39) one (12/39) to several (27/39) exons. Finally, in 4 patients, the methionine start codon was mutated (M1V, M1R, M1I, c.1–16_c.1del17), which may result in use of an alternative start codon and in one patient, a balanced translocation was found, t(14;17)(q32;q11.2) [6]. Although rare, the contribution of gross chromosomal aberrations might be slightly underestimated in the current study. An overview of the spectrum of mutations is depicted in figure 2.

The nomenclature of the mutations is based on *NF1* mRNA sequence NM_000267.1, with 1 being the first nucleotide from the methionine startcodon. The *NF1* exons are named according to the most widely used and best known nomenclature, which does not use strictly consecutive numbers (see [4]).

Advantages of Using an RNA-Based Core Assay

RNA-based mutation detection techniques are a powerful, reliable and efficient means to identify mutations, especially in large and complex genes expressed in easily accessible cells such as lymphocytes and are more sensitive,

especially in genes with a mutational spectrum showing a high prevalence of splicing mutations not residing at the canonically conserved GT/AG splice sites [2, 6, 10]. Moreover, the approach is very cost-effective: indeed for the *NF1* gene the entire coding region can be analyzed in only 3–5 large overlapping fragments using ~18–20 sequencing primers, instead of the 57 amplicons needed if all exons are analyzed at the gDNA level. Full benefit of the advantages of the RNA-based assays is however only possible if the necessary precautions are taken to prevent illegitimate splicing as well as nonsense-mediated mRNA decay.

Correct Classification of Mutations that Affect Splicing but Mimic Nonsense, Missense or Silent Mutations

In this study, we identified a total of 63 *different* exonic mutations in 192 patients, affecting correct splicing. Seventeen mimic nonsense mutations at the gDNA, 34 missense, 10 silent alterations and 1 a frameshift mutation, c.6792delC, leading to in-frame skipping of exon 37. Whereas the mutations mimicking a nonsense mutation would still overall be classified as 'pathogenic' in a gDNA direct sequencing approach, their classification and assessment of effect would be incorrect, which eventually may impact downstream studies such as genotype-phenotype correlations. The decisive evaluation of the 'missense' and 'silent' alterations would not be possible without a functional evaluation of the effect of these alterations on the splicing of the exons harboring the alterations.

One class of exonic alterations create de novo splice sites, which are used despite the presence of the intact wild-type splice sites and result in the loss of a part of the exon. c.1466A>G ('p.Y489C') in exon 10b exemplifies this class. It is the most recurrent minor lesion mutation in our cohort (30/1770) and creates a novel 5′ splice site (ss) that is used instead of the authentic wild-type and leads to skipping of the last 62 nucleotides of exon 10b [14]. Other examples include c.2709G>A ('p.V903V'), c.3278T>A ('p.V1093E') and c.3831C>T ('p.G1277G') [10].

Another class of exonic alterations leads to exon skipping most likely by disruption/creation of splicing regulatory elements such as exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSs). Data strongly support the notion that p.Y2264X (c.6792C>A or C>G), one of the more frequently recurring mutations resulting in exon 37 skipping [15], acts through the disruption of an ESE [16]. Other examples include p.R304X, p.W336X and p.E1907X. Although ESE/ESS prediction programs have proven to be useful for understanding the mechanisms of mutation-induced exon skipping, experimental data from patient tissues remains pivotal to assess the effect on splicing of silent or conservative amino acid changes.

Avoidance of the Detection of Rare Benign Intronic Variants with No Significance

Intronic sequences without functional impact diverge faster and accumulate more variation in the population [17]. These rare variants, besides the other more commonly observed SNPs, will come to attention by gDNA-based exon-by-exon sequencing, which usually assesses the intronic regions flanking each exon from –30 to +30, however their significance will be unknown. An effect on splicing can only be safely assumed for alterations affecting the conserved canonical GT/AG dinucleotides. As all new intronic variants found in this region may or may not impact correct splicing, they will need further formal evaluation by RNA-based analysis of the region of interest starting from a new blood draw.

Detection of Deep Intronic Splice Mutations

In this study we identified 23 different deep intronic mutations in 36 unrelated patients. Eighteen of them were 'private' mutations only observed in one unrelated patient/family. These mutations alter a single nucleotide often within very large introns, creating de novo 5′ or 3′ intronic splice sites that are used in conjunction with an already available intronic 'partner' cryptic splice site leading to inclusion of a cryptic exon. One deep intronic mutation, c.5749 + 332A>G in intron 30, reported previously in three independent studies [10, 18, 19], was identified in 7 unrelated patients in this study. This mutation creates a strong de novo 5′ss and leads to the insertion of a 177-bp cryptic exon between exons 30 and 31. Interestingly, the 3′ss at c.5749 + 154, used by c.5749 + 332A>G to create the 177-bp cryptic exon is predicted to be very weak by the currently available in silico prediction tools [10]. This finding has two immediate implications: it suggests that other intronic sequences help to define this cryptic exon [20] and demonstrates that the available in silico prediction tools (so far) lack the power to predict the outcome of an array of variants that would be encountered by direct sequencing of entire introns and to decide based on these predictions which of these variants should be pursued as likely pathogenic.

Another novel deep intronic splice mutation in the 17.7 kb long intron 6, i.e. c.888 + 651t>a, is illustrated in figure 3. According to the splice site prediction by neural network (SSPNN) program (http://www.fruitfly.org/seq_tools/splice.html) this 'private' mutation, found as de novo mutation in a sporadic patient with spinal NF, creates a strong 3′ss that is used in conjunction with a cryptic strong 5′ss leading to inclusion of a cryptic 132-bp exon containing a premature stop codon. Interestingly, the cDNA data indicate that the cryptic exon only gets included in part of the mutant alleles, indicative of poor cryptic exon recognition at least in the investigated tissue.

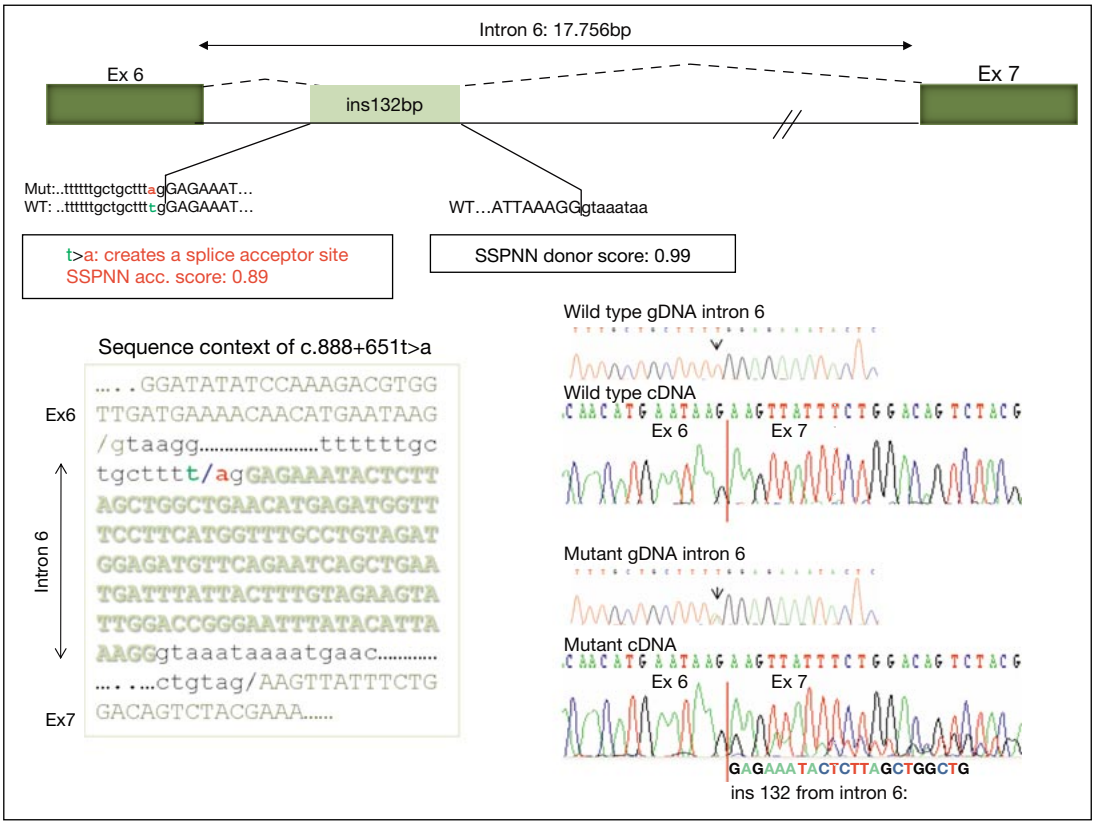


Fig. 3. Deep intronic de novo splice mutation in the 17.7 kb long intron 6, i.e. c.888 + 651t>a, leading to exonisation of 132 bp from intron 6 due to the use of a newly created splice acceptor site that gets used in conjunction with a cryptic splice donor site.

Avoidance of Amplification of Non-Processed Pseudogenes

In addition to the functional *NF1* gene, over 30 non-processed pseudogenes are present in the human genome [1]. The pseudogenes on chromosomes 2, 14 and 22, containing the middle part of the *NF1* gene, without the GAP-encoding segment, have a sequence homology of more than 95%. Hence, the design of primers to *specifically* and *uniquely* amplify those *NF1* exons that also have been duplicated during evolution and are part of these pseudogenes, has been a real challenge and greatly impeded accurate mutation analysis solely based on DNA-dependent methods in the past. This challenge has recently become more manageable due to the public availability of the sequences of the entire human genome as well as through more powerful primer design and

homology search software [21]. Nevertheless, it is advisable when designing primers for gDNA sequencing to allow the amplification of at least 30 nucleotides of the adjacent intronic sequences. Primers too close to the exon-intron border (see e.g. primer pair for exon 6 [21]) will miss splicing mutations outside the canonical GT/AG splice site dinucleotides. Furthermore, primer pairs with only one mismatch at the terminal bases between one or more of the *NF1* pseudogenes and the real *NF1* gene may still coamplify some pseudogene sequences, since the extension of the primers from the pseudogene template may continue if terminal mismatching has weak-destabilizing effect.

Pathogenic Versus Rare Benign Variant

The distinction between a pathogenic and rare benign variant is of utmost importance when mutation analysis is performed as clinical testing and this distinction is, apart from the silent and deep intronic sequence changes, particularly challenging for missense alterations.

In the absence of functional assays, rigorous criteria must be applied before a novel missense alteration in the *NF1* gene can be unequivocally classified as the disease causing mutation in order to avoid diagnostic errors. Sometimes, a final decision will necessarily need to be suspended until additional data from larger cohorts of patients become available. Importantly, we have found missense mutations spread over the entire gene from exon 1 to exon 46. The missense mutations were not restricted uniquely to any of the so far more characterized regions with known or putative functions (GRD, CSRD, Sec14). No missense alterations were detected (so far?) in the exons 5, 9br, 23a, 34, 35, 37, 39, 42–45, 47–49.

We propose the following criteria for the evaluation of missense alterations: (i) absence of any other possible deleterious mutation after comprehensive analysis of the whole coding region. This is a necessary but insufficient criterion. This analysis *must* include screening for a total gene deletion, smaller dosage alterations (one to multiple exons deletions/duplications) and splice mutations including deep intronic mutations affecting splicing. 'Missense' mutations shown to have an effect on splicing if RNA-based mutation analysis is performed, can be considered to be deleterious; (ii) absence of the sequence alteration in a large number of unrelated control samples. This is a necessary but insufficient criterion. We *still* find novel benign variants on the wild-type *NF1* allele, inherited from the unaffected parent, even after sequencing the entire coding region in >3000 patients. Furthermore, no sufficient data are available from all ethnic backgrounds; (iii) alterations at amino acids that have been conserved over a long evolutionary distance in the *NF1* orthologs of *Pan*

troglodytes, Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio, Canis familiaris, Anopheles gambiae, Takifugu rubripes and *Drosophila melanogaster* have a high likelihood of being deleterious. In addition, algorithms that differentiate one variant from another at a given codon based on chemical differences (such as PolyPhen, SIFT or Grantham score A-GVGD), can add further support; (iv) however, finally and most importantly, clinical and molecular genetic assessment of the relevant family members is needed before a final conclusion should be made for all novel missense alterations. Although NF1 is a progressive and phenotypically highly variable disorder, the disease is fully penetrant. In a sporadic patient, the missense mutation needs to be *proven* to be a de novo event and clinical evaluation of both parents needs to confirm absence of signs of NF1 in them. De novo occurrence of a missense alteration after comprehensive mutation analysis, along with other supportive data such as evolutionary conservation and Grantham score, provides the strongest argument in favor of pathogenicity at this time. In a familial patient, the missense mutation needs to be proven to segregate with the disorder in the family by analysis of at least one affected relative. The latter argues that the missense alteration segregates with the symptoms in the family, but does not provide in itself proof of its pathogenicity.

Recurrent Mutations with a Frequency >0.5% in the NF1 Patient Population

1,017 different *NF1* mutations were identified in this cohort of 1,770 unrelated patients in whom a mutation was found. 813 out of these 1,017 mutations were singular mutations, indicating that ~46% of the patients (813/1,770) carry a 'private mutation'. We expect that the number of novel mutations will further steadily increase as more patients get analyzed and that we will continue to witness that almost half of the newly analyzed patients carry a private mutation.

In our cohort, 89 unrelated patients carried microdeletions that fall in at least 4 different types/size classes (all encompassing the entire *NF1* gene with or without additional flanking genes). 868 patients carried 200 different recurrent minor lesion mutations and intragenic copy number changes found twice to 30 times in our cohort.

Twenty-nine of these *different nonmicrodeletion* mutations residing in 23 different exons each occurred in >0.5% of the patients (ranging from 9/1,770 to 30/1,770) and together account for 413/1,770 of the mutations (23.3%) (table 1 and fig. 4). Another 10 different mutations residing in 7 different exons/introns occurred in >0.3% and <0.5% of the patients and account for 68/1,770 of the mutations (4%) (table 1 and fig. 4). Fifty-one different mutations found in 167

Table 1. Summary of all recurrent mutations presenting in >0.3% of the patients in this cohort

Mutation	Exon/ intron	Type/ effect	No. of patients
Mutations found in >0.5% of the patients			
c.499_502delTGTT	E4b	frameshift	22
c.574C>T; p.R192X	E4b	nonsense	10
c.910C>T; p.R304X	E7	nonsense/splice	12
c.1246C>T; R416X	E9	nonsense	13
c.1318C>T; p.R440X	E10a	nonsense	12
c.1381C>T; p.R461X	E10a	nonsense	10
c.1466A>G; 'Y489C'	E10b	splice	30
c.1541_1542delAG	E10c	frameshift	13
c.1756_1759delACTA	E12a	frameshift	22
c.1885G>A; 'p.G629R'	E12b	splice	10
c.2033dupC	E13	frameshift	20
c.2041C>T; p.R681X	E13	nonsense	10
c.2446C>T; p.R816X	E15	nonsense	15
c.2970_2972delAAT; p.991delM	E17	1 AA deletion	14
c.3457_3460delCTCA	E20	frameshift	14
c.3721C>T; p.R1241X	E22	nonsense	10
c.3826C>T; p.R1276X	E22	nonsense	13
c.4084C>T; p.R1362X	E23-2	nonsense	16
c.4267A>G; p.K1423E	E24	missense	17
c.4537C>T; p.R1513X	E27a	nonsense	18
c.5242C>T; p.R1748X	E29	nonsense	13
c.5425C>T; p.R1809C	E29	missense	13
c.5546G>A; 'p.R1849Q'	E29	splice	17
c.5839C>T; p.R1947X	E31	nonsense	10
c.6709C>T; p.R2237X	E36	nonsense	9
c.6792C>A; 'p.Y2264X'	E37	splice	16
c.7096_7101delAACTTT; p.2366_2367delNF	E39	2 AA deletion	9
c.7486C>T; p.R2496X	E42	nonsense	9
c.7846C>T; p.R2616X	E45	nonsense	16
Mutations found in >0.3% but <0.5% of the patients			
c.1019_1020delCT	E7	frameshift	6
c.2325+1G>A	I14	splice	7
c.3827G>A; p.R1276Q	E22	missense	6
c.3916C>T; p.R1306X	E23-1	nonsense	7
c.5749+332A>G	I30	deep intronic splice	7
c.6789_6792delTTAC	E37	frameshift	6
c.6791dupA	E37	frameshift	8

Table 1. (continued)

Mutation	Exon/ intron	Type/ effect	No. of patients
c.6792C>G; 'p.Y2264X'	E37	splice	7
c.7267dupA	E41	frameshift	6
c.7285C>T; p.R2429X	E41	nonsense	8

Mutations that can be explained by spontaneous methyl-cytosine deamination-mediated C>T/G>A transitions at 19 of the 120 CpG dinucleotides of the *NF1* coding region are shown in bold.

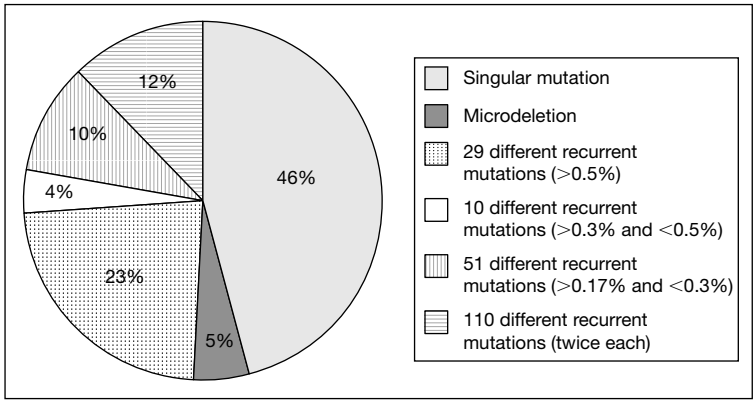


Fig. 4. Overview of the fraction of recurrent versus singular mutations in the cohort of 1,770 unrelated patients.

unrelated patients, occurred 3 to 5 times in this cohort and 110 different mutations were identified twice in this cohort.

Of the 29 recurring mutations with a prevalence of >0.5%, 19 can be explained by spontaneous methyl-cytosine deamination-mediated C>T/G>A transitions at 19 of the 120 CpG dinucleotides of the *NF1* coding region, see mutations presented as bold in table 1. Three deletions are tandem-repeat mediated deletions (c.499_502delTGTT, c.1756delACTA and c.7096_7101delAACTTT). Mutation c.2033dupC can be caused by DNA-polymerase slippage within a C_7-homonucleotide tract. p.991delM is suggested to be mediated by palindrome correction [22]. Mutation c.1541_1542delAG may be explained by

excision of two mispaired nucleotides in an inverted repeat [23]. c.1466A>G and c.4267A>G are probably induced by spontaneous adenine deamination at the sense strand [24]. p.Y2264X in exon 37 is located within a hypermutable region in exon 37 containing a 'quasi-symmetric' element [25]. The reason for the recurrence of c.3457_3460delCTCA remains so far unexplained.

Final Remarks

We have described and defined the overall unbiased spectrum of constitutional *NF1* mutations as found in a large cohort of 1,770 *unrelated* patients analyzed in the Medical Genomics Laboratory at UAB using a comprehensive mutation analysis consisting of an RNA-based core assay supplemented with methods to identify *NF1* microdeletions as well as smaller copy number changes. We do not expect to see further significant fluctuations in the *percentages* contributed by the different types of mutations by analyzing larger cohorts of patients. However, the spectrum of mutations within each of the subtypes (e.g. missense, deep intronic splice mutations, etc.) will need to be defined/described in more detail to further our understanding of the genomics of the *NF1* gene. This spectrum will serve as a baseline to compare against specific *subgroups* of patients presenting with variant forms/subtypes of NF1. It will also allow exploring in further depth whether the spectrum of second hit mutations as identified in different cells/tumors differs from the constitutional spectrum.

There is a need for a *reliable, specific* and *sensitive NF1* mutational detection approach, to help resolve diagnostic dilemmas using clinical genetic testing in patients not fulfilling the NIH diagnostic criteria, especially young children but also atypical patients, such as patients with spinal neurofibromatosis or segmental NF. Only about half of patients with sporadic *NF1* mutations (i.e. founder patients) fulfill the NIH diagnostic criteria by one year of age and *still* 5% will not fulfill these criteria by the age of 8 years [26]. CAL-spots are often the first signs of NF1, increasing in number during the first years of life. Waiting for more symptoms to appear with time in order to ascertain the diagnosis on a clinical basis can be very stressful for families. Furthermore, distinction between NF1 and the recently reported autosomal dominant *NF1-like disorder*, caused by mutations in the *SPRED1* gene and characterized by multiple CAL-spots, freckling, macrocephaly but absence of cutaneous neurofibromas, will become essential once the symptoms and complications associated with this new disorder are documented in more detail [27].

Earlier diagnosis allows to offer genetic counseling to parents and relatives earlier as well as to initiate interventions for learning or developmental problems sooner. Earlier diagnosis will become even more important once more

therapeutic options become available. A direct genetic test may help to establish the diagnosis earlier, especially in sporadic patients, but *only* when the testing has a high sensitivity, i.e. finds the mutation in (almost) all patients that eventually will fulfill the NIH criteria (low false negative results) *and*, equally important, does not confuse a benign variant with a pathogenic mutation (no false positive results).

References

1 Luijten M, Redeker S, Minoshima S, Shimizu N, Westerveld A, Hulsebos TJ: Duplication and transposition of the *NF1* pseudogene regions on chromosomes 2, 14, and 22. Hum Genet 2001;109: 109–116.

2 Ars E, Serra E, García J, Kruyer H, Gaona A, et al: Mutations affecting mRNA splicing are the most common molecular defects in patients with neurofibromatosis type 1. Hum Mol Genet 2000;9:237–247.

3 Fahsold R, Hoffmeyer S, Mischung C, Gille C, Kucukceylan N, et al: Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 2000;66:790–818.

4 Han SS, Cooper DN, Upadhyaya MN: Evaluation of denaturing high performance liquid chromatography (DHPLC) for the mutational analysis of the neurofibromatosis type 1 *(NF1)* gene. Hum Genet 2001;109:487–497.

5 Heim RA, Kam-Morgan LNW, Binnie CG, Corns DD, Cayouette MC, et al: Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. Hum Mol Genet 1995;4:975–981.

6 Messiaen L, Callens T, Mortier G, Beysen D, Vandenbroucke I, et al: Exhaustive mutation analysis of the *NF1* gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. Hum Mutat 2000;15:541–555.

7 Wimmer K, Eckart M, Rehder H, Fonatsch C: Illegitimate splicing of the *NF1* gene in healthy individuals mimics mutation-induced splicing alterations in NF1 patients. Hum Genet 2000;106: 311–313.

8 Vandenbroucke I, van Doorn R, Callens T, Cobben JM, Starink TM, Messiaen L: Genetic and clinical mosaicism in a patient with neurofibromatosis type 1. Hum Genet 2004;114:284–290.

9 Wimmer K, Yao S, Claes K, Kehrer-Sawatzki H, Tinschert S, et al: Spectrum of single- and multiexon NF1 copy number changes in a cohort of 1,100 unselected NF1 patients. Genes Chromosomes Cancer 2006;45:265–276.

10 Wimmer K, Roca X, Beiglbock H, Callens T, Etzler J, et al: Extensive in silico analysis of NF1 splicing defects uncovers determinants for splicing outcome upon 5′ splice-site disruption. Hum Mutat 2007;28:599–612.

11 De Raedt T, Brems H, Lopez-Correa C, Vermeesch JR, Marynen P, Legius E: Genomic organization and evolution of the *NF1* microdeletion region. Genomics 2004;84:346–360.

12 Jenne DE, Tinschert S, Dorschner MO, Hameister H, Stephens K, Kehrer-Sawatzki H: Complete physical map and gene content of the human NF1 tumor suppressor region in human and mouse. Genes Chromosomes Cancer 2003;37:111–120.

13 Kehrer-Sawatzki H, Kluwe L, Sandig C, Kohn M, Wimmer K, et al: High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the *JJAZ1* gene. Am J Hum Genet 2004;75:410–423.

14 Messiaen LM, Callens T, Roux KJ, Mortier GR, De Paepe A, et al: Exon 10b of the *NF1* gene represents a mutational hotspot and harbors a recurrent missense mutation Y489C associated with aberrant splicing. Genet Med 1999;1:248–253.

15 Messiaen L, Callens T, De Paepe A, Craen M, Mortier G: Characterisation of two different nonsense mutations, C6792A and C6792G, causing skipping of exon 37 in the *NF1* gene. Hum Genet 1997;101:75–80.

16 Zatkova A, Messiaen L, Vandenbroucke I, Wieser R, Fonatsch C, et al: Disruption of exonic splicing enhancer elements is the principal cause of exon skipping associated with seven nonsense or missense alleles of NF1. Hum Mutat 2004;24:491–501.
17 Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, et al: Initial sequencing and comparative analysis of the mouse genome. Nature 2002;420:520–562.
18 Ars E, Kruyer H, Morell M, Pros E, Serra E, et al: Recurrent mutations in the *NF1* gene are common among neurofibromatosis type 1 patients. J Med Genet 2003;40:e82.
19 Perrin G, Morris MA, Antonarakis SE, Boltshauser E, Hutter P: Two novel mutations affecting mRNA splicing of the neurofibromatosis type 1 (*NF1*) gene. Hum Mutat 1996;7:172–175.
20 Raponi M, Upadhyaya M, Baralle D: Functional splicing assay shows a pathogenic intronic mutation in neurofibromatosis type 1 (NF1) due to intronic sequence exonization. Hum Mutat 2006;27:294–295.
21 Bausch B, Borozdin W, Mautner VF, Hoffmann MM, Boehm D, et al: Germline *NF1* mutational spectra and loss-of-heterozygosity analyses in patients with pheochromocytoma and neurofibromatosis type 1. J Clin Endocrinol Metab 2007;92:2784–2792.
22 Upadhyaya M, Huson SM, Davies M, Thomas N, Chuzhanova N, et al: An absence of cutaneous neurofibromas associated with a 3-bp inframe deletion in exon 17 of the *NF1* gene (c.2970—2972 delAAT): evidence of a clinically significant NF1 genotype-phenotype correlation. Am J Hum Genet 2007;80:140–151.
23 Ball EV, Stenson PD, Abeysinghe SS, Krawczak M, Cooper DN, Chuzhanova NA: Microdeletions and microinsertions causing human genetic disease: common mechanisms of mutagenesis and the role of local DNA sequence complexity. Hum Mutat 2005;26:205–213.
24 Lindahl T: Instability and decay of the primary structure of DNA. Nature 1993;362:709–715.
25 Robinson PN, Böddrich A, Peters H, Tinschert S, Buske A, et al: Two recurrent nonsense mutations and a 4 bp deletion in a quasi-symmetric element in exon 37 of the *NF1* gene. Hum Genet 1995;96:95–98.
26 DeBella K, Szudek J, Friedman JM: Use of the National Institutes of Health criteria for diagnosis of neurofibromatosis 1 in children. Pediatrics 2000;105:608–614.
27 Brems H, Chmara M, Sahbatou M, Denayer E, Taniguchi K, et al: Germline loss-of-function mutations in *SPRED1* cause a neurofibromatosis 1-like phenotype. Nat Genet 2007;39:1120–1126.

Ludwine M. Messiaen
Department of Genetics at UAB, Medical Genomics Laboratory
720 20th Street S
Birmingham – 35294 Alabama (USA)
Tel. +1 205 996 2915, Fax +1 205 996 2929, E-Mail lmessiaen@genetics.uab.edu

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 78–88

Clinical Phenotypes in Patients with *NF1* Microdeletions

S. Tinschert

Institut für Klinische Genetik, Medizinische Fakultät Carl Gustav Carus, TU Dresden, Dresden, Germany

Abstract

The *NF1* microdeletion syndrome is caused by haploinsufficiency of the *NF1* gene and neighbouring genes in its flanking regions. Most patients carry a constitutional type-1 1.4-Mb deletion comprising 14 genes. The second recurrent type-2 1.2-Mb deletion comprising 13 genes is usually present in a mosaic status resulting in milder phenotypes. Only a few patients with unusually sized *NF1* microdeletions have been described. A comprehensive genotype/phenotype correlation is lacking. The *NF1* microdeletion syndrome, in comparison to patients with intragenic *NF1* mutations, is characterized by a more severe clinical phenotype associated with a dysmorphic, coarse facial appearance, cognitive disabilities, growth disturbances, cardiovascular abnormalities, connective tissue abnormalities, skeletal abnormalities, the impression of earlier onset and large number of cutaneous neurofibromas, and a higher lifetime risk for the development of malignant peripheral nerve sheath tumours (MPNSTs). Mental retardation, dysmorphisms, and cardiac anomalies are the most observed extra NF1 clinical signs in patients with *NF1* microdeletions. If present, they should lead to the suspicion of an *NF1* microdeletion syndrome.

The first patient with a large constitutional *NF1* deletion was reported in 1992 [1]. It was presumed that mental impairment and facial dysmorphism of this patient may result from haploinsufficiency involving both the *NF1* gene and contiguous genetic material. Since then it was shown that about 5% of individuals with neurofibromatosis 1 (NF1) carry a deletion in 17q11.2 that encompasses the *NF1* gene and neighbouring genes lying in its flanking regions [2, 3]. Numerous reports on a more severe phenotype in these patients than in those with intragenic *NF1* mutations have been published [2, 4–24]. The condition caused by haploinsufficiency of the *NF1* gene and contiguous genes is now known as the '*NF1* microdeletion syndrome'. This syndrome, in comparison to

patients with intragenic mutations, is characterized by a severe manifestation associated with facial dysmorphism, learning disabilities, mental retardation, connective tissue abnormalities, cardiovascular abnormalities, and early onset and large number of cutaneous neurofibromas, together with a higher lifetime risk for the development of malignant peripheral nerve sheath tumours (MPNSTs) [9, 15, 20, 21].

Microdeletions (Large Deletions) of the *NF1*-Gene

Most of the large (genomic) *NF1* deletions are microdeletions from the cytogenetic point of view and are not detectable by the use of standard cytogenetic techniques. The terms 'large deletion' and 'microdeletion' are synonymical in this context.

The genomic architecture of 17q11 containing the *NF1* gene is characterized by several segmental duplications (also called repeated sequences (REPs), low copy repeats (LCRs), or paralogous sequences) that increase the susceptibility of genomic rearrangements resulting in deletions and duplications of the interval between the segmental duplications.

Two major types of *NF1* microdeletions have been identified. Both are caused by recombinations between low copy repeat sequences of the *NF1* gene region. The most common type-1 deletion encompasses 1.4 Mb and comprises 14 genes. It is mediated by recombination between the proximal to the *NF1* gene located LCR-P (NF1REPa) and the distal to the *NF1* gene located LCR-D (NF1REPc) [13, 25–28]. The less frequent type-2 deletion spans 1.2 Mb and comprises 13 of these genes. It is mediated by recombination between the *SUZ12* (*JJAZ1*) gene and its pseudogene, which are flanked by NF1REPc and NF1REPa, respectively [29, 30].

Whereas the type-1 deletions are constitutional deletions that arise by interchromosomal non-allelic homologous recombinations during maternal meiosis, the type-2 deletions arise by postzygotic intrachromosomal non-allelic homologous recombinations during mitosis, leading to somatic mosaics, meaning that the deletion is present in some but not in all cells [29–31]. All type-2 mosaic deletions have been found in female patients.

Constitutional type-2 deletions have so far been reported only in two patients. Therefore, it is not known if type-2 deletions result in a clinical phenotype being different from type-1 deletions. In both cases, the deletions were inherited from a mother who carried a mosaic deletion [29, 30].

Furthermore, besides the recurrent type-1 and type-2 deletions, a few patients with unusually sized *NF1* microdeletions have been described [1, 2, 5, 13, 15, 18, 21, 22, 32–35]. Whereas most of these atypical *NF1* microdeletions

are larger than 2 Mb ranging to 7 Mb, also some shorter deletions overlapping partially with the common 1.4-Mb interval have been reported.

In addition to fluorescence in situ hybridisation, the standard method to detect *NF1* microdeletions, other methods such as multiplex ligation-dependent probe amplification (MLPA) and locus specific microarray-CGH analysis have been shown to be suitable methods in deletion detection [30, 35, 36].

Genotype/Phenotype Correlations in Patients with *NF1* Microdeletions

Numerous studies have checked for genotype/phenotype correlations in NF1 [37, 38]. However, the wide diversity of *NF1* mutation types on the one hand and the wide range of the phenotypic spectrum of NF1 on the other hand have made it difficult to establish genotype/phenotype correlations. With the one most recently detected exception that a 3-bp deletion in exon 17 of the *NF1* gene is associated with a very mild NF1 phenotype lacking neurofibromas in most patients, genotype/phenotype correlation has been found only in patients with *NF1* microdeletions [39].

It has been suggested that genes located in the deleted region in 17q11.2 act as modifier genes and that several manifestations could be attributed to their hemizygosity. Fourteen genes have been identified in the common 1.4-Mb deletion interval. Their individual contribution to the phenotype however is unclear. Of particular interest with regard to genotype-phenotype correlations are unusually sized *NF1* microdeletions, which overlap only partially with the common 1.4-Mb deletion interval. Despite a large number of patients with known *NF1* microdeletions, the role of putative modifier genes has been difficult to assess because the number of patients reported with both comprehensive clinical information and good molecular characterisation of deletion size is small. So, the correlation between size of deletion and clinical phenotype remains incompletely defined.

Haploinsufficiency of the *OMG* and/or *CDK5R1* genes has been proposed to be implicated in learning disability, and haploinsufficiency of the *JJAZ1* and *CENTA2* genes in development of cardiovascular anomalies [21, 40].

Phenotypic Manifestations

In two large studies the incidences of several clinical features in patients with the *NF1* microdeletion syndrome and patients with intragenic *NF1* mutations were compared [21, 23]. The studies included 92 [21] and 157 patients

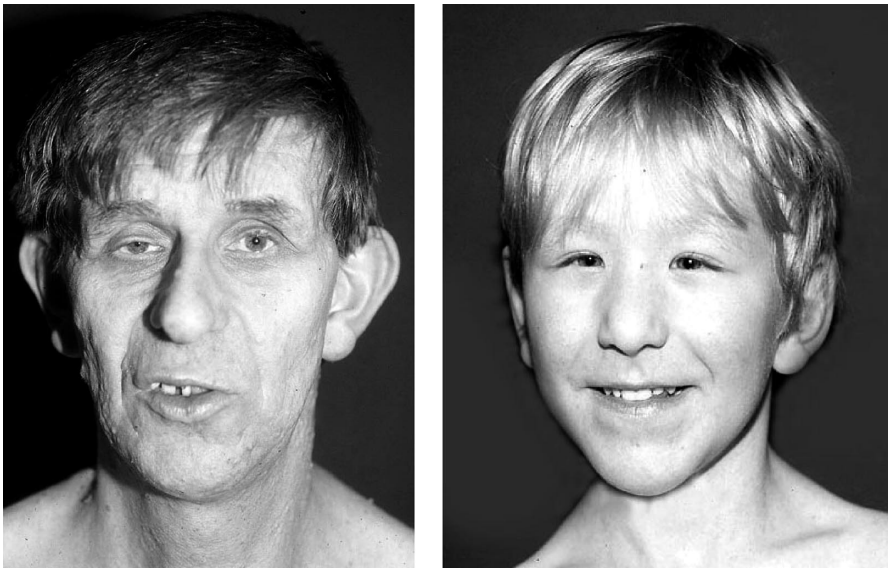


Fig. 1. Patients with *NF1* microdeletions. Left: Affected father (WEM) at 40 years of age. Right: His affected son (WER) at five years of age. Note facial coarsening with prominent nose and fleshy nasal tip, more pronounced in the father. Informed consent for publication of photographs was obtained.

[23], respectively and were based on an extensive literature review, and own observations. Two other comparative studies investigated single clinical signs (cognitive ability, MPNST risk and height growth, respectively) [20, 41, 42].

Craniofacial Dysmorphism

Dysmorphic craniofacial features (one or more) are significantly more frequent in patients with *NF1* microdeletions than in the general NF1 population (78–75% vs. 5–15%) [21, 23]. The most commonly noticed features were: hypertelorism, coarse face, broad, fleshy nose, broad nasal bridge, ptosis, downslanting palpebral fissures, and micrognathia. There is a common characteristic of a coarse dysmorphic face in the majority of patients, so that the general impression seems more specific than single dysmorphic signs [9, 21] (fig. 1). Presumably that there are other genes or modifiers influencing the pathways, resulting in craniofacial dysmorphism [21].

Patients with an *NF1* microdeletion are often described with dysmorphic features resembling Noonan syndrome (craniofacial dysmorphism, broad

and/or webbed neck, short stature, cardiac defect). Sometimes the same patient was described in multiple reports. In the following 14 patients Noonan syndrome-like appearance was noted at least once: UWA 166–1, [4, 8, 13], 166–2, [4, 8, 13], UWA169–1 [4, 8, 13], UWA 69–3 [5, 43], UWA 106–3 [1, 5, 13], UWA 123–3 [5, 13], UWA 128–3 [5, 13], UWA 160–1 [7, 13], UWA 176–1 [13], P-13 [2], 3–1, 3–2 [44], P-1 [23], P-7 [45].

Based on 157 patients with *NF1* microdeletions and reports on dysmorphisms in 118 of them [23], the incidence of Noonan syndrome-like features can be calculated to be 12% (14 of 118). In a systematic study of NF1 families, 9 of 94 individuals (9.5%) had signs of Noonan syndrome. Two patients carried an *NF1* microdeletion. Thus, the incidence of Noonan syndrome-like features in patients with classical NF1 was 7 of 92 (7.6%) [44].

Recently, the clinical overlap of NF1 and Noonan syndrome was elucidated. Both the gene products of *NF1* and of *PTPN11* (the causative gene in about half of the patients with Noonan syndrome) are involved in the signal transduction pathway of Ras. While SHP-2, the *PTPN11* gene product, is an upstream activator of Ras, neurofibromin (encoded by *NF1*) functions as an inactivating Ras-GTPase [46–48].

Weaver syndrome-like features were suspected in four patients with *NF1* microdeletions: a mother and her son [2, 49], and in three unrelated boys COS, ERS, and PFA [42]. For two of them (COS and PFA) the break points of type-1 deletions have been verified by deletion junction-specific PCRs. It was proposed that Weaver-like features belong to the phenotypic spectrum associated with the common *NF1* microdeletions [42].

Intellectual Impairment

Learning disabilities and attention deficit disorder are frequently observed in NF1 patients but mental retardation (MR) is not frequent [50]. Studies reveal mean full-scale IQ (FSIQ) scores between 88 and 99, thus within one standard deviation (=15) of the normal population [50].

Recently, it was confirmed that average intelligence in the group of individuals with *NF1* microdeletions is lower than in the group of NF1 individuals without a microdeletion [41], but MR was not as frequent as expected based on previous reports in the literature. An average full scale IQ difference of 12.5 points has been reported between the two groups (mean FSIQ in the *NF1* microdeletion group was 76.0; in the non-microdeletion group 88.5). Cognitive deficiency and/or developmental delay of various degrees, ranging from learning disability to severe mental retardation was found in 70% (83/118) of patients with microdeletions exceeding the 30–65% [51] expected in the general NF1

population [23]. Of 26 patients with known type-1 deletions 12 (46.1%) had mental retardation but only two (7.7%) had moderate to severe mental retardation. Because all of four patients with a deletion extending telomerically to the NF1REPb mapping close to the *NF1* 5′ region are affected by moderate or severe mental retardation, it was hypothesized that haploinsufficiency of one or more genes distally to NF1REPb, such as *CDK5R1*, plays a critical role in brain function or development, thus accounting for severe mental retardation in patients carrying such deletions [21].

It was suggested that increased frequency of MR in NF1 patients with microdeletions could be explained with a higher rate of brain malformations in this group [52]. However, this data was shown to be controversial.

Growth

In contrast to macrocephaly and short stature which are regarded distinctive traits of NF1 resulting from *NF1* haploinsufficiency, advanced body height is not part of the phenotypic spectrum of NF1. Anthropometric measurements and growth charts for the general NF1 population showed that height growth was nearly normal during childhood but decreased with increasing age [53–56].

Patients with the common *NF1* microdeletions display distinct growth anomalies. In contrast to classical NF1 patients, childhood overgrowth occurs in these patients [42]. In addition, evidence of bone age acceleration in *NF1* microdeletion patients was found [42]. This contrasted with findings in classical NF1 patients whose prepubertal bone age is delayed by 1–2 years [53].

Published data on height growth of patients with *NF1* microdeletions are sparse and an age dependency of their growth patterns has not been reported. Tall stature was found in 12% (19 of 157) of patients with *NF1* microdeletions [23].

Cardiovascular Malformations

Microdeletion syndromes are often associated with congenital heart defects [57]. This applies to *NF1* microdeletion syndrome, too [21]. It was shown that cardiovascular malformations are significantly more frequent in NF1 patients with microdeletions than in those without (20 vs. 2.1%) [21, 58]. The structural heart defects included pulmonic stenosis, atrial/ventricular septal defects, valve defects, hypertrophic cardiopathy and patent ductus arteriosus. These findings underline the importance of cardiological investigations as pointed out by Friedman and coworkers [59] for all patients with NF1 and of special care for those with microdeletions.

Connective Tissue Abnormalities

Connective tissue involvement is part of the clinical spectrum of the classic NF1 phenotype. Scoliosis, bone dysplasia, and vascular anomalies are common features.

Connective tissue abnormalities were present in 12% (19 of the 157) of patients with *NF1* microdeletions [23]. The most common feature was joint laxity (11 patients), followed by soft, fleshy palms (7 patients). Six patients including three from Venturin's group had mitral valve prolapse, in one patient secondary complication of bacterial endocarditis occurred, one patient had aortic dissection [6, 8, 19, 21].

Although comparison between NF1 patients with *NF1* microdeletions and those without microdeletions is difficult, the phenotype in the former group seems to be more pronounced [23].

Skeletal Anomalies

Skeletal anomalies were found to be relatively common in patients with *NF1* microdeletion [23]. Significant anomalies were found in 42 of 157 patients. The most common findings were scoliosis (18/42), pectus excavatum (14/42), and brachydactyly (9/42).

In a prior study no statistical differences in frequencies of scoliosis and pectus deformity in patients with microdeletions and patients of general NF1 population were shown [21]. The frequencies for scoliosis were 15% and 10 to 30%, respectively and for pectus deformity 17 and 20%, respectively.

Neurofibromas

The observation of an early onset of dermal neurofibromas (under the age of 10) and an increased number of such tumours relative to age was one of the first signs reported in connection with *NF1* microdeletions [1, 5, 13]. This general impression has been confirmed in many reports.

Despite the absence of precise data on number and onset of neurofibromas in most patients with *NF1* microdeletions, a quantification of tumours often as 'multiple' or 'numerous' was given for 145 of 157 patients reviewed by Mensink and coworkers [23]. Multiple subcutaneous neurofibromas were described in 61% (89 of 145). It has been suggested that one or several genes located in the common 1.4 Mb region act as modifier genes and that their hemizygosity might enhance neurofibroma growth.

Plexiform neurofibromas were present in 24% (34 of 141) [23] and 28% (25 of 88) [21]. This portion is similar to the 25–30% in the general NF1 population [21].

Malignancies

The lifetime risk for malignant peripheral nerve sheath tumour (MPNST) in individuals with NF1 was estimated to be 8–13% in a population-based study using a dual approach for ascertainment (NF1 register and Cancer registry) [60]. It was shown that NF1 patients with microdeletions have a substantially higher lifetime risk for the development of MPNSTs (16–26%) compared with the general NF1 group [20]. MPNSTs were reported in 19 of 145 (13%) of patients with *NF1* microdeletions [23].

A routine testing of all NF1 patients for the presence of a microdeletion and follow-up of those with microdeletions with a higher level of suspicion for MPNSTs has been proposed [20].

Conclusion

Although a large number of patients with *NF1* microdeletions have been reported in the last years, the precise clinical phenotype and correlations between specific clinical phenotypes and the deletion sizes remain to be defined. Future reports on patients with *NF1* microdeletions should include comprehensive clinical and molecular information.

The knowledge of specific phenotypic manifestations in patients with microdeletions is important in clinical management of this group of NF1 patients. Further, associations between phenotype and genotype shed light on the pathogenesis of specific features and may lead to the identification of genes in neighbourhood of the *NF1* gene or other modifying factors responsible for specific manifestations.

References

1 Kayes LM, Riccardi VM, Burke W, Bennett RL, Stephens K: Large de novo DNA deletion in a patient with sporadic neurofibromatosis 1, mental retardation, and dysmorphism. J Med Genet 1992;29:686–690.

2 Cnossen MH, van der Est MN, Breuning MH, van Asperen CJ, Breslau-Siderius EJ, et al: Deletions spanning the neurofibromatosis type 1 gene: implications for genotype-phenotype correlations in neurofibromatosis type 1? Hum Mutat 1997;9:458–464.

3 Kluwe L, Siebert R, Gesk S, Friedrich RE, Tinschert S, et al: Screening 500 unselected neurofibromatosis 1 patients for deletions of the *NF1* gene. Hum Mutat 2004;23:111–116.

4 Kaplan P, Rosenblatt B: A distinctive facial appearance in neurofibromatosis von Recklinghausen. Am J Med Genet 1985;21:463–470.

5 Kayes LM, Burke W, Riccardi VM, Bennett R, Ehrlich P, et al: Deletions spanning the neurofibromatosis 1 gene: identification and phenotype of five patients. Am J Hum Genet 1994;54:424–436.

6 Wu BL, Schneider GH, Korf BR: Deletion of the entire *NF1* gene causing distinct manifestations in a family. Am J Med Genet 1997;69:98–101.

7 Leppig KA, Viskochil D, Neil S, Rubenstein A, Johnson VP, et al: The detection of contiguous gene deletions at the neurofibromatosis 1 locus with fluorescence in situ hybridization. Cytogenet Cell Genet 1996;72:95–98.

8 Leppig KA, Kaplan P, Viskochil D, Weaver M, Ortenberg J, Stephens K: Familial neurofibromatosis 1 microdeletions: cosegregation with distinct facial phenotype and early onset of cutaneous neurofibromata. Am J Med Genet 1997;73:197–204.

9 Tonsgard JH, Yelavarthi KK, Cushner S, Short MP, Lindgren V: Do *NF1* gene deletions result in a characteristic phenotype? Am J Med Genet 1997;73:80–86.

10 Valero MC, Pascual-Castroviejo I, Velasco E, Moreno F, Hernández-Chico C: Identification of de novo deletions at the *NF1* gene: no preferential paternal origin and phenotypic analysis of patients. Hum Genet 1997;99:720–726.

11 Rasmussen SA, Colman SD, Ho VT, Abernathy CR, Arn PH, et al: Constitutional and mosaic large *NF1* gene deletions in neurofibromatosis type 1. J Med Genet 1998;35:468–471.

12 Upadhyaya M, Ruggieri M, Maynard J, Osborn M, Hartog C, et al: Gross deletions of the neurofibromatosis type 1 (*NF1*) gene are predominantly of maternal origin and commonly associated with a learning disability, dysmorphic features and developmental delay. Hum Genet 1998;102:591–597.

13 Dorschner MO, Sybert VP, Weaver M, Pletcher BA, Stephens K: *NF1* microdeletion breakpoints are clustered at flanking repetitive sequences. Hum Mol Genet 2000;9:35–46.

14 Lopez Correa C, Brems H, Lazaro C, Marynen P, Legius E: Unequal meiotic crossover: a frequent cause of *NF1* microdeletions. Am J Hum Genet 2000;66:1969–1974.

15 Riva P, Corrado L, Natacci F, Castorina P, Wu BL, et al: *NF1* microdeletion syndrome: refined FISH characterization of sporadic and familial deletions with locus-specific probes. Am J Hum Genet 2000;66:100–109.

16 Ruggieri M, D'Arrigo G, Abbate M, Distefano A, Upadhyaya M: Multiple coronary artery aneurysms in a child with neurofibromatosis type 1. Eur J Pediatr 2000;159:477–480.

17 Lampe AK, Seymour G, Thompson PW, Toutain A, Lynch SA: Familial neurofibromatosis microdeletion syndrome complicated by rhabdomyosarcoma. Arch Dis Child 2002;87:444–445.

18 Kehrer-Sawatzki H, Tinschert S, Jenne DE: Heterogeneity of breakpoints in non-LCR-mediated large constitutional deletions of the 17q11.2 *NF1* tumour suppressor region. J Med Genet 2003;40:e116.

19 Oktenli C, Saglam M, Demirbas S, Thompson P, Upadhyaya M, et al: A large deletion (1.5 Mb) encompassing the neurofibromatosis type 1 (*NF1*) gene in a patient with sporadic NF1 associated with dysmorphism, mental retardation, and unusual ocular and skeletal features. Clin Dysmorphol 2003;12:199–201.

20 De Raedt T, Brems H, Wolkenstein P, Vidaud D, Pilotti S, et al: Elevated risk for MPNST in NF1 microdeletion patients. Am J Hum Genet 2003;72:1288–1292.

21 Venturin M, Guarnieri P, Natacci F, Stabile M, Tenconi R, et al: Mental retardation and cardiovascular malformations in *NF1* microdeleted patients point to candidate genes in 17q11.2. J Med Genet 2004;41:35–41.

22 Kehrer-Sawatzki H, Kluwe L, Fünsterer C, Mautner VF: Extensively high load of internal tumors determined by whole body MRI scanning in a patient with neurofibromatosis type 1 and a non-LCR-mediated 2-Mb deletion in 17q11.2. Hum Genet 2005;116:466–475.

23 Mensink KA, Ketterling RP, Flynn HC, Knudson RA, Lindor NM, et al: Connective tissue dysplasia in five new patients with *NF1* microdeletions: further expansion of phenotype and review of the literature. J Med Genet 2006;43:e8.

24 Lopez-Correa C, Brems H, Lazaro C, Marynen P, Legius E: Unequal meiotic crossover: a frequent cause of *NF1* microdeletions. Am J Hum Genet 2000;66:1969–1974.
25 Lopez Correa C, Brems H, Lazaro C, Estivill X, Clementi M, et al: Molecular studies in 20 submicroscopic neurofibromatosis type 1 gene deletions. Hum Mutat 1999;14:387–393.
26 Jenne DE, Tinschert S, Stegmann E, Reimann H, Nürnberg P, et al: A common set of at least 11 functional genes is lost in the majority of NF1 patients with gross deletions. Genomics 2000;66: 93–97.
27 Jenne DE, Tinschert S, Reimann H, Lasinger W, Thiel G, Hameister H, Kehrer-Sawatzki H: Molecular characterization and gene content of breakpoint boundaries in patients with neurofibromatosis type 1 with 17q11.2 microdeletions. Am J Hum Genet 2001;69:516–527.
28 De Raedt T, Maertens O, Chmara M, Brems H, Heyns I, et al: Somatic loss of wild type *NF1* allele in neurofibromas: Comparison of *NF1* microdeletion and non-microdeletion patients. Genes Chromosomes Cancer 2006;45:893–904.
29 Petek E, Jenne DE, Smolle J, Binder B, Lasinger W, et al: Mitotic recombination mediated by the *JJAZF1 (KIAA0160)* gene causing somatic mosaicism and a new type of constitutional NF1 microdeletion in two children of a mosaic female with only few manifestations. J Med Genet 2003;40:520–525.
30 Kehrer-Sawatzki H, Kluwe L, Sandig C, Kohn M, Wimmer K, et al: High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the *JJAZ1* gene. Am J Hum Genet 2004;75:410–423.
31 Lopez-Correa C, Dorschner M, Brems H, Lázaro C, Clementi M, et al: Recombination hotspot in *NF1* microdeletion patients. Hum Mol Genet 2001;10:1387–1392.
32 Upadhyaya M, Roberts SH, Maynard J, Sorour E, Thompson PW, et al: A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay. J Med Genet 1996;33:148–152.
33 Jenne DE, Tinschert S, Dorschner MO, Hameister H, Stephens K, Kehrer-Sawatzki H: Complete physical map and gene content of the human NF1 tumor suppressor region in human and mouse. Genes Chromosomes Cancer 2003;37:111–120.
34 Venturin M, Gervasini C, Orzan F, Bentivegna A, Corrado L, et al: Evidence for non-homologous end joining and non-allelic homologous recombination in atypical *NF1* microdeletions. Hum Genet 2004;115:69–80.
35 Mantripragada KK, Thuresson AC, Piotrowski A, Díaz de Ståhl T, Menzel U, et al: Identification of novel deletion breakpoints bordered by segmental duplications in the *NF1* locus using high resolution array-CGH. J Med Genet 2006;43:28–38.
36 Wimmer K, Yao S, Claes K, Kehrer-Sawatzki H, Tinschert S, et al: Spectrum of single- and multiexon *NF1* copy number changes in a cohort of 1,100 unselected NF1 patients. Genes Chromosomes Cancer 2006;45:265–276.
37 Easton DF, Ponder MA, Huson SM, Ponder BA: An analysis of variation in expression of neurofibromatosis (NF) type 1 (NF1): evidence for modifying genes. Am J Hum Genet 1993;53:305–313.
38 Castle B, Baser ME, Huson SM, Cooper DN, Upadhyaya M: Evaluation of genotype-phenotype correlations in neurofibromatosis type 1. J Med Genet 2003;40:e109.
39 Upadhyaya M, Huson SM, Davies M, Thomas N, Chuzhanova N, et al: An absence of cutaneous neurofibromas associated with a 3-bp inframe deletion in exon 17 of the *NF1* gene (c.2970–2972 delAAT): evidence of a clinically significant NF1 genotype-phenotype correlation. Am J Hum Genet 2007;80:140–151.
40 Venturin M, Bentivegna A, Moroni R, Larizza L, Riva P: Evidence by expression analysis of candidate genes for congenital heart defects in the *NF1* microdeletion interval. Ann Hum Genet 2005;69(Pt 5):508–516.
41 Descheemaeker MJ, Roelandts K, De Raedt T, Brems H, Fryns JP, Legius E: Intelligence in individuals with a neurofibromatosis type 1 microdeletion. Am J Med Genet A 2004;131:325–326.
42 Spiegel M, Oexle K, Horn D, Windt E, Buske A, et al: Childhood overgrowth in patients with common *NF1* microdeletions. Eur J Hum Genet 2005;13:883–888.
43 Riccardi V, Eichner J: Neurofibromatosis: Phenotype, Natural History, and Pathogenesis, ed 1. The Johns Hopkins University Press, Baltimore and London 1986.

44 Colley A, Donnai D, Evans DG: Neurofibromatosis/Noonan phenotype: a variable feature of type 1 neurofibromatosis. Clin Genet 1996;49:59–64.
45 Hüffmeier U, Zenker M, Hoyer J, Fahsold R, Rauch A: A variable combination of features of Noonan syndrome and neurofibromatosis type I are caused by mutations in the *NF1* gene. Am J Med Genet A 2006;140:2749–2756.
46 Phillips RA, Hunter JL, Eccleston JF, Webb MR: The mechanism of Ras GTPase activation by neurofibromin. Biochemistry 2003;42:3956–3965.
47 Tartaglia M, Gelb BD: Noonan syndrome and related disorders: genetics and pathogenesis. Annu Rev Genomics Hum Genet 2005;6:45–68.
48 Gelb BD, Tartaglia M: Noonan syndrome and related disorders: dysregulated RAS-mitogen activated protein kinase signal transduction. Hum Mol Genet 2006;15 Spec No 2:R220–R226.
49 van Asperen CJ, Overweg-Plandsoen WC, Cnossen MH, van Tijn DA, Hennekam RC: Familial neurofibromatosis type 1 associated with an overgrowth syndrome resembling Weaver syndrome. J Med Genet 1998;35:323–327.
50 North KN, Riccardi V, Samango-Sprouse C, Ferner R, Moore B, et al: Cognitive function and academic performance in neurofibromatosis. 1: consensus statement from the NF1 Cognitive Disorders Task Force. Neurology 1997;48:1121–1127.
51 North K: Neurofibromatosis type 1. Am J Med Genet 2000;97:119–127.
52 Korf BR, Schneider G, Poussaint TY: Structural anomalies revealed by neuroimaging studies in the brains of patients with neurofibromatosis type 1 and large deletions. Genet Med 1999;1: 136–140.
53 Carmi D, Shohat M, Metzker A, Dickerman Z: Growth, puberty, and endocrine functions in patients with sporadic or familial neurofibromatosis type 1: a longitudinal study. Pediatrics 1999; 103:1257–1262.
54 Clementi M, Milani S, Mammi I, Boni S, Monciotti C, Tenconi R: Neurofibromatosis type 1 growth charts. Am J Med Genet 1999;87:317–323.
55 Szudek J, Birch P, Friedman JM: Growth charts for young children with neurofibromatosis 1 (NF1). Am J Med Genet 2000;92:224–228.
56 Virdis R, Street ME, Bandello MA, Tripodi C, Donadio A, et al: Growth and pubertal disorders in neurofibromatosis type 1. J Pediatr Endocrinol Metab 2003;16 Suppl 2:289–292.
57 Gelb BD: Recent advances in the understanding of genetic causes of congenital heart defects. Front Biosci 2000;5:D321–D333.
58 Lin AE, Birch PH, Korf BR, Tenconi R, Niimura M, et al: Cardiovascular malformations and other cardiovascular abnormalities in neurofibromatosis 1. Am J Med Genet 2000;95:108–117.
59 Friedman JM, Arbiser J, Epstein JA, Gutmann DH, Huot SJ, Lin AE, McManus B, Korf BR: Cardiovascular disease in neurofibromatosis 1: report of the NF1 Cardiovascular Task Force. Genet Med 2002;4:105–111.
60 Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A: Malignant peripheral nerve sheath tumours in neurofibromatosis 1. J Med Genet 2002;39:311–314.

Sigrid Tinschert
Institut für Klinische Genetik
Medizinische Fakultät Carl Gustav Carus, TU Dresden
Fetscherstraße 74, DE–01307 Dresden (Germany)
Tel. +49 351 458 4278, Fax +49 351 458 4316, E-Mail sigrid.tinschert@tu-dresden.de

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 89–102

Structure of the *NF1* Gene Region and Mechanisms Underlying Gross *NF1* Deletions

H. Kehrer-Sawatzki

Institute of Human Genetics, University of Ulm, Ulm, Germany

Abstract

Duplicated sequences such as the NF1REPs and the *SUZ12* sequences located within the *NF1* gene region predispose to gross *NF1* deletions, which constitute the most frequent recurrent mutations in NF1. Type-1 deletions of 1.4 Mb are mediated by non-allelic homologous recombination (NAHR) between the highly homologous NF1REPs in 17q11.2 and are the most common types of *NF1* gene deletion. Two distinctive NAHR recombination hotspots have been identified within the NF1REPs. Interestingly, they are paralogous to a hotspot of allelic homologous recombination (AHR) on chromosome 19. These findings imply the sequence conservation of recombination hotspots within paralogues on chromosomes 17 and 19, sequences which diverged at least 6 Mya. Furthermore, NAHR leading to pathological genomic rearrangements, and AHR occurring regularly during meiosis, seem to share many features such as frequent gene conversion close to sites of crossover. Less common than type-1 deletions are type-2 deletions which have breakpoints in the *SUZ12* gene and its pseudogene. The majority of type-2 deletions are associated with somatic mosaicism and are caused by mitotic NAHR. Obviously, positional preference exists in the *NF1* gene region with regard to mitotic versus meiotic recombination which is suggestive of different sequence requirements for both processes. In addition to the segmental duplications, copy number variations have been suggested to occur in the *NF1* gene region, which may further contribute to its instability.

Approximately 5% of all patients with neurofibromatosis type 1 (NF1) have large deletions in 17q11.2, which include the *NF1* gene and its flanking regions [1–3]. The majority of the *NF1* deletions encompass 1.4 Mb but they are often termed ‘microdeletions’ because they are too small to be identified by classical chromosome analysis. The standard method to detect *NF1*

microdeletions is fluorescence in situ hybridization (FISH) but, additionally, other methods such as multiplex ligation-dependent probe amplification (MLPA) and microarray analysis proved to be very useful [4, 5]. From the clinical point of view, *NF1* microdeletions are of great interest as they are frequently associated with a more severe manifestation of symptoms than observed in the general population of NF1 patients with intragenic mutations. This suggests that modifier genes may be located in the *NF1* gene region. The hemizygosity of one or several of these genes, in addition to the loss of the *NF1* gene, could directly or indirectly cause a more severe manifestation of the disease.

The deletion-associated phenotype includes an early onset of neurofibroma growth and a heavy burden of these tumors [reviewed in 6, 7]. Further *NF1* deletions are associated with a higher risk for malignant peripheral nerve sheath tumors, which are often life threatening with a very poor prognosis [8]. The average intelligence of *NF1* microdeletion patients is lower than in NF1 individuals without a microdeletion [9]. Additionally, patients with *NF1* microdeletions frequently have cardiac anomalies, connective tissue dysplasia, skeletal malformations and dysmorphic facial features [reviewed by 6, 7]. Furthermore, advanced height growth as well as accelerated carpal bone age is part of the phenotypic spectrum in patients with *NF1* microdeletions [10]. However, except for the assessment of cognitive abilities, comprehensive and standardized phenotype/genotype correlations in a large cohort of patients with precisely characterized deletion boundaries have not so far been performed. Furthermore, it would be necessary to include in these kinds of comparisons sex- and age-matched patients with intragenic *NF1* mutations, and this has not yet been done. It is therefore still unclear how significant the association between large *NF1* deletions and a severe phenotype is and to which extent the phenotype is influenced by the size of the deletion.

The *NF1* gene region has a complicated structure due to several segmental duplications that increase the susceptibility of genomic rearrangements such as microdeletions. Therefore, *NF1* deletions belong to the group of genomic disorders which are caused by the respective local genomic architecture [11–15]. Genomic disorders frequently result from recurrent DNA rearrangements mediated by non-allelic homologous recombination (NAHR) between low copy repeats (LCRs). LCRs usually encompass 10–400 kb of genomic DNA and exhibit more than 95% sequence identity. If they are located in direct orientation, they provide the substrates for NAHR resulting in deletions or duplications of the intervening sequences. The clinical phenotype associated with these genomic disorders is due to an abnormal dosage of gene(s) located within the rearranged regions. For many genomic disorders, reciprocal microduplication and microdeletion syndromes have been identified. As yet, however, no patients with duplications of the *NF1* gene region have been reported. This may be due

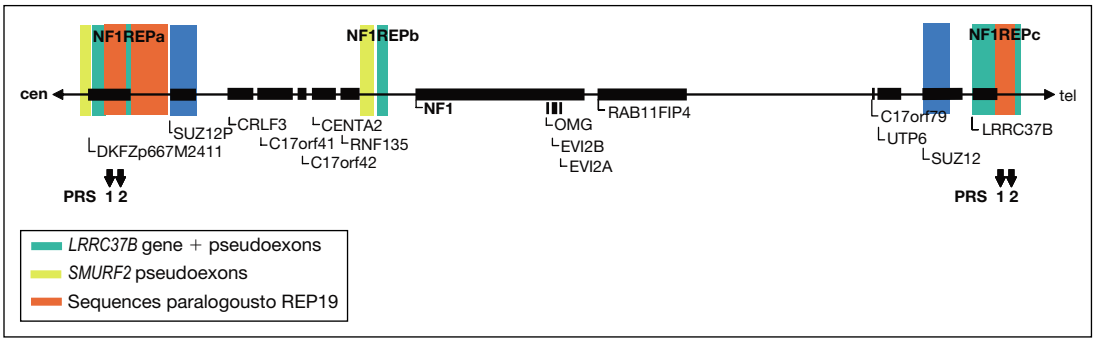


Fig. 1. Schematic representation of the *NF1* gene region. The genes located between and within the NF1REPs are indicated as black boxes with their designation given above. The position of the recombination hotspots PRS1 and 2 are indicated by arrows.

to a detection bias because the clinical phenotype associated with an *NF1* gene duplication is still unknown.

In the following, the structure of the *NF1* gene region, in particular of the LCRs located there and the mechanisms underlying these deletions, are discussed. Beyond increasing our knowledge about the *NF1* microdeletions themselves, the various studies cited have contributed considerably to our present understanding of the genesis of de novo genomic rearrangements and disorders.

Segmental Duplications in the *NF1* Gene Region

The *NF1* gene region is flanked by large segmental duplications with a high degree of homology (~96% sequence identity). These duplications have hampered the mapping of this region particularly at the time when not all genomic clones had been completely sequenced. Now, however, the genomic map of the *NF1* gene region is well established [16–18]. The proximal *NF1* low copy repeat (LCR) is located ~400 kb centromeric to the *NF1* gene. This duplicon, also termed NF1REPa, has high homology to NF1REPb, mapping close to the 5′ region of the *NF1* gene and to NF1REPc located distal to the *NF1* gene (fig. 1). The NF1REPs contain the *LRRC37B* gene and several pseudo-exons thereof. The functional *LRRC37B* gene is located in the distal NF1REPc. In addition to duplicated *LRRC37B* exons, NF1REPa contains exons of the *SMURF2* gene, whose functional copy is located at 17q24. Interestingly, the duplicated *SMURF2* exons 4–6 and exons 9–11 of the *LRRC37B* gene within NF1REPa are expressed and spliced together in one transcript (DKFZp667M2411)

suggesting that a new gene has been generated by the duplication and insertion events that had formed NF1REPa [17]. However, it is unknown whether this gene is functional or a pseudogene.

NF1REPa and NF1REPc additionally encompass sequences with homology to genomic segments originally located at 19p13.1 including parts of the intergenic segments between the *LPHN1* and the *CD97* genes as well as pseudo-exons of the *LPHN1* gene [16–18]. The region on chromosome 19, which contains sequences paralogous to the NF1REPs on chromosome 17, has been called REP19 [19].

In addition to the NF1REPs, further duplicated sequences are located in the *NF1* gene region. Separated by 40 kb of unduplicated sequence, the NF1REPc is flanked by the functional *SUZ12* gene, which encompasses 16 exons (fig. 1). A pseudogene copy of the *SUZ12P* gene containing exons 1–9 is located distal to NF1REPa. The protein encoded by the functional *SUZ12* gene is an important component of the Polycomb group of proteins which form complexes to regulate histone-methylation and gene silencing (table 1).

The complex structure of the LCRs in the human *NF1* gene region suggests that they originate from multiple rearrangements including duplications and transpositions. Comparative FISH analysis revealed that the orangutan and the rhesus macaque do not have sequences with homology to chromosome 19 in their *NF1* gene region. Thus, the transposition of the paralogous sequences from chromosome 19 to chromosome 17 must have occurred after the separation of the orangutan from the common ancestral lineage leading to humans, 7–9 Mya [17]. Interestingly, the murine *Nf1* gene region does not contain any segmental duplications at all [16].

Types of *NF1* Microdeletions

The most common *NF1* microdeletion type spans 1.4 Mb and is mediated by NAHR between the NF1REPs a and c [19–22]. In these type-1 deletions, two preferred regions of NAHR have been observed, PRS2 and PRS1 (fig. 1) [17, 18]. Of 60 type-1 deletions investigated, 40 had breakpoints in the 3.4 kb spanning PRS2 and 13/60 had breakpoints in PRS1 (1.8 kb) [19]. This clearly shows the presence of hotspots of NAHR in NF1REPa and c.

Type-2 deletions are the second most common type of recurrent *NF1* microdeletion. In these cases, the breakpoints are located within the *SUZ12* sequences which flank the NF1REPs (fig. 1). Type-2 deletions encompass 1.2 Mb and only three of them have been characterized so far with respect to the precise breakpoint localization [23, 24]. Thus, it is still unknown whether a preferred NAHR region occurs in the *SUZ12* sequences or if breakpoint

Table 1. Summary of the functional genes located in the *NF1* gene region between and within the NF1REPa and NF1REPc

Gene	Function of the encoded protein
DKFZp667M2411	The function of the encoded protein termed hypothetical protein LOC147172 is unknown. It maps to the NF1REPa.
CRLF3	The cytokine receptor like factor 3 has a fibronectin type III domain but its detailed function is unknown. Increased expression of *CRLF3* has been observed in cutaneous squamous cell carcinoma
ATAD5 (C17ORF41)	It associates with Rad9, is involved in the Bcl2 pathway and in a Rad9-related damage checkpoint important in determining whether DNA damage will be tolerated or whether the damaged cells will be eliminated by apoptosis
C17orf42	The function of this nuclear protein is unknown.
CENTA2	Centaurin alpha 2 contains an N-terminal ARF-GAP-like zinc finger motif, followed by 2 pleckstrin homology domains. Highly expressed in placenta, spleen, kidney, skeletal muscle, and adrenal gland. It binds to inositol 1,3,4,5-tetrakisphosphate and phosphatidylinositol 3,4,5-trisphosphate as an adaptor protein
RNF135	The function of the encoded protein which contains a zinc finger RING-type domain is unknown.
NF1	Neurofibromin is a GTPase activating protein with a SEC14 domain, modulates cytoskeleton-mediated-processes and acts as tumor suppressor.
OMG	The oligodendrocyte-myelin glycoprotein is highly expressed in the central nervous system and binds to the NOGO receptor A. This complex is a potent inhibitor of neurite outgrowth in cultured neurons. Interfering with the OMGP/NOGO receptor pathway may allow lesioned axons to regenerate after injury in vivo
EVI2B	The protein encoded by the ecotropic viral integration site 2B gene is an integral plasma membrane protein highly expressed in bone marrow, spleen and thymus. It is involved in cell differentiation and aberrantly expressed in chronic lymphocytic leukemia [54, 55].
EVI2A	The ecotropic viral integration site 2A protein is integral to the plasma membrane, has receptor activity and is highly expressed in bone marrow, spleen and thymus like *EVI2B*. In contrast to *EVI2B, EVI2A* is also highly expressed in brain and spinal cord. It may function as an oncogene in retrovirus-induced myeloid tumors.
RAB11FIP4	RAB11 family interacting protein 4 is strongly expressed in the brain and functions in the delivery of recycling endosomes to the cleavage furrow, binds to Arf6 and is together with Rab11 essential for completion of abscission, the terminal step of cytokinesis

Table 1. (continued)

Gene	Function of the encoded protein
C17orf79	The molecular function of the chromosome 17 open reading frame 79 encoded protein is unknown.
UTP6	The protein is homolog to the small subunit (SSU) processome component in yeast and has been identified as hepatocellular carcinoma-associated antigen 66
SUZ12	SUZ12 is a component of the Polycomb group complexes 2, 3, and 4, which are critical for proper embryonic development. Suz12 target promoters are transcription factors and homeobox proteins predominating in embryonal cells and glycoproteins and immunoglobulin-related proteins in adult tumors Mice lacking *Suz12* are not viable and die during early postimplantation stages displaying severe developmental and proliferative defects and specific loss of di- and trimethylated H3K27. SUZ12 is essential for the histone methyltransferase activity and the silencing function of the EED-EZH2 complex in vivo
LRRC37B	The function of the leucine rich repeat containing 37B protein is unknown. It is expressed in testis.

heterogeneity exists. Interestingly, type-2 deletions are frequently associated with somatic mosaicism of normal cells and those with the microdeletion. All mosaic patients identified so far have been females [24].

In addition to the recurrent type-1 and type-2 deletions, non-recurrent atypical deletions of different sizes have been observed. Currently, 19 atypical large *NF1* deletions have been reported [reviewed in 5, 25, 26]. Most of these deletions have unique breakpoints, except for three deletions characterized by Mantripragada et al. [5] that would appear to display spatially similar proximal breakpoints in intron 1 of the *NF1* gene. Several of these atypical *NF1* microdeletions are larger than 2 Mb although also some smaller deletions have been reported which partially overlap with the common 1.4 Mb deletion interval [reviewed in 26].

Mechanisms Underlying *NF1* Microdeletions

Type-1 *NF1* microdeletions are mediated by NAHR during maternal meiosis I [27, 28]. As already mentioned, the recombination breakpoints in type-1 *NF1* deletions are not randomly distributed but rather are located in two distinctive hotspots, PRS1 and PRS2 (fig. 1) [18, 19]. Similarly, a narrow NAHR hotspot of 600 bp has been observed in Charcot-Marie-Tooth disease 1A (CMT1A) and hereditary neuropathy with pressure palsies (HNPP) associated with microduplications and microdeletions in 17p11.2 [29, 30]. The narrow concentration of recombination hotspots in regions of 1–2 kb has also been observed in 'normal' allelic homologous recombination (AHR) during meiosis [31, 32]. Another similarity between AHR and NAHR is the frequent occurrence of gene conversion in breakpoint regions [22, 33–35]. This suggests that AHR and NAHR have many features in common. Interestingly, the position of recombination hotspots also seems to be conserved when paralogous sequences are compared. Population genetic data have revealed a hotspot of AHR in the REP19 sequences on chromosome 19, in regions paralogous to PRS1 and PRS2 of the NF1REPs [19]. This indicates conservation of recombination hotspots during AHR in REP19 and during NAHR in the NF1REPs leading to *NF1* microdeletions. The position of recombination hotspots in the paralogous sequences seems to be quite robust despite the long divergence times of these LCRs. The NF1REPs and REP19 diverged over 6 Mya, prior to the human-chimpanzee split. The conservation of recombination hotspots in paralogous sequences is quite remarkable since hotspots for recombination are generally not highly conserved between humans and chimpanzees [36–38]. Obviously, patterns of recombination between paralogous sequences in humans are more stable than those patterns between human and chimpanzee orthologues. The reason for this

difference is unclear but may be associated with interspecies deviations in epigenetic regulation or in the recombination machinery itself [19].

In contrast to type-1 deletions, which are constitutional deletions mediated by interchromosomal NAHR during maternal meiosis, type-2 deletions arise by intrachromosomal postzygotic mitotic recombination leading to somatic mosaicism between normal cells and those with the deletion [23, 24]. Thus, type-1 and type-2 deletions clearly differ from one another, not only with regard to breakpoint localization (NF1REPs versus *SUZ12* sequences), but also mechanistically. The reasons for the preferred occurrence of mitotic recombination in the *SUZ12* sequence and not in the closely flanking NF1REPs are unknown. Presumably, the sequence requirements for mitotic recombination are different from those favouring meiotic recombination. Interestingly, an AHR hotspot is not found in the *SUZ12* sequences, but instead within the NF1REPs according to the International HapMap project data (http://www.hapmap.org/index.html.en).

The breakpoints of the four type-2 deletions characterized so far appear to have been mediated by NAHR, but also type-2 deletions with non-homologous breakpoints have been identified [Steinmann et al., submitted]. This implies that not only NAHR but also a non-homology driven mechanism, such as non-homologous end joining (NHEJ) are involved in the genesis of type-2 deletions. NHEJ plays a prominent role in the non-recurrent rearrangements such as the atypical NF1 deletions [5, 6, 25, 26, 39]. In contrast to the common 1.4 Mb type-1 deletions, which are predominantly caused by interchromosomal recombination during maternal meiosis [28], six atypical deletions characterized in this regard occurred on the paternal chromosome by an intrachromosomal mechanism [16, 22, 25, 26, 40–42]. Non-homology driven mechanisms appear to occur predominantly during meiotic cell division in spermatogenesis.

Genes Located within and between the *NF1* LCRs

Fifteen functional genes are located within and between the NF1REPa and NF1REPc (fig. 1). Whilst type-1 deletions are associated with hemizygosity of 14 genes, type-2 deletions lead to the loss of only 13 genes, since the *LRRC37B* gene is not included within the bounds of type-2 deletions. So far, it is unknown whether type-2 deletions are associated with a different clinical phenotype as compared to type-1 deletions. Only two patients with constitutional type-2 deletions have so far been reported. In both cases, the deletions were inherited from a mosaic mother [23, 24]. Since many patients with large deletions have been reported to suffer from a high load of neurofibromas, it has been suggested that one or several genes located in the common 1.4 Mb region act as modifier genes and that their hemizygosity might enhance neurofibroma growth. However, little

is known about the function of these genes, apart from the *SUZ12* gene and the *NF1* gene itself (table 1). Deduced from its function, the SUZ12 protein is a good candidate for being a modifier of the deletion-associated phenotype. SUZ12 is part of the Polycomb group complex involved in the trimethylation of histone H3 on lysine 27, which has a widespread impact on many cellular processes such as differentiation, stem cell self-renewal and tumorigenesis [43–45].

Of particular interest with regard to genotype/phenotype correlations might be atypical deletions, which overlap only partially with the common 1.4 Mb deletion interval. Currently, eight deletions of this kind have been identified [reviewed in 26]. However, genotype-phenotype comparisons in these patients with atypical deletions partially overlapping the commonly deleted 1.4 Mb interval do not identify a specific deleted region that is associated with an increased number of neurofibromas.

Copy Number Variations in the *NF1* Gene Region

An important question is whether, in addition to the presence of segmental duplications, other structural features might exist that could serve to enhance the frequency of NAHR in the *NF1* gene region. As shown for other genomic disorders, polymorphic inversions of the respective regions facilitate chromosomal rearrangements in the offspring of inversion carriers (table 2). Genome-wide comparisons have revealed that polymorphic inversions are quite common and widespread throughout the human genome [46]. However, variant inversions of the *NF1* gene region have not so far been reported.

In addition to inversions, copy number variants (CNVs) might facilitate NAHR. CNVs have turned out to be a frequent source of normal human genomic variation. They include deletions, insertions and duplications larger than 1 kb and up to several Mb, and are found ubiquitously in the human genome [47, 48 and references therein]. Interestingly, three genome-wide studies performed to generate a global map of copy number variation in the human genome have indicated that CNVs also exist in the *NF1* gene region (table 3). One of the two identified CNVs is a duplication of the *NF1* gene [48, 49]. The second CNV represents a duplication of a 316-kb segment including the *SUZ12* gene and NF1REPc [47, 49]. The frequency of these variants in the normal population is currently unknown. Further analysis is needed to investigate whether these duplications are found at a high frequency in transmitting parents of patients with *NF1* microdeletions. This would help to establish whether these variants increase directly the susceptibility to NAHR events causing *NF1* microdeletions, most likely by misalignment or pairing of non-allelic but homologous sequences.

Table 2. Variant inversions predisposing to human disease

Disease/ structural rearrangement	Localization	Size of the inversion[b]	Frequency of the inversion in		Reference
			transmitting parents	controls	
Williams-Beuren syndrome (microdeletion)	7q11.23	1.5 Mb	25–33%	5%	58
Angelman syndrome (microdeletion)	15q11→q13	4 Mb	67%	9%	59
Sotos syndrome (*NSD1*) (microdeletion)	5q35.3	1.9 Mb	100%	67–75%	60
inv (dup)(8p), +der(8p)	8p23	nd	100%	26[d]	61
t(4;8)(p16;p23)[a]	4p16 8p23	nd nd	100%	12.5% 26[d]	62
17q21.31 microdeletion syndrome	17q21.31	900 kb	unknown[c]	20% of Europeans (H2 haplotype)	63–66

[a]Parents of individuals with t(4;8)(p16;p23) were shown to be heterozygous carriers of inversions at both 4p16 and 8p23. In the offspring of carriers, unbalanced karyotypes lead to Wolf-Hirschhorn syndrome if the der(4) chromosome is inherited.

[b]nd: not defined.

[c]The frequency of the H2 inversion haplotype in transmitting parents is not yet known since only 5 families have been analysed. However, in 4 of these 5 families, the transmitting parents were carriers of the inversion-specific haplotype [64, 65].

[d]2.5% are double heterozygotes.

Conclusion

Although the *NF1* gene region and microdeletions thereof have been studied in detail, many questions still remain open. First, a comprehensive genotype/phenotype correlation is lacking but this would be necessary to corroborate whether or not large deletions are significantly more often associated with a severe phenotype than has been observed in patients with intragenic *NF1* mutations. Further, the consequences of the size of the deletion should be addressed in this regard. These analyses would be very important for genetic counseling and the treatment of patients with *NF1* deletions. Second, the molecular basis of positional preference of mitotic versus meiotic non-allelic recombination in the *NF1* gene region is unexplained. Furthermore, it should be determined whether certain inherited haplotypes influence the risk of microdeletions. The answers to the latter two

Table 3. Copy number variants (CNVs) identified in the *NF1* gene region

Type of CNV	Coordinates in the human genome assembly 18	Genes included in the CNV	Number of individuals identified with this CNV	Method applied	Reference
Gain	27,245,834–27,562,095	*SUZ12, C17orf40, LRRC37B, RHOT1*	1 African (Yoruba)	Whole genome tiling path CGH array	47
Loss	~26,576,214–26,734,130	*NF1*	5	Whole genome tiling path CGH array	48
Gain	~26,576,214–26,734,130	*NF1*	1	Whole genome tiling path CGH array	48
Gain	insertion start at 26686418	*NF1*	1	Genome assembly comparsion[a]	49
Gain	insertion start at 27500745	*SUZ12*, *C17orf40, LRRC37B, RHOT1*	1	Genome assembly comparsion[a]	49

[a]In this study two human genome assemblies were compared by direct alignments (Celera's R27c compilation and the Build 35 reference sequence).

questions would contribute considerably to our understanding of the molecular mechanisms underlying *NF1* microdeletions and probably genomic disorders in general.

References

1 Cnossen MH, van der Est MN, Breuning MH, van Asperen CJ, Breslau-Siderius EJ, et al: Deletions spanning the neurofibromatosis type 1 gene: implications for genotype-phenotype correlations in neurofibromatosis type 1? Hum Mutat 1997;9:458–464.

2 Rasmussen SA, Colman SD, Ho VT, Abernathy CR, Arn PH, et al: Constitutional and mosaic large *NF1* gene deletions in neurofibromatosis type 1. J Med Genet 1998;35:468–471.

3 Kluwe L, Siebert R, Gesk S, Friedrich RE, Tinschert S, Kehrer-Sawatzki H, Mautner V-F: Screening of 500 unselected Neurofibromatosis 1 patients for deletions of the NF1 gene. Hum Mutat 2004;23:111–116.

4 Wimmer K, Yao S, Claes K, Kehrer-Sawatzki H, Tinschert S, et al: Spectrum of single- and multiexon NF1 copy number changes in a cohort of 1,100 unselected NF1 patients. Genes Chromosomes Cancer 2006;45:265–276.

5 Mantripragada KK, Thuresson AC, Piotrowski A, Diaz de Stahl T, Menzel U, et al: Identification of novel deletion breakpoints bordered by segmental duplications in the *NF1* locus using high resolution array-CGH. J Med Genet 2006;43:28–38.

6 Venturin M, Guarnieri P, Natacci F, Stabile M, Tenconi R, et al: Mental retardation and cardiovascular malformations in *NF1* microdeleted patients point to candidate genes in 17q11.2. J Med Genet 2004;41:35–41.

7 Mensink KA, Ketterling RP, Flynn HC, Knudson RA, Lindor NM, et al: Connective tissue dysplasia in five new patients with *NF1* microdeletions: further expansion of phenotype and review of the literature. J Med Genet 2006;43:e8.

8 De Raedt T, Brems H, Wolkenstein P, Vidaud D, Pilotti S, et al: Elevated risk for MPNST in NF1 microdeletion patients. Am J Hum Genet 2003;72:1288–1292.

9 Descheemaeker MJ, Roelandts K, De Raedt T, Brems H, Fryns JP, Legius E: Intelligence in individuals with a neurofibromatosis type 1 microdeletion. Am J Med Genet 2004;131A:325–326.

10 Spiegel M, Oexle K, Horn D, Windt E, Buske A, et al: Childhood overgrowth in patients with common NF1 microdeletions. Eur J Hum Genet 2005;13:883–888.

11 Emanuel BS, Shaikh TH: Segmental duplications: an ‘expanding’ role in genomic instability and disease. Nat Rev Genet 2001;2:791–800.

12 Inoue K, Lupski JR: Molecular mechanisms for genomic disorders. Annu Rev Genomics Hum Genet 2002;3:199–242.

13 Stankiewicz P, Lupski JR: Genome architecture, rearrangements and genomic disorders. Trends Genet 2002;18:74–82.

14 Shaw CJ, Lupski JR: Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. Hum Mol Genet 2004;13:R57–R64.

15 Lupski JR, Stankiewicz P: Genomic disorders: molecular mechanisms for rearrangements and conveyed phenotypes. PLoS Genet 2005;1:e49.

16 Jenne DE, Tinschert S, Dorschner MO, Hameister H, Stephens K, Kehrer-Sawatzki H: Complete physical map and gene content of the human NF1 tumor suppressor region in human and mouse. Genes Chromosomes Cancer 2003;37:111–120.

17 De Raedt T, Brems H, Lopez-Correa C, Vermeesch JR, Marynen P, Legius E: Genomic organization and evolution of the NF1 microdeletion region. Genomics 2004;84:346–360.

18 Forbes SH, Dorschner MO, Le R, Stephens K: Genomic context of paralogous recombination hotspots mediating recurrent NF1 region microdeletion. Genes Chromosomes Cancer 2004;41:12–25.

19 De Raedt T, Stephens M, Heyns I, Brems H, Thijs D, et al: Conservation of hotspots for recombination in low-copy repeats associated with the *NF1* microdeletion. Nat Genet 2006;38:1419–1423.

20 Dorschner MO, Sybert VP, Weaver M, Pletcher BA, Stephens K: *NF1* microdeletion breakpoints are clustered at flanking repetitive sequences. Hum Mol Genet 2000;9:35–46.

21 Jenne DE, Tinschert S, Reimann H, Lasinger W, Thiel G, Hameister H, Kehrer-Sawatzki H: Molecular characterization and gene content of breakpoint boundaries in patients with neurofibromatosis type 1 with 17q11.2 microdeletions. Am J Hum Genet 2001;69:516–527.

22 López-Correa C, Dorschner M, Brems H, Lazaro C, Clementi M, et al: Recombination hotspot in NF1 microdeletion patients. Hum Mol Genet 2001;10:1387–1392.

23 Petek E, Jenne DE, Smolle J, Binder B, Lasinger W, et al: Mitotic recombination mediated by the *JJAZF1* (KIAA0160) gene causing somatic mosaicism and a new type of constitutional NF1 microdeletion in two children of a mosaic female with only few manifestations. J Med Genet 2003;40:520–525.

24 Kehrer-Sawatzki H, Kluwe L, Sandig C, Kohn M, Wimmer K, et al: High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the *JJAZ1* gene. Am J Hum Genet 2004;75:410–423.

25 Kehrer-Sawatzki H, Kluwe L, Funsterer C, Mautner VF: Extensively high load of internal tumors determined by whole body MRI scanning in a patient with neurofibromatosis type 1 and a non-LCR-mediated 2-Mb deletion in 17q11.2. Hum Genet 2005;116:466–475.

26 Kehrer-Sawatzki H, Schmid E, Kluwe L, Funsterer C, Mautner VF: Absence of cutaneous neurofibromas in an NF1 patient with an atypical deletion partially overlapping the classical 1.4 Mb microdeleted region. Am J Hum Genet 2008:146:691–699.
27 Upadhyaya M, Ruggieri M, Maynard J, Osborn M, Hartog C, et al: Gross deletions of the neurofibromatosis type 1 (*NF1*) gene are predominantly of maternal origin and commonly associated with a learning disability, dysmorphic features and developmental delay. Hum Genet 1998;102: 591–597.
28 López-Correa C, Brems H, Lazaro C, Marynen P, Legius E: Unequal meiotic crossover: a frequent cause of *NF1* microdeletions. Am J Hum Genet 2000;66:1969–1974.
29 Reiter LT, Murakami T, Koeuth T, Pentao L, Muzny DM, Gibbs RA, Lupski JR: A recombination hotspot responsible for two inherited peripheral neuropathies is located near a mariner transposon-like element. Nat Genet 1996;12:288–297.
30 Lindsay SJ, Khajavi M, Lupski JR, Hurles ME: A chromosomal rearrangement hotspot can be identified from population genetic variation and is coincident with a hotspot for allelic recombination. Am J Hum Genet 2006;79:890–902.
31 Jeffreys AJ, Kauppi L, Neumann R: Intensely punctate meiotic recombination in the class II region of the major histocompatibility complex. Nat Genet 2001;29:217–222.
32 McVean GAT, Myers SR, Hunt S, Deloukas P, Bentley DR, Donnelly P: The fine-scale structure of recombination rate variation in the human genome. Science 2004;304:581–584.
33 Hurles M: Gene conversion homogenizes the CMT1A paralogous repeats. BMC Genomics 2001;2:11.
34 Bi W, Park SS, Shaw CJ, Withers MA, Patel PI, Lupski JR: Reciprocal crossovers and a positional preference for strand exchange in recombination events resulting in deletion or duplication of chromosome 17p11.2. Am J Hum Genet 2003;73:1302–1315.
35 Jeffreys AJ, May CA: Intense and highly localized gene conversion activity in human meiotic crossover hot spots. Nat Genet 2004;36:151–156.
36 Winckler W, Myers SR, Richter DJ, Onofrio RC, McDonald GJ, et al: Comparison of fine-scale recombination rates in humans and chimpanzees. Science 2005;308:107–111.
37 Ptak SE, Roeder AD, Stephens M, Gilad Y, Pääbo S, Przeworski M: Absence of the TAP2 human recombination hotspot in chimpanzees. PLoS Biol 2004;2:e155.
38 Ptak SE, Hinds DA, Koehler K, Nickel B, Patil N, et al: Fine-scale recombination patterns differ between chimpanzees and humans. Nat Genet 2005;37:429–434.
39 Kehrer-Sawatzki H, Tinschert S, Jenne DE: Heterogeneity of breakpoints in non-LCR-mediated large constitutional deletions of the 17q11.2 NF1 tumour suppressor region. J Med Genet 2003; 40:E116.
40 Kayes LM, Burke W, Riccardi VM, Benett R, Ehrlich P, Rubinstein A, Stephens K: Deletions spanning the neurofibromatosis I gene: identification and phenotype of five patients. Am J Hum Genet 1994;54:424–436.
41 Upadhyaya M, Roberts SH, Maynard J, Sorour E, Thompson PW, et al: A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay. J Med Genet 1996;33:148–152.
42 López Correa C, Brems H, Lazaro C, Estivill X, Clementi M, et al: Molecular studies in 20 submicroscopic neurofibromatosis type 1 gene deletions. Hum Mutat 1999;14:387–393.
43 Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E, Helin K: Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. EMBO J 2004;23:4061–4071.
44 de la Cruz CC, Fang J, Plath K, Worringer KA, Nusinow DA, Zhang Y, Panning B: Developmental regulation of Suz 12 localization. Chromosoma 2005;114:183–192.
45 Squazzo SL, O'Geen H, Komashko VM, Krig SR, Jin VX, et al: Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome Res 2006;16:890–900.
46 Tuzun E, Sharp AJ, Bailey JA, Kaul R, Morrison VA, et al: Fine-scale structural variation of the human genome. Nat Genet 2005;37:727–732.
47 Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, et al: Global variation in copy number in the human genome. Nature 2006;444:444–454.
48 Wong KK, Deleeuw RJ, Dosanjh NS, Kimm LR, Cheng Z, et al: A comprehensive analysis of common copy-number variations in the human genome. Am J Hum Genet 2007;80:91–104.

49 Khaja R, Zhang J, Macdonald JR, He Y, Joseph-George AM, et al: Genome assembly comparison identifies structural variants in the human genome. Nat Genet 2006;38:1413–1418.
50 Dang C, Gottschling M, Manning K, O'Currain E, Schneider S, et al: Identification of dysregulated genes in cutaneous squamous cell carcinoma. Oncol Rep 2006;16:513–519.
51 Ishii H, Inageta T, Mimori K, Saito T, Sasaki H, et al: Frag1, a homolog of alternative replication factor C subunits, links replication stress surveillance with apoptosis. Proc Natl Acad Sci USA 2005;102:9655–9660.
52 Hanck T, Stricker R, Sedehizade F, Reiser G: Identification of gene structure and subcellular localization of human centaurin alpha 2, and p42IP4, a family of two highly homologous, Ins 1,3,4,5-P4-/PtdIns 3,4,5-P3-binding, adapter proteins. J Neurochem 2004;88:326–336.
53 Wang Y, Han KJ, Pang XW, Vaughan HA, Qu W, et al: Large scale identification of human hepatocellular carcinoma-associated antigens by autoantibodies. J Immunol 2002;169:1102–1109.
54 Kaufmann D, Gruener S, Braun F, Stark M, Griesser J, Hoffmeyer S, Bartelt B: EVI2B, a gene lying in an intron of the neurofibromatosis type 1 (*NF1*) gene, is as the *NF1* gene involved in differentiation of melanocytes and keratinocytes and is overexpressed in cells derived from NF1 neurofibromas. DNA Cell Biol 1999;18:345–356.
55 Aalto Y, El-Rifa W, Vilpo L, Ollila J, Nagy B, et al: Distinct gene expression profiling in chronic lymphocytic leukemia with 11q23 deletion. Leukemia 2001;15:1721–1728.
56 Fielding AB, Schonteich E, Matheson J, Wilson G, Yu X, et al: Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. EMBO J 2005;24:3389–3399.
57 Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z: Oligodendrocyte-myelin glycoprotein in a Nogo receptor ligand that inhibits neurite outgrowth. Nature 2002;417:941–944.
58 Osborne LR, Li M, Pober B, Chitayat D, Bodurtha J, et al: A 1.5 million-base pair inversion polymorphism in families with Williams-Beuren syndrome. Nat Genet 2001;29:321–325.
59 Gimelli G, Pujana MA, Patricelli MG, Russo S, Giardino D, et al: Genomic inversions of human chromosome 15q11–q13 in mothers of Angelman syndrome patients with class II (BP2/3) deletions. Hum Mol Genet 2003;12:849–858.
60 Visser R, Shimokawa O, Harada N, Kinoshita A, Ohta T, Niikawa N, Matsumoto N: Identification of a 3.0-kb major recombination hotspot in patients with Sotos syndrome who carry a common 1.9-Mb microdeletion. Am J Hum Genet 2005;76:52–67.
61 Giglio S, Broman KW, Matsumoto N, Calvari V, Gimelli G, et al: Olfactory receptor-gene clusters, genomic-inversion polymorphisms, and common chromosome rearrangements. Am J Hum Genet 2001;68:874–883.
62 Giglio S, Calvari V, Gregato G, Gimelli G, Camanini S, et al: Heterozygous submicroscopic inversions involving olfactory receptor–gene clusters mediate the recurrent t(4;8) (p16; p23) translocation. Am J Hum Genet 2002;71:276–285.
63 Stefansson H, Helgason A, Thorleifsson G, Steinthorsdottir V, Masson G, et al: A common inversion under selection in Europeans. Nat Genet 2005;37:129–137.
64 Koolen DA, Vissers LE, Pfundt R, de Leeuw N, Knight SJ, et al: A new chromosome 17q21.31 microdeletion syndrome associated with a common inversion polymorphism. Nat Genet 2006;38:999–1001.
65 Sharp AJ, Hansen S, Selzer RR, Cheng Z, Regan R, et al: Discovery of previously unidentified genomic disorders from the duplication architecture of the human genome. Nat Genet 2006;38:1038–1042.
66 Shaw-Smith C, Pittman AM, Willatt L, Martin H, Rickman L, et al: Microdeletion encompassing *MAPT* at chromosome 17q21.3 is associated with developmental delay and learning disability. Nat Genet 2006;38:1032–1037.

Hildegard Kehrer-Sawatzki
Institute of Human Genetics, University of Ulm
Albert-Einstein-Allee 11
DE–89081 Ulm (Germany)
Tel. +49 731 5006 5422, Fax +49 731 5006 5402, E-Mail hildegard.kehrer-sawatzki@uni-ulm.de

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 103–112

NF1 Gene Evolution in Mammals

G. Assum, C. Schmegner

Institut für Humangenetik, Universität Ulm, Ulm, Germany

Abstract

In the review presented here three aspects of the *NF1* gene evolution in mammals are considered: (1) Comparisons of amino acid sequences of neurofibromin of human, chimpanzee, dog, mouse and rat demonstrate a very high structural and functional constraint, which led to the evolutionary conservation of almost all amino acids of the protein. The only exception is a small part encoded by exon 12b of the *NF1* gene. This constraint seems to be higher in nonrodent than in rodent species. (2) The structural analysis of the *NF1* gene region in various mammalian species revealed a number of chromosomal rearrangements which altered the order of genes in the direct neighborhood of *NF1*. Despite these rearrangements the isochore structure at the locus remained conserved. (3) SNP analysis in different human populations showed a very high degree of linkage disequilibrium throughout the whole *NF1* gene. This fact enabled the unambiguous deduction of haplotypes from genotypes after resequencing of intronic parts of *NF1* in a European population. The resulting haplotype structure revealed no signs of positive selection of *NF1* during the recent evolution of anatomically modern humans. Taken together these facts show that the evolution of *NF1* in mammals is clearly dominated by a strong selective pressure on the correct structure and function of neurofibromin.

Evolution of the Coding Sequences of *NF1*

The evolution of the *NF1* gene in mammals is clearly dominated by a strong selective pressure on the correct function of the gene product, neurofibromin. This can be demonstrated for distantly related mammalian species, for the more recent evolution in primate species, and in human populations.

A remarkably high degree of *NF1* sequence conservation between human and mouse was first described by Bernards et al. [1], who found more than 98% sequence identity between the neurofibromin amino acid sequences of these two species. Only a small part of the protein, namely the 52 amino acids in human and the 54 in mouse that are encoded by *NF1* exon 12b, showed a higher

Table 1. Sequence conservation between human, dog, mouse and rat

Species	*NF1*[a]				*OMGP*		*EVI2B*[a]	
	% identity			K_A/K_S	% identity	K_A/K_S	% identity	K_A/K_S
	overall	without exon 12b	only exon 12b					
human/dog	99.4	99.6	88.9	0.02	95.9	0.177	74.5	0.85
human/mouse	98.4	98.9	72.2	0.025	88.8	0.266	55.7	0.462
dog/mouse	98.3	98.8	74.1	0.024	89.5	0.215	52.7	1.02
human/rat	98.5	99.0	77.8	0.023	89.5	0.248	–	–
dog/rat	98.4	98.9	77.8	0.021	89.5	0.238	–	–
mouse/rat	98.6	99.0	83.3	0.042	94.8	0.228	–	–

[a] K_A: nonsynonymous basepair exchanges per nonsynonymous site; K_S: synonymous basepair exchanges per synonymous site.

degree of divergence. Exon 12b also carries the only insertion/deletion variant found in the whole protein of both species. We now added further data about the neurofibromin of the dog and the rat to this comparison. As presented in table 1, these data underline the very high level of sequence identity of neurofibromin of all four species; the sequence conservation especially becomes evident when it is compared to the data obtained for *OMGP* and *EVI2B*, two of the three smaller genes embedded in exon 27b of *NF1*. The part of the protein that is encoded by exon 12b is again the only exception. Amino acid sequence comparisons for this part of the protein resulted for all four species in severely reduced values of sequence identity when compared to the rest of the protein. Nevertheless even that part of neurofibromin is under a functional constraint: In addition to a still relatively high degree of evolutionary conservation, a missense mutation (C1194T; S665F) leading to neurofibromatosis type 1 is described in at least one patient [2]; the amino acid affected by this mutation is conserved in all four species.

A further measure for the selective constraint acting upon neurofibromin are the K_A/K_S values (given in table 1). These values result from a comparison of the nonsynonymous basepair exchanges per nonsynonymous site (K_A) with the synonymous basepair exchanges per synonymous site (K_S) between two species. Sequences which are devoid of selective pressures should result in K_A/K_S values around one. Values lower than one result from forces of purifying

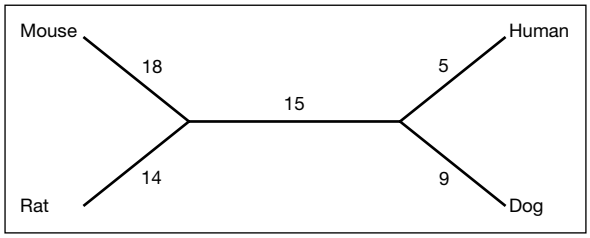


Fig. 1. Amino acid exchanges derived from a comparison of neurofibromin sequences from human, dog, mouse and rat. The numbers indicate the distribution of the altogether 61 divergent sites between the four species.

selection acting upon a gene (for review see [3]). The remarkably low values obtained for the *NF1* gene for all comparisons between the four species demonstrate a strong functional and structural constraint on almost all sites of this large protein.

Analysis of sequences from more than two species enables the assignment of mutations leading to amino acid exchanges to individual lineages. The comparison of neurofibromin sequences from the four species mentioned above led to the identification of altogether 68 divergent sites. Seven of these divergent sites could not be assigned unambiguously to individual lineages because more than two different amino acids were present at the respective sites in the four species; five of these seven sites are located in the relatively variable part of neurofibromin encoded by exon 12b. The results obtained for the remaining 61 divergent sites are given in figure 1. As depicted there, human and dog group together and are separated from the two rodent species by 15 sites where they together diverge from both mouse and rat. This fact points to a closer relationship between primates and carnivores than between either primates and rodents or carnivores and rodents, and corroborates results recently published by Cannarozzi et al. [4]. Using chicken sequences as an outgroup, 13 of these 15 sites could be assigned to the rodent lineage, one to the lineage leading to primates and carnivores, and one site could not be assigned unambiguously. This striking difference in the lengths of the branches leading to rodents on the one side and to carnivores and primates on the other may result from differences in the functional and structural constraint on neurofibromin present in the three lineages. This interpretation is substantiated by the relatively high number of 32 divergent sites between mouse and rat compared to only 14 divergent sites found in the human/dog comparison. The higher degree of divergence found between the rodent species compared to human and dog becomes even more prominent when the divergence times between the lineages are considered. The primate and

Table 2. Nonsynonymous human SNPs of *NF1* in the GenBank database

SNP ID	Polymorphic basepairs	Polymorphic amino acids	Population frequency
rs1051841	1689 A/G	496 I/M	100% G in 252 chromosomes
rs9907627	3476 A/G	1089 K/E	100% G in 836 chromosomes
rs17884349	4412 C/T	1401 H/Y	98.9% C in 174 chromosomes
rs9891398	7206 C/A	2332 A/D	100% A in 828 chromosomes
rs17885083	7679 C/G	2490 L/V	99.4% G in 180 chromosomes

carnivore lineages diverged from each other 75 million years ago, whereas the mouse and rat lineages diverged only twelve to 24 million years ago [5, 6]. The strong functional and structural constraint on the neurofibromin protein in primates is further underscored by the 100% identity of the amino acid sequences of human and chimpanzee (divergence time six million years), and by the absence of nonsynonymous SNPs with minor allele frequencies above 1% in the human population (all nonsynonymous human SNPs present in the GenBank database are listed in table 2). In summary these data show that the constraint on neurofibromin in rodents may not be as high as in other mammalian species, thereby allowing for amino acid exchanges at more sites of the protein.

Evolution of the *NF1* Gene Region

During primate evolution a number of chromosomal rearrangements occurred which severely altered the gene order in the *NF1* gene region and affected the immediate neighborhood of *NF1*. A comparison of the gene order of the respective regions in mouse, dog, rhesus macaque, chimpanzee and human, as taken from the NCBI map viewer, is given in figure 2. As mouse and dog, two species belonging to two different groups of mammals, show the same order of genes, the state of the region in these species may be taken as ancestral. A first inversion of an approximately 3-Mb region between the genes *WSB1* and *GOSR1*, located immediately upstream of *NF1*, occurred early during primate evolution and is present in all primate species tested. This inversion may therefore be specific for either all primates or for old world monkeys only. A further complex rearrangement that may be explained by three inversion events, resulted in an exchange of a 700-kb region located between *NF1* and *SUZ12* and a 200-kb region between *CLRF3* and *RNF135*, and led to the gene order found in human and chimpanzee. Whether this rearrangement is specific only

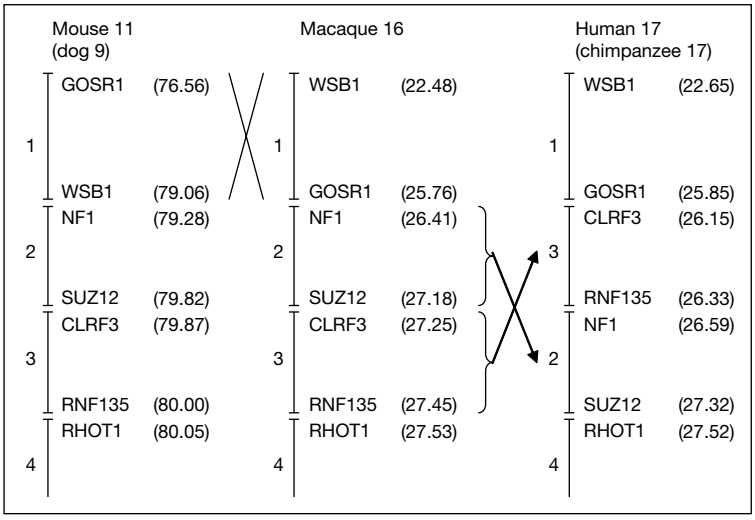


Fig. 2. Comparative gene order in the *NF1* gene region of mouse, macaque and human. Rearrangements between the four major blocks numbered 1–4 are indicated; the position on the respective chromosome is given in parenthesis.

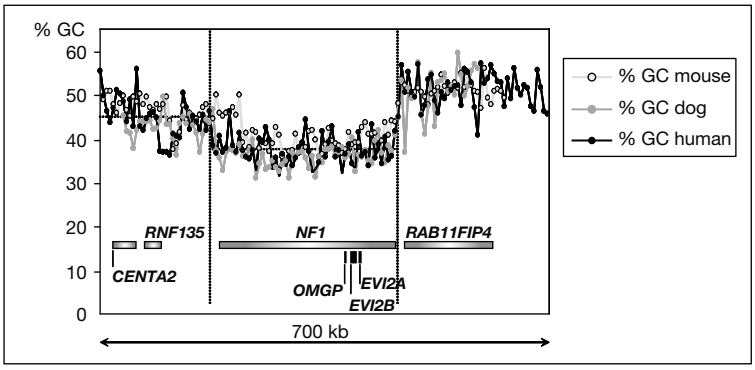


Fig. 3. GC-content curves for the *NF1* gene region of human, dog and mouse. Below these curves, the position of the genes in the region is indicated.

for these two species or for the hominid group as a whole cannot be told due to lacking sequence information. Despite these rearrangements the sequence composition as reflected in the isochore structure of the region, remained conserved in all species tested. As an example the long range GC content of the *NF1* gene region in mouse, dog and human is given in figure 3.

Isochores, i.e. long stretches of a few hundred kb to several Mb of DNA with a relatively homogeneous GC content, are fundamentally structural and functional units of the mammalian genome [7, 8]. In all species tested here the *NF1* gene in its whole length is GC-poor with less than 40% GC. Immediately upstream of the *NF1* gene the long range GC content of the DNA sequences raises to 45% in all species irrespective of the fact that *NF1* is neighbored by three different genes in the five species. Downstream, in the intergenic region between *NF1* and *RAB11FIP4*, the GC content raises to over 50% in all species, with a defined transition zone of only 5 kb between the neighboring isochores. Therefore, the sequence composition in the *NF1* gene region is an impressive example for the conservation of the isochore structure throughout mammalian evolution. This conservation has been demonstrated before for several loci in a number of species [7] and for the whole genomes of mouse and human [9]. Moreover the isochore boundary between *NF1* and *RAB11FIP4* is also a boundary between regions with low and high recombination frequencies [10] and between replication time zones [11, 12], with *RAB11FIP4* being replicated early and *NF1* late in the S phase of the cell cycle. This astonishing conservation of the composition of noncoding sequences demonstrates the existence of forces which exert their effects to stabilize the structure of the mammalian genome. Three mechanisms are under discussion as candidates for these forces: the regional variation of mutation biases, and two forces which lead to biases in the fixation rates of different mutations in a population – namely direct selection on the GC content and biased gene conversion (for review see [13]).

For the *NF1* gene region as well as for other regions in the human genome, a bias in the mutational input can be excluded as reason for the stability of the isochore pattern [14–17]. The analysis of the pattern of polymorphic sites, which can be taken as a proxy for the mutation pattern, demonstrated a higher probability for a GC basepair to mutate into an AT basepair than vice versa in both isochores tested, irrespective of the GC content. This mutation bias directly explained the GC content of the GC-poor isochore, whereas it is supposed to decrease in the GC content of the GC-rich isochore over time. However, the analysis of divergent sites between human and chimpanzee (when compared to gorilla as an outgroup member) showed equal numbers of substitutions of GC basepairs by AT basepairs and AT basepairs by GC basepairs in both isochores [14]. This fact points to an evolutionarily stable GC content of the GC-poor and the GC-rich isochore and directly demonstrates the action of a fixation bias in favor of mutations that replace AT by GC basepairs in the GC-rich isochore. Whether the fixation bias, which could also be demonstrated for other genomic regions [18–21], is the result of selection on the GC content or of biased gene conversion cannot be deduced from these data. The results of these

analyses also revealed differences in the SNP densities and in the human chimpanzee divergence between the two analyzed isochores of the *NF1* gene region. Both the SNP densities and the interspecies divergence were found to be significantly lower in the noncoding parts of the GC-poor *NF1* gene than in the neighboring GC-rich *RAB11FIP4* gene. These facts point to differences in the mutation rates in the two isochores with a relatively low rate in the GC-poor *NF1* gene and a higher rate in the GC-rich isochore, and corroborate results published earlier for other genomic regions [22]. Therefore the relatively high frequency of sporadic cases of neurofibromatosis type 1 (1 in 7,000 newborns) may not be due to a high mutation rate per basepair in the *NF1* gene but may result from the large size of the gene and the high number of sites which lead to the disease when mutated. An alternative explanation comes from the assumption of the presence of evolutionarily conserved regulatory elements in *NF1* introns. However the latter explanation seems to be unlikely because 25 kb of sequences were analyzed to obtain the SNP-densities and several hundred kb for the interspecies divergence.

Evolution of the *NF1* Gene in Human Populations

Genetic variability data derived from genomic loci that show a sufficient number of polymorphic sites in combination with a very low recombination rate can be used to infer the demographic history of the anatomically modern human (AMH). The *NF1* gene region is predisposed for that kind of analysis as the whole *NF1* gene is located in an approximately 300-kb region with long range linkage disequilibrium (LD) [10, 23–25]. In centromeric direction the long range LD ends within the 87 kb of intergenic sequences between *NF1* and the *RNF135* gene; the telomeric boundary is located in the intergenic region between *NF1* and the *RAB11FIP4* gene. Besides *NF1* three further genes (*EVI2A*, *EVI2B* and *OMGP*) that are embedded in its intron 27b are located in the region of high LD.

A combined resequencing and SNP typing project of *NF1* sequences in a European population [26] identified an adequate amount of polymorphic sites that due to the low recombination rate allowed the unambiguous deduction of haplotypes from genotypes. A differentiated analysis of these haplotypes disclosed the presence of two well separated subgroups of *NF1* sequences. In a phylogenetic tree of European *NF1* sequences these two subgroups appear as two well separated, old branches (fig. 4). *NF1* sequences within the same subgroup show relatively little variability.

One possible explanation for the deep split in the phylogenetic tree is the action of balancing selection, that may lead to the retention of two lineages for

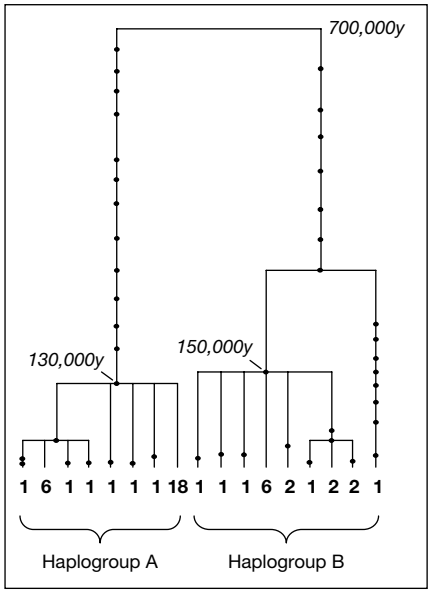


Fig. 4. Phylogenetic tree derived from European *NF1* sequences. The tree can be subdivided into two major subgroups of haplotypes, haplogroups A and B. Mutations on the branches are indicated by black circles. Bold numbers below the tree give the number of identical haplotypes observed. Italicized dates give the TMRCA in years for the whole sample and for both subgroups.

an extended period of time (for review see [27]). All genes located in the region of high LD (i.e. *NF1*, *OMGP*, *EVI2A* and *EVI2B*) may in case of ongoing selection influence the pattern of variability in the whole sequence block. A prerequisite for balancing selection is the presence of a functional variant upon which selection can act. But resequencing of the four genes in a sample of probands did not reveal any coding variant explaining the observed haplotype pattern. Thus the action of balancing selection cannot be excluded, but there is no good evidence for this type of selection. Therefore demographic factors must be considered as a further possible explanation for the deep split observed in the phylogenetic tree. A statistical analysis revealed a constant population size and a time to the most recent common ancestor (TMRCA) of 700,000 years for the whole sample, a value well within the range of other autosomal and X-chromosomal loci [28–31]. In contrast, a separate analysis of the two subgroups indicated a growing population and TMRCAs between 130,000 and 150,000 years. Taken together these data lead to a model that the recent European population went through a bottleneck followed by a population expansion that started 130,000 to 150,000 years before present. This time frame coincides with the time range for the transition to the AMH; hence the postulated population bottleneck could reflect this speciation event. But concerning demographic factors, one must keep in mind that for the depicted phylogenetic tree only one

European population was analyzed. A global survey of *NF1* haplotypes (data not shown) revealed that the phylogenetic tree of the European *NF1* haplotypes represents just a small part of a larger phylogenetic tree. African haplotypes show the highest diversity, and the most ancestral haplotypes of both European subgroups are found exclusively in Africa. Hence the reduction to a few branches as it is observed in Europe seems to result from bottlenecks which occurred during the emigration of AMHs out of Africa. Of course, these emigrants could have left Africa in timely separated waves of emigration, so that the recent European *NF1* haplotypes represent an admixture of two ancient African populations. In summary the data show that the *NF1* gene has not been under positive selection during the recent evolution of the AMH, but shows signs of a neutral evolution in human populations.

References

1 Bernards A, Snijders AJ, Hannigan GE, Murthy AE, Gusella JF: Mouse neurofibromatosis type 1 cDNA sequence reveals high degree of conservation of both coding and non-coding mRNA segments. Hum Mol Genet 1993;2:645–650.

2 Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, et al: Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 2000;66:790–818.

3 Kreitman M: Methods to detect selection in populations with applications to the human. Annu Rev Genomics Hum Genet 2000;1:539–559.

4 Cannarozzi G, Schneider A, Gonnet G: A phylogenomic study of human, dog, and mouse. PLoS Comput Biol 2007;3:e2.

5 Springer MS, Murphy WJ, Eizirik E, O'Brien SJ: Placental mammal diversification and the Cretaceous-Tertiary boundary. Proc Natl Acad Sci USA 2003;100:1056–1061.

6 Rat Genome Sequencing Consortium: Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature 2004;428:493–521.

7 Bernardi G: Isochores and the evolutionary genomics of vertebrates. Gene 2000;241:3–17.

8 Costantini M, Clay O, Auletta F, Bernardi G: An isochore map of human chromosomes. Genome Res 2006;16:536–541.

9 Mouse Genome Sequencing Consortium: Initial sequencing and comparative analysis of the mouse genome. Nature 2002;420:520–562.

10 Eisenbarth I, Vogel G, Krone W, Vogel W, Assum G: An isochore transition in the *NF1* gene region coincides with a switch in the extent of linkage disequilibrium. Am J Hum Genet 2000;67:873–880.

11 Schmegner C, Berger A, Vogel W, Hameister H, Assum G: An isochore transition zone in the *NF1* gene region is a conserved landmark of chromosome structure and function. Genomics 2005;86:439–445.

12 Schmegner C, Hameister H, Vogel W, Assum G: Isochores and replication time zones: a perfect match. Cytogenet Genome Res 2007;116:167–172.

13 Eyre-Walker A, Hurst LD: The evolution of isochores. Nat Rev Genet 2001;2:549–555.

14 Schmegner C, Hoegel J, Vogel W, Assum G: The rate, not the spectrum, of base pair substitutions changes at a GC-content transition in the human *NF1* gene region: implications for the evolution of the mammalian genome structure. Genetics 2007;175:421–428.

15 Eyre-Walker A: Evidence of selection on silent site base composition in mammals: potential implications for the evolution of isochores and junk DNA. Genetics 1999;152:675–683.

16 Smith NG, Eyre-Walker A: Synonymous codon bias is not caused by mutation bias in G+C-rich genes in humans. Mol Biol Evol 2001;18:982–986.
17 Alvarez-Valin F, Lamolle G, Bernardi G: Isochores, GC3 and mutation biases in the human genome. Gene 2002;300:161–168.
18 Duret L, Semon M, Piganeau G, Mouchiroud D, Galtier N: Vanishing GC-rich isochores in mammalian genomes. Genetics 2002;162:1837–1847.
19 Lercher MJ, Smith NG, Eyre-Walker A, Hurst LD: The evolution of isochores: evidence from SNP frequency distributions. Genetics 2002;162:1805–1810.
20 Lercher MJ, Hurst LD: Can mutation or fixation biases explain the allele frequency distribution of human single nucleotide polymorphisms (SNPs)? Gene 2002;300:53–58.
21 Webster MT, Smith NG: Fixation biases affecting human SNPs. Trends Genet 2004;20:122–126.
22 Smith NG, Webster MT, Ellegren H: Deterministic mutation rate variation in the human genome. Genome Res 2002;12:1350–1356.
23 Jorde LB, Watkins WS, Viskochil D, O'Connell P, Ward K: Linkage disequilibrium in the neurofibromatosis 1 (NF1) region: implications for gene mapping. Am J Hum Genet 1993;53: 1038–1050.
24 Messiaen L, De Bie S, Moens T, Van den EA, Leroy J: Lack of independence between five DNA polymorphisms in the *NF1* gene. Hum Mol Genet 1993;2:485.
25 Purandare SM, Cawthon R, Nelson LM, Sawada S, Watkins WS, et al: Genotyping of PCR-based polymorphisms and linkage-disequilibrium analysis at the NF1 locus. Am J Hum Genet 1996;59:159–166.
26 Schmegner C, Hoegel J, Vogel W, Assum G: Genetic variability in a genomic region with long-range linkage disequilibrium reveals traces of a bottleneck in the history of the European population. Hum Genet 2005;118:276–286.
27 Excoffier L: Human demographic history: refining the recent African origin model. Curr Opin Genet Dev 2002;12:675–682.
28 Kaessmann H, Heissig F, von Haeseler A, Paabo S: DNA sequence variation in a non-coding region of low recombination on the human X chromosome. Nat Genet 1999;22:78–81.
29 Labuda D, Zietkiewicz E, Yotova V: Archaic lineages in the history of modern humans. Genetics 2000;156:799–808.
30 Zhao Z, Jin L, Fu YX, Ramsay M, Jenkins T, et al: Worldwide DNA sequence variation in a 10-kilobase noncoding region on human chromosome 22. Proc Natl Acad Sci USA 2000;97:11354–11358.
31 Yu N, Zhao Z, Fu YX, Sambuughin N, Ramsay M, et al: Global patterns of human DNA sequence variation in a 10-kb region on chromosome 1. Mol Biol Evol 2001;18:214–222.

Günter Assum
Institut für Humangenetik, Universität Ulm
Albert-Einstein-Allee 11
DE–89081 Ulm (Germany)
Tel. +49 (0)731 5006 5460, Fax +49 (0)731 5006 5402, E-Mail guenter.assum@uni-ulm.de

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 113–128

Structure and Function of Neurofibromin

S. Welti, I. D'Angelo, K. Scheffzek

European Molecular Biology Laboratory (EMBL) Heidelberg, Heidelberg, Germany

Abstract

The *NF1* gene encodes the giant protein neurofibromin (320 kDa), whose activity is impaired in patients with neurofibromatosis type 1 (NF1). Understanding the biochemical functions of neurofibromin is a key requirement for understanding NF1-associated disease mechanisms at the molecular level and is essential for developing efficient therapeutics against NF1. The RasGAP activity of neurofibromin has long been known and investigated in biochemical and structural detail, essentially suggesting that Arg1276 acts as the so-called arginine finger that contacts the GTP/GDP-binding site to stabilize the transition state of the GTPase reaction [1–7]. Structural proteomics strategies have recently uncovered a novel structural module that is composed of an N-terminal domain related to the Sec14-like lipid binding module, associated with a previously undetected pleckstrin homology (PH)-like domain. The functional importance of this module is underscored by a considerable number of missense mutations found in this protein segment in NF1 patients. In this chapter we summarize the current knowledge about the molecular structure and function of neurofibromin with focus on the central portions whose 3-dimensional structures are now known.

Neurofibromin is the protein product of the *NF1* gene and was originally identified as a cytosolic protein with a molecular weight of approximately 280 kDa [8, 9], which could be immunoprecipitated in a 500-kDa protein complex. From studies in various vertebrates expression is ubiquitous from the onset of organogenesis to mid-stage embryonic development, while in the adult organism it is expressed predominantly in neuronal cells of the brain [10]. Homozygous deletion of the *Nf1* gene is lethal in mice [11] and leads to size defects in the fruit fly *Drosophila melanogaster* [12], suggesting roles in organismal development. The subcellular localization of neurofibromin is cell type dependent; the protein can be found associated with the plasma membrane [13], the endoplasmic reticulum [14] and also colocalized with mitochondria [15]. How localization is spatiotemporally regulated in the various cell types remains largely unclear.

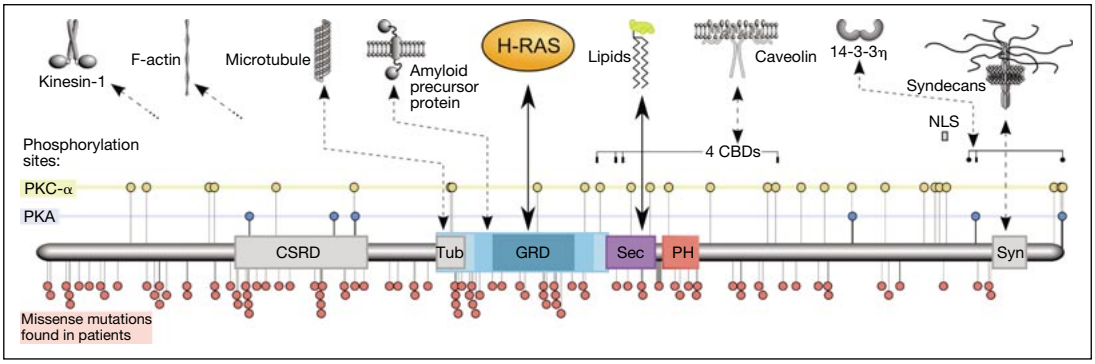


Fig. 1. Domain scheme of neurofibromin with reported protein-ligand interactions [19, 20, 23–25, 89, 101, 102]. Colored domains and interaction partners are confirmed by structural analysis, grey ones indicated by biochemical experiments. Red circles mark the positions of missense mutations found in patients [30, 32–38, 75–77, 90–97] (including amino acid deletions and the tandem duplication TD1699–1713). Additional features are potential phosphorylation sites for PKA [21, 98] (blue circles) and PKC-α [22] (yellow circles, only serine phosphorylation), a bipartite nuclear localization sequence (NLS) [99, 100] and several caveolin binding motifs (CBDs). The GRD part indicated in dark blue is the minimal GAP domain [5], in mid-blue the crystallized fragment [2] and in light blue the whole fragment with sequence homology to the p120-GAP protein, overlapping with the Tub and Sec domain [19]. CSRD: Cystein and serine rich domain; Tub: tubulin binding region; Sec: Sec14-like domain; PH: PH-like domain.

Neurofibromin acts as a Ras-specific GTPase activating protein (RasGAP) [16–18] downregulating the biological activity of normal but not oncogenic Ras proteins. The RasGAP activity is mediated by a central portion, termed GAP related domain (GRD, 44 kDa) and remains so far the only clearly defined biochemical function. In search for functions of neurofibromin beyond the RasGAP activity a number of interaction partners other than Ras have been identified using a variety of biochemical approaches including the yeast two hybrid system. These include tubulin [19], kinesin-1 [20], protein kinase A (PKA) [21] and C (PKC) [22], Syndecan [23], Caveolin [24] and the amyloid precursor protein [25]. While regions of neurofibromin involved in those interactions have been reported, the biological significance of the respective protein-protein interactions remains unclear.

The original domain scheme with a central GRD flanked by largely helical elements has been expanded by the prediction of a lipid-binding Sec14p-like domain (NF1-Sec) [26] at the C-terminal end of the GRD and more recently by the discovery of a pleckstrin homology-like domain (NF1-PH) adjacent to and interacting with the NF1-Sec portion (see below), as derived from the crystal structure of a bipartite module composed of NF1-Sec and NF1-PH [27] (fig. 1).

In this chapter we will review the current knowledge of neurofibromin functions and mechanisms with focus on aspects, for which structure-function relationships have been studied.

The GAP Related Domain and the Mechanism of GTPase Activation

Neurofibromin down-regulates the biological activity of normal but not oncogenic Ras proteins by acting as a GTPase activating protein (GAP). The RasGAP activity is encoded by the central GRD [16–18], which has been reported to cover a segment of 300–400 residues but can be narrowed down to a 230-residue fragment corresponding to the structural catalytic domain [4, 5]. The 3-dimensional structure of the GRD presents an entirely helical protein with a groove in the surface [2, 4], which is lined by the conserved residues and accommodates Ras in the structure of the Ras-RasGAP complex (fig. 2a). This structure is similar to the GAP domain of the homologous p120GAP complexed with Ras • GDP • Aluminium fluoride [1], which mimics the γ-phosphate during the transition state of the phosphotransfer reaction. In the complex a peptide segment, termed 'finger loop' lines the active site of Ras presenting a conserved arginine residue (Arg1276, Arg789 in p120GAP), termed 'arginine finger', which contacts the γ-phosphate mimicking AlFx by its guanidinium group to stabilize the transition state of the GTPase reaction. In addition, the so-called switch regions of Ras that play an important role in the transition between GTP-bound ON- and GDP-bound OFF-states, are stabilized by portions of the GAP domain [1]. Exchanging Arg1276 for alanine or even lysine (which partly retains the ability to form polar contacts) leads to a 2000-fold decrease in catalytic activity. Arginine 1391 of the FLR-finger print motif has been originally suspected to be the major catalytic player but turned out to have a significant but less prominent role in GAP-catalysis [6, 28] (fig. 2b).

Missense mutations derived from NF1 patients have been identified in the GRD. They are located in the area of the Ras-binding region as well as in regions that appear to play a role for protein stability rather than its biochemical function. In general, the effect of a substitution depends on the biochemical nature and the spatial location of the residues involved. Mutations can have functional (catalytic, ion-binding etc.) or structural consequences, which can lead to subtle geometric distortions of important protein parts or alterations in the connectivity of hydrogen bond networks, resulting in functionally unfavorable orientations of key residues. For example, replacement of a constrained glycine (the smallest residue) within a hydrophobic core for the bulky phenylalanine or tryptophan, most likely leads to severe steric problems. Similarly, alterations that replace the positively charged arginine/lysine for a glutamic or

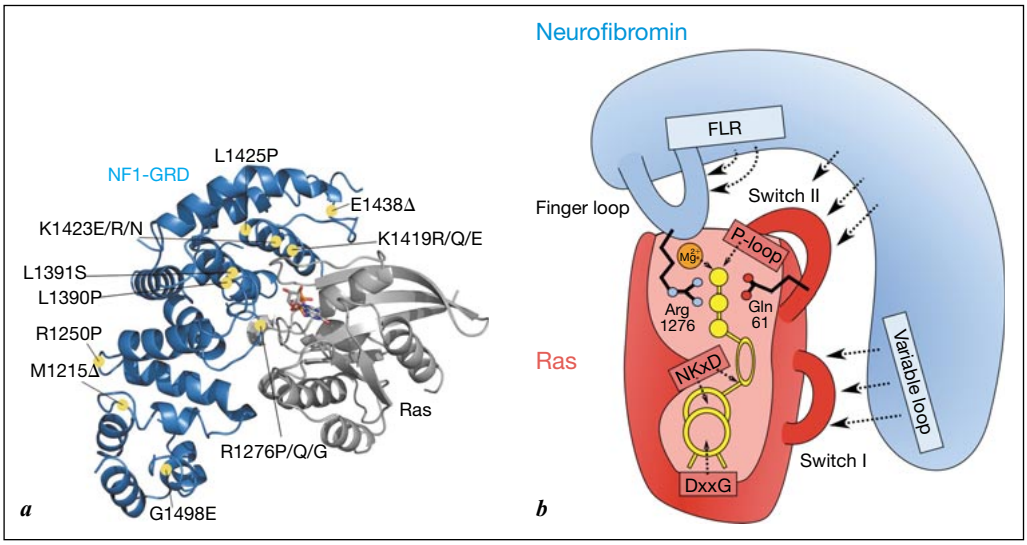


Fig. 2. (***a***) Ribbon representation of GRD and Ras modeled as complex, based on the p120GAP • Ras structure [1]. Yellow dots indicate the location of GRD missense mutations found in patients. The nucleotide is displayed as stick model. (***b***) Cartoon of the Ras-RasGAP complex showing the interactions leading to Ras activation. Upon binding of neurofibromin, the GTPase activity of Ras is strongly enhanced by complementation of the active site with the finger loop, delivering the critical arginine 1276. The GTP (yellow) is recognized, bound and oriented in the active site by the NKxD- and DxxG-motifs, an Mg^{2+} ion and the P-loop. Glutamine 61 of Ras is contributing to the catalysis reaction by positioning the phosphate accepting water molecule or the nucleophile derived from it. The FLR motif of neurofibromin is reinforcing the finger-loop, while the variable loop and additional residues stabilize the switch I and II regions of Ras, supporting a conformation favourable for efficient GTP hydrolysis.

aspartic acid are likely to generate electrostatic repulsion that may affect structure, ligand binding or catalysis. When considering fragments or domains of a protein, one has to take into account that mutations on the surface may be fatal because they disrupt physiological interactions within the context of the whole protein. A summary of missense mutations within the GAP- and Sec14-PH domains of neurofibromin is given in tables 1 and 2.

Of paramount importance for neurofibromin is the occurrence of a mutation substituting Arg1276 for proline in a patient with severe malignant Schwannoma. Biochemical analysis of the recombinant mutant GRD indicated 8000-fold loss of catalytic activity with Ras-binding affinity similar to that of the wild-type GRD, together with the genetic analysis indicating that loss of GAP activity is sufficient to cause NF1 [29]. The FLR-finger print derived

Table 1. Patient derived missense mutations in the GAP related domain (GRD)

Missense mutation	Location	Potential or demonstrated effect	Reference
R1204W	GAPex	not visible in the structure, geometric distortions due to the insertion of a bulky residue	[32]
M1215△	GAPex	domain destabilization, structural distortions	[33]
R1250P	GRD, distal of Ras-binding region	structural distortions, destabilization of the 'finger loop'	[33]
R1276P/Q/G	active site, Arg-finger	GAP stimulated GTP hydrolysis 500–8000-fold compromised	[29, 33, 34]
R1325G	backside of Ras binding groove	not visible in the structure, could destabilize Ras binding groove and weaken Ras binding	[35]
L1390P	FLR-motif	reduction of GAP stimulated GTP hydrolysis, stabilization of Arg-finger-loop impaired	[35]
R1391S	FLR-motif	GAP stimulated GTP hydrolysis 300-fold compromised, stabilization of Arg-finger-loop impaired	[30]
K1419Q	Ras binding groove	weakens Ras binding, likely ionic interaction with Ras-Glu37 disrupted	[30]
K1423E/R/N	Ras binding groove	Ras Switch II region not stabilized GAP stimulated GTP hydrolysis 200–400 fold compromised	[31, 36, 37]
L1425P	Ras binding groove	destabilization of Ras binding groove, weakens Ras binding	[38]
E1438△	variable loop	Ras Switch I region not stabilized improper Ras docking likely	[34]
S1468G	close to C-terminus of GRD	not visible in the structure, unlikely to affect GAP activity but eventually another neurofibromin domain	[30]
G1498E	close to C-terminus of GRD	not visible in the structure, unlikely to affect GAP activity but eventually another neurofibromin domain	[32]

Table 2. Patient derived missense mutations in the NF1-Sec-PH domains

Missense mutation	Location	Potential effect	Reference
I1584V	cage backside, core	destabilization of NF1-Sec cage structure	[33]
R1590W	interface region, surface	destabilization of domain interaction and structures	[74]
V1621R	cage backside, core	destabilization of NF1-Sec cage structure	[75]
1658-59IY△	cage inside/backside, core	destabilization of NF1-Sec cage structure	[76]
N1662K	interface region, core	destabilization of domain interaction and structures	[24]
TD1699-1713	NF1-Sec/PH linker	prevention of proper domain interaction	[77]
K1750△	NF1-PH protrusion	alteration of lipid binding cage access regulation	[33]
A1764S	NF1-PH core	destabilization of NF1-PH domain	[36]
T1787M	NF1-PH surface	no apparent effect, eventually affecting neighboring NF1 domains	[35]

Arg1391 has been found to be substituted for serine in NF1 patients and the mutant protein has been reported to be 300-fold less active than the wild-type [30], which is consistent with the supplementary role of this residue in GAP-accelerated GTP-hydrolysis. The roles of various regions of the GRD have been investigated in biochemical detail, identifying the finger loop and the FLR region as catalytic and binding determinants, while the so-called variable loop region seems to account for the specificity of the Ras-RasGAP interaction [7]. In this context it is interesting to note that Lys1423, which has been found somatically mutated to glutamate in NF1 patients [31], is contacting acidic residues of the switch I region. A glutamate in that position is likely to disrupt the complex preventing productive Ras-RasGAP interaction, consistent with no detectable association of the two proteins [7].

The Phospholipid-Binding NF1-Sec-PH Module

NF1-Sec

The Sec14-homologous (NF1-Sec) and the novel PH-like (NF1-PH) domains of neurofibromin are located adjacent to the C-terminus of the GRD, spanning residues 1560–1698 and 1715–1816, respectively (fig. 1) [27]. Both domains are in contact with each other and undergo additional interactions via an atypical protrusion derived from NF1-PH, extending on top of the NF1-Sec domain [27, 39].

NF1-Sec adopts a fold common to the Sec14p-like protein family also known as CRAL-TRIO domain. Sec14p-like proteins are unrelated to other known phosphatidylinositol transfer proteins (PITPs) and show a large diversification concerning physiological function and ligand specificity. They are involved in various cellular processes like the generation of secretory trans Golgi network (TGN) vesicles [40, 41], cholesterol biosynthesis [42, 43], vitamin E homeostasis and the visual cycles of rod and cone. Several members are associated with diseases such as α-Tocopherol transfer protein (α-TTP, AVED syndrome; vitamin E deficiency) [43, 44], caytaxin (human caiman ataxia) [45–47] and cellular retinaldehyde binding protein (CRALBP, retinitis pigmentosa/punctata albescens, Bothnia dystrophy, fundus albipunctatus, Newfoundland rod/cone dystrophy) [48, 49]. Other PITPs are usually PtdIns/PtdCho transfer or binding proteins [40, 50–52].

Structurally, the CRAL-TRIO motif of NF1-Sec folds into a globular Sec14p-like lipid binding cage [27, 43, 44, 53], that is closed by an amphipathic helical segment, termed ‘lid’ in NF1-Sec [27, 54]. The cavity is lined with mostly hydrophobic residues binding (3-*sn*-phosphatidyl)-ethanolamine (PtdEtn), -choline (PtdCho) and 1-(3-*sn*-phosphatidyl)-*sn*-glycerol (PtdGro) molecules

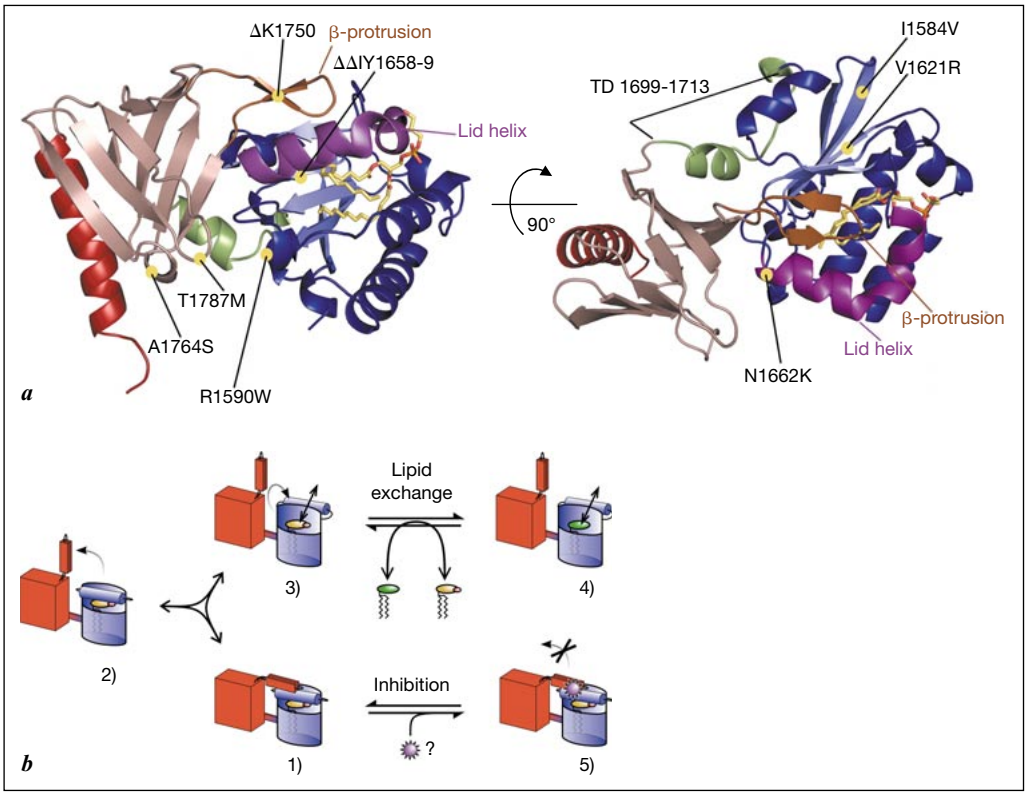


Fig. 3. (***a***) Ribbon representation of the NF1-Sec (blue-violet) and -PH domains (red-brown) in side and top view, with missense mutations found in patients indicated as yellow dots. Single amino acid deletions are indicated with Δ. A phosphatidylethanolamine (PE) ligand is bound in the NF1-Sec lipid binding cage, displayed as stick model. The lid helix (coloured violet) is in a closed conformation, with the protrusion derived from the NF1-PH domain (coloured brown) stacked on top. (***b***) Cartoon of the NF1-Sec-PH module, illustrating the proposed structural changes leading to the exchange of bound glycerophospholipid ligands. Starting from the observed structure (1), both, protrusion and lid-helix, are thought to move either stepwise ($1 > 2 > 3$) or in a concerted fashion ($1 > 3$) into an open conformation (3). This could allow the exchange of lipid molecules between the NF1-Sec cage and a membrane (4). The exchange reaction can be inhibited by binding of an inhibitor to the protrusion/lid helix interface, probably preventing a rearrangement of the protrusion segment (5).

(fig. 3a). While the fatty acid tails of the lipid ligand are buried inside the lipid binding cage, the polar head group is stabilized by hydrogen bonds with the main chain of Phe1642 and the guanidinium group of Arg1684, the counterparts of which in Sec14-like proteins are thought to be important for the binding of phospholipid head groups (fig. 3a). By shielding the hydrophobic tails of the lipid

ligands in the described manner, Sec14-like domains are able to shuttle lipids between membrane compartments through the aqueous medium [41, 55–58]. The ligand uptake is thought to be achieved through the lid helix entering the membrane and extracting a single lipid in a 'bulldozer'-like mechanism [54] and seals the lipid binding cage during the transfer. In the structural context of NF1-Sec-PH the conformation of the lid-helix renders the Sec14 portion in a closed conformation that appears to be supported by a structural element derived from the neighbouring PH-like domain.

NF1-PH

The NF1-PH module (residues 1699–1816) is a novel feature of neurofibromin only recognized as a PH-like domain after structural comparison with the contents of protein data bank (www.rcsb.org). It shows a typical β-sandwich fold with a protrusion inserted between the strands β3 and β4, interacting closely with NF1-Sec [27, 39] (see below, fig. 3a).

In general, PH domains are structural scaffolds able to accommodate binding sites for various ligand types including PIPs, polyproline helices, phosphotyrosine binding motifs and protein ligands [59, 60]. PH-like domains are associated with numerous biological functions for their host proteins, including membrane localization via PIP binding by classic PH domains [61–66], scaffold activities by protein binding (Homer-Vessel EVH1 domains) [67, 68] or binding to phosphotyrosine peptide stretches (PTB domains) [59, 69, 70], modulation of actin cytoskeleton dynamics and coupling to signal transduction pathways (EVH1 domains in Ena/VASP- and Wiscott-Aldrich syndrome protein families) [67, 68, 71] and assembly/disassembly of Ran-importin/exportin complexes in nucleocytoplasmic transport (RanBD domains) [59, 72, 73]. Given the diverse activities associated with PH domains and the fact that NF1-PH has no sequence similarity or close structural relation to other members of the PH superfamily, it is difficult to predict its precise function.

Despite the apparently tight interaction between the PH-derived protrusion and the lid of NF1-Sec, the lipid cargo can be exchanged in vitro, demonstrating that the lipid binding cage is likely to be able to adopt an open conformation, at least in the isolated module. Interestingly, mutational analysis of the lid helix–β-protrusion interface in combination with PIP binding assays revealed a second ligand binding site for PIPs (fig. 3a), consisting of residues derived from both NF1-Sec and -PH. A bound PIP could therefore be able to stabilize a closed conformation of the lipid binding cage, which is corroborated by experiments showing that soluble PIP head groups can inhibit the exchange of NF1-Sec bound lipids [27, 39] (fig. 3b). Taken together the domain-interface between the Sec14- and the PH-like portions suggests a regulatory communication between the two domains, the details of which are still to be elucidated.

The effect of most of the patient mutations found in the NF1-Sec-PH module is currently unclear. While for example the Ile1584 → Val or the Ala1764 → Ser mutations suggest minor structural alterations because of their locations and the mild change in chemical properties, the Val1621 → Arg and the Asn1662 → Lys substitutions represent more drastic changes with respect to size and chemical environment. Of particular interest are a number of mutations in prominent parts of NF1-Sec-PH, including a single residue deletion (ΔLys1750) in the protrusion potentially regulating access to the lipid binding cage [33], a duplication of the linker region connecting the two domains (TD1699–1713) [77] and a missense mutation (Asn1662 → Lys) of a residue presumably involved in hinge movements of the helical lid-segment [24]. These are likely to interfere with ligand binding although they did not affect binding of the phospholipids investigated by the authors [39].

What are the Cellular Functions of the Sec14-PH Module?

Although structural and biochemical data suggest the presence of two ligand binding sites and an interface region, supporting a regulatory interaction of NF1-Sec and NF1-PH, the meaning of this arrangement in a cellular context is still not clear. The NF1-Sec-PH module was found to bind PtdEtn and PtdGro with the lipid binding site located inside the Sec14-portion. In mammalian cells PtdEtn accounts for 15% of total phospholipids in certain neuronal cells and 45% in some *Drosophila melanogaster* tissues expressing an orthologue of neurofibromin. In contrast, PtdGro is restricted to mitochondrial membranes where it occurs as an intermediate of cardiolipin synthesis and thus is present only in small amounts [57, 78]. Therefore PtdEtn seems to be a more likely physiological ligand in this type of cells.

Ras proteins, the targets of neurofibromin, usually control several different signalling pathways and relay the incoming signal by direct interactions with effectors. Remarkably, not all subordinate signalling pathways get activated at a time, but only a signal-specific subset. This selective activation was shown to be dependent on the subcellular localization of Ras [79]. As a consequence, correct localization of RasGEFs and GAPs like neurofibromin, should result in the selective regulation of specific Ras pathways.

Taking into account that Ras is membrane anchored, it is therefore tempting to speculate that neurofibromin harbours membrane targeting activities. Indeed Sec14-like domains are present in characteristic patterns in a number of other signal regulatory proteins including RhoGAPs, RhoGEFs and PTPases [80–83]. In several of these, the Sec14-portion affects subcellular localization of the proteins, although the molecular mechanisms behind these findings remain unclear. It is however interesting to note, that the observed localization in the case of the proto-oncogenic RhoGEF Dbs involves a direct interaction

between the Sec14-portion at the N-terminus and the PH-domain that in this case is located at the C-terminus of the protein [81].

The occurrence of the PH-like domain in neurofibromin is particularly intriguing. While biochemical/structural studies suggest that mechanistically it may regulate lipid exchange of the Sec14-lipid binding cage, the nature of a physiological ligand that would perform such a function in vivo is currently unclear. Following the ligand classification above, a phospholipid-, polyproline- or phosphotyrosine binding activity hosted by NF1-PH is rather unlikely given the low sequence similarity to such domains. However, this lack of sequence similarity is not unique among PH-like domains and could indicate a protein ligand, as observed for the likewise structurally discovered PH-like domains of BEACH domain containing proteins [84], TFIIH [85], Pob3 [86], and Ran-BD [73]. All these proteins are unrelated and involved in specific tasks as different as nuclear import, transcription initiation and membrane dynamics. Unfortunately, this leaves no consensus feature that could restrict the range of likely NF1-PH functions any further, nor does it rule out an indirect membrane targeting activity via a membrane bound protein interaction partner. In addition, one has to keep in mind that PH-like domains may also bind simultaneously to two different ligands, as in the case of PLC-ß2 in which the PH-domain binds PIPs, $G_{\beta\gamma}$ subunits and GNPBs of the Rho family [87, 88].

Concluding Remarks

Alterations of the *NF1* gene, encoding the giant neurofibromin protein, are responsible for the pathogenesis of neurofibromatosis type I. Neurofibromin is a RasGAP and is therefore located at a central position of several signal transduction pathways. Structural and biochemical studies have established the GAP mechanism as active site complementation of Ras mediated by the GAP derived arginine finger and identified the key players. Recent studies have extended the neurofibromin part known in 3D to about 25% of the whole protein, revealing a glycerophospholipid binding Sec14- and a closely interacting PH-like domain that has evaded previous discovery by other methods.

One of the major obstacles in the biochemical characterization of neurofibromin is the routine expression and purification of the protein and the notorious insolubility of most fragments produced in heterologous expression systems [Bonneau and Scheffzek, unpublished]. It has to be considered that neurofibromin hosts additional features beside the GAP activity, given the size of the protein, the distribution of missense mutations in patients (fig. 1) and the fact that it acts on several signal transduction pathways requiring sophisticated regulation. This could include membrane localization features for translocation

to selected pools of the membrane anchored target protein Ras, several ligand docking sites to connect to upstream signalling pathways and GRD regulating domains for signal processing and the production of a fine tuned output.

Acknowledgements

For the work on neurofibromin the authors acknowledge financial support by the medical research program of the American Department of Defense and by the Peter and Traudl Engelhorn Stiftung (Penzberg, Germany). The authors are furthermore grateful for financial support of the Deutsche Forschungsgemeinschaft (DFG).

References

1 Scheffzek K, Ahmadian MR, Kabsch W, Wiesmuller L, Lautwein A, et al: The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science 1997;277:333–338.
2 Scheffzek K, Ahmadian MR, Wiesmuller L, Kabsch W, Stege P, et al: Structural analysis of the GAP-related domain from neurofibromin and its implications. EMBO J 1998;17:4313–4327.
3 Scheffzek K, Ahmadian MR, Wittinghofer A: GTPase-activating proteins: helping hands to complement an active site. Trends Biochem Sci 1998;23:257–262.
4 Scheffzek K, Lautwein A, Kabsch W, Ahmadian MR, Wittinghofer A: Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. Nature 1996;384:591–596.
5 Ahmadian MR, Wiesmuller L, Lautwein A, Bischoff FR, Wittinghofer A: Structural differences in the minimal catalytic domains of the GTPase-activating proteins p120GAP and neurofibromin. J Biol Chem 1996;271:16409–16415.
6 Ahmadian MR, Stege P, Scheffzek K, Wittinghofer A: Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. Nat Struct Biol 1997;4:686–689.
7 Ahmadian MR, Kiel C, Stege P, Scheffzek K: Structural fingerprints of the Ras-GTPase activating proteins neurofibromin and p120GAP. J Mol Biol 2003;329:699–710.
8 DeClue JE, Cohen BD, Lowy DR: Identification and characterization of the neurofibromatosis type 1 protein product. Proc Natl Acad Sci USA 1991;88:9914–9918.
9 Gutmann DH, Collins FS: The neurofibromatosis type 1 gene and its protein product, neurofibromin. Neuron 1993;10:335–343.
10 Shen MH, Harper PS, Upadhyaya M: Molecular genetics of neurofibromatosis type 1 (NF1). J Med Genet 1996;33:2–17.
11 Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumour predisposition in mice heterozygous for a targeted mutation in *Nf1*. Nat Genet 1994;7:353–361.
12 The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, et al: Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. Science 1997;276:791–794.
13 Malhotra R, Ratner N: Localization of neurofibromin to keratinocytes and melanocytes in developing rat and human skin. J Invest Dermatol 1994;102:812–818.
14 Nordlund M, Gu X, Shipley MT, Ratner N: Neurofibromin is enriched in the endoplasmic reticulum of CNS neurons. J Neurosci 1993;13:1588–1600.
15 Roudebush M, Slabe T, Sundaram V, Hoppel CL, Golubic M, Stacey DW: Neurofibromin colocalizes with mitochondria in cultured cells. Exp Cell Res 1997;236:161–172.
16 Martin GA, Viskochil D, Bollag G, McCabe PC, Crosier WJ, et al: The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell 1990;63:843–849.

17 Ballester R, Marchuk D, Boguski M, Saulino A, Letcher R, et al: The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell 1990;63:851–859.
18 Xu GF, Lin B, Tanaka K, Dunn D, Wood D, et al: The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. Cell 1990;63:835–841.
19 Bollag G, McCormick F, Clark R: Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. EMBO J 1993;12:1923–1927.
20 Hakimi MA, Speicher DW, Shiekhattar R: The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway of neurofibromatosis. J Biol Chem 2002;277:36909–36912.
21 Izawa I, Tamaki N, Saya H: Phosphorylation of neurofibromatosis type 1 gene product (neurofibromin) by cAMP-dependent protein kinase. FEBS Lett 1996;382:53–59.
22 Mangoura D, Sun Y, Li C, Singh D, Gutmann DH, et al: Phosphorylation of neurofibromin by PKC is a possible molecular switch in EGF receptor signaling in neural cells. Oncogene 2006;25:735–745.
23 Hsueh YP, Roberts AM, Volta M, Sheng M, Roberts RG: Bipartite interaction between neurofibromatosis type I protein (neurofibromin) and syndecan transmembrane heparan sulfate proteoglycans. J Neurosci 2001;21:3764–3770.
24 Boyanapalli M, Lahoud OB, Messiaen L, Kim B, Anderle de Sylor MS, et al: Neurofibromin binds to caveolin-1 and regulates ras, FAK, and Akt. Biochem Biophys Res Commun 2006;340:1200–1208.
25 De Schepper S, Boucneau JM, Westbroek W, Mommaas M, Onderwater J, et al: Neurofibromatosis type 1 protein and amyloid precursor protein interact in normal human melanocytes and colocalize with melanosomes. J Invest Dermatol 2006;126:653–659.
26 Aravind L, Neuwald AF, Ponting CP: Sec14p-like domains in NF1 and Dbl-like proteins indicate lipid regulation of Ras and Rho signaling. Curr Biol 1999;9:R195–R197.
27 D'Angelo I, Welti S, Bonneau F, Scheffzek K: A novel bipartite phospholipid-binding module in the neurofibromatosis type 1 protein. EMBO Rep 2006;7:174–179.
28 Sermon BA, Lowe PN, Strom M, Eccleston JF: The importance of two conserved arginine residues for catalysis by the ras GTPase-activating protein, neurofibromin. J Biol Chem 1998;273:9480–9485.
29 Klose A, Ahmadian MR, Schuelke M, Scheffzek K, Hoffmeyer S, et al: Selective disactivation of neurofibromin GAP activity in neurofibromatosis type 1. Hum Mol Genet 1998;7:1261–1268.
30 Upadhyaya M, Osborn MJ, Maynard J, Kim MR, Tamanoi F, Cooper DN: Mutational and functional analysis of the neurofibromatosis type 1 *(NF1)* gene. Hum Genet 1997;99:88–92.
31 Li Y, Bollag G, Clark R, Stevens J, Conroy L, et al: Somatic mutations in the neurofibromatosis 1 gene in human tumors. Cell 1992;69:275–281.
32 Ars E, Kruyer H, Morell M, Pros E, Serra E, et al: Recurrent mutations in the *NF1* gene are common among neurofibromatosis type 1 patients. J Med Genet 2003;40:e82.
33 Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, et al: Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 2000;66:790–818.
34 Mattocks C, Baralle D, Tarpey P, ffrench-Constant C, Bobrow M, Whittaker J: Automated comparative sequence analysis identifies mutations in 89% of NF1 patients and confirms a mutation cluster in exons 11–17 distinct from the GAP related domain. J Med Genet 2004;41:e48.
35 Lee MJ, Su YN, You HL, Chiou SC, Lin LC, et al: Identification of forty-five novel and twenty-three known *NF1* mutations in Chinese patients with neurofibromatosis type 1. Hum Mutat 2006;27:832.
36 Han SS, Cooper DN, Upadhyaya MN: Evaluation of denaturing high performance liquid chromatography (DHPLC) for the mutational analysis of the neurofibromatosis type 1 *(NF1)* gene. Hum Genet 2001;109:487–497.
37 De Luca A, Buccino A, Gianni D, Mangino M, Giustini S, et al: *NF1* gene analysis based on DHPLC. Hum Mutat 2003;21:171–172.
38 Peters H, Hess D, Fahsold R, Schulke M: A novel mutation L1425P in the GAP-region of the *NF1* gene detected by temperature gradient gel electrophoresis (TGGE). Mutation in brief no. 230. Online. Hum Mutat 1999;13:337.

39 Welti S, Fraterman S, D'Angelo I, Wilm M, Scheffzek K: The sec14 homology module of neurofibromin binds cellular glycerophospholipids: mass spectrometry and structure of a lipid complex. J Mol Biol 2007;366:551–562.

40 Phillips SE, Vincent P, Rizzieri KE, Schaaf G, Bankaitis VA, Gaucher EA: The diverse biological functions of phosphatidylinositol transfer proteins in eukaryotes. Crit Rev Biochem Mol Biol 2006; 41:21–49.

41 Bankaitis VA, Aitken JR, Cleves AE, Dowhan W: An essential role for a phospholipid transfer protein in yeast Golgi function. Nature 1990;347:561–562.

42 Stocker A, Tomizaki T, Schulze-Briese C, Baumann U: Crystal structure of the human supernatant protein factor. Structure 2002;10:1533–1540.

43 Stocker A, Baumann U: Supernatant protein factor in complex with RRR-alpha-tocopherylquinone: a link between oxidized Vitamin E and cholesterol biosynthesis. J Mol Biol 2003;332: 759–765.

44 Min KC, Kovall RA, Hendrickson WA: Crystal structure of human alpha-tocopherol transfer protein bound to its ligand: implications for ataxia with vitamin E deficiency. Proc Natl Acad Sci USA 2003;100:14713–14718.

45 Bomar JM, Benke PJ, Slattery EL, Puttagunta R, Taylor LP, et al: Mutations in a novel gene encoding a CRAL-TRIO domain cause human Cayman ataxia and ataxia/dystonia in the jittery mouse. Nat Genet 2003;35:264–269.

46 Hayakawa Y, Itoh M, Yamada A, Mitsuda T, Nakagawa T: Expression and localization of Cayman ataxia-related protein, Caytaxin, is regulated in a developmental- and spatial-dependent manner. Brain Res 2007;1129:100–109.

47 Xiao J, Gong S, Ledoux MS: Caytaxin deficiency disrupts signaling pathways in cerebellar cortex. Neuroscience 2007;144:439–461.

48 Crabb JW, Gaur VP, Garwin GG, Marx SV, Chapline C, et al: Topological and epitope mapping of the cellular retinaldehyde-binding protein from retina. J Biol Chem 1991;266:16674–16683.

49 Saari JC, Crabb JW: Focus on molecules: cellular retinaldehyde-binding protein (CRALBP). Exp Eye Res 2005;81:245–246.

50 Allen-Baume V, Segui B, Cockcroft S: Current thoughts on the phosphatidylinositol transfer protein family. FEBS Lett 2002;531:74–80.

51 Wirtz KW: Phospholipid transfer proteins in perspective. FEBS Lett 2006;580:5436–5441.

52 Alb JG Jr, Cortese JD, Phillips SE, Albin RL, Nagy TR, et al: Mice lacking phosphatidylinositol transfer protein-alpha exhibit spinocerebellar degeneration, intestinal and hepatic steatosis, and hypoglycemia. J Biol Chem 2003;278:33501–33518.

53 Meier R, Tomizaki T, Schulze-Briese C, Baumann U, Stocker A: The molecular basis of vitamin E retention: structure of human alpha-tocopherol transfer protein. J Mol Biol 2003;331:725–734.

54 Sha B, Phillips SE, Bankaitis VA, Luo M: Crystal structure of the *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein. Nature 1998;391:506–510.

55 Cleves A, McGee T, Bankaitis V: Phospholipid transfer proteins: a biological debut. Trends Cell Biol 1991;1:30–34.

56 Wirtz KW, Zilversmit DB: Participation of soluble liver proteins in the exchange of membrane phospholipids. Biochim Biophys Acta 1969;193:105–116.

57 Daum G, Paltauf F: Isolation and partial characterization of a phospholipid transfer protein from yeast cytosol. Biochim Biophys Acta 1984;794:385–391.

58 Aitken JF, van Heusden GP, Temkin M, Dowhan W: The gene encoding the phosphatidylinositol transfer protein is essential for cell growth. J Biol Chem 1990;265:4711–4717.

59 Blomberg N, Baraldi E, Nilges M, Saraste M: The PH superfold: a structural scaffold for multiple functions. Trends Biochem Sci 1999;24:441–445.

60 Lemmon MA: Pleckstrin homology domains: not just for phosphoinositides. Biochem Soc Trans 2004;32:707–711.

61 Itoh T, De Camilli P: Membrane trafficking: dual-key strategy. Nature 2004;429:141–143.

62 Lemmon MA, Ferguson KM, Abrams CS: Pleckstrin homology domains and the cytoskeleton. FEBS Lett 2002;513:71–76.

63 Baumeister MA, Rossman KL, Sondek J, Lemmon MA: The Dbs PH domain contributes independently to membrane targeting and regulation of guanine nucleotide-exchange activity. Biochem J 2006;400:563–572.

64 Godi A, Di Campli A, Konstantakopoulos A, Di Tullio G, Alessi DR, et al: FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat Cell Biol 2004;6:393–404.
65 Varnai P, Lin X, Lee SB, Tuymetova G, Bondeva T, et al: Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130. J Biol Chem 2002;277:27412–27422.
66 Di Paolo G, De Camilli P: Phosphoinositides in cell regulation and membrane dynamics. Nature 2006;443:651–657.
67 Ball LJ, Jarchau T, Oschkinat H, Walter U: EVH1 domains: structure, function and interactions. FEBS Lett 2002;513:45–52.
68 Duncan RS, Hwang SY, Koulen P: Effects of Vesl/Homer proteins on intracellular signaling. Exp Biol Med (Maywood) 2005;230:527–535.
69 Uhlik MT, Temple B, Bencharit S, Kimple AJ, Siderovski DP, Johnson GL: Structural and evolutionary division of phosphotyrosine binding (PTB) domains. J Mol Biol 2005;345:1–20.
70 Yan KS, Kuti M, Zhou MM: PTB or not PTB – that is the question. FEBS Lett 2002;513:67–70.
71 Takenawa T, Suetsugu S: The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol 2007;8:37–48.
72 Fried H, Kutay U: Nucleocytoplasmic transport: taking an inventory. Cell Mol Life Sci 2003;60:1659–1688.
73 Pemberton LF, Paschal BM: Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic 2005;6:187–198.
74 Upadhyaya M, Maynard J, Osborn M, Harper PS: Six novel mutations in the neurofibromatosis type 1 *(NF1)* gene. Hum Mutat 1997;10:248–250.
75 Jeong SY, Park SJ, Kim HJ: The spectrum of *NF1* mutations in Korean patients with neurofibromatosis type 1. J Korean Med Sci 2006;21:107–112.
76 Wu R, Lopez-Correa C, Rutkowski JL, Baumbach LL, Glover TW, Legius E: Germline mutations in NF1 patients with malignancies. Genes Chromosomes Cancer 1999;26:376–380.
77 Tassabehji M, Strachan T, Sharland M, Colley A, Donnai D, et al: Tandem duplication within a neurofibromatosis type 1 *(NF1)* gene exon in a family with features of Watson syndrome and Noonan syndrome. Am J Hum Genet 1993;53:90–95.
78 Chen D, Zhang XY, Shi Y: Identification and functional characterization of hCLS1, a human cardiolipin synthase localized in mitochondria. Biochem J 2006;398:169–176.
79 Chang EC, Philips MR: Spatial segregation of Ras signaling: new evidence from fission yeast. Cell Cycle 2006;5:1936–1939.
80 Ueda S, Kataoka T, Satoh T: Role of the Sec14-like domain of Dbl family exchange factors in the regulation of Rho family GTPases in different subcellular sites. Cell Signal 2004;16:899–906.
81 Kostenko EV, Mahon GM, Cheng L, Whitehead IP: The Sec14 homology domain regulates the cellular distribution and transforming activity of the Rho-specific guanine nucleotide exchange factor Dbs. J Biol Chem 2005;280:2807–2817.
82 Sirokmany G, Szidonya L, Kaldi K, Gaborik Z, Ligeti E, Geiszt M: Sec14 homology domain targets p50RhoGAP to endosomes and provides a link between Rab and Rho GTPases. J Biol Chem 2006;281:6096–6105.
83 Zhao R, Fu X, Li Q, Krantz SB, Zhao ZJ: Specific interaction of protein tyrosine phosphatase-MEG2 with phosphatidylserine. J Biol Chem 2003;278:22609–22614.
84 Gebauer D, Li J, Jogl G, Shen Y, Myszka DG, Tong L: Crystal structure of the PH-BEACH domains of human LRBA/BGL. Biochemistry 2004;43:14873–14880.
85 Gervais V, Lamour V, Jawhari A, Frindel F, Wasielewski E, et al: TFIIH contains a PH domain involved in DNA nucleotide excision repair. Nat Struct Mol Biol 2004;11:616–622.
86 VanDemark AP, Blanksma M, Ferris E, Heroux A, Hill CP, Formosa T: The structure of the yFACT Pob3-M domain, its interaction with the DNA replication factor RPA, and a potential role in nucleosome deposition. Mol Cell 2006;22:363–374.
87 Philip F, Guo Y, Scarlata S: Multiple roles of pleckstrin homology domains in phospholipase Cbeta function. FEBS Lett 2002;531:28–32.
88 Illenberger D, Schwald F, Pimmer D, Binder W, Maier G, et al: Stimulation of phospholipase C-beta2 by the Rho GTPases Cdc42Hs and Rac1. EMBO J 1998;17:6241–6249.

▶89 Lin YL, Lei YT, Hong CJ, Hsueh YP: Syndecan-2 induces filopodia and dendritic spine formation via the neurofibromin-PKA-Ena/VASP pathway. J Cell Biol 2007;177:829–841.
▶90 Bausch B, Borozdin W, Mautner VF, Hoffmann MM, Boehm D, et al: Germline *NF1* mutational spectra and loss-of-heterozygosity analyses in patients with pheochromocytoma and neurofibromatosis type 1. J Clin Endocrinol Metab 2007;92:2784–2792.
▶91 Griffiths S, Thompson P, Frayling I, Upadhyaya M: Molecular diagnosis of neurofibromatosis type 1:2 years experience. Fam Cancer 2007;6:21–34.
▶92 Hudson J, Wu CL, Tassabehji M, Summers EM, Simon S, et al: Novel and recurrent mutations in the neurofibromatosis type 1 *(NF1)* gene. Hum Mutat 1997;9:366–367.
▶93 Kluwe L, Friedrich RE, Korf B, Fahsold R, Mautner VF: *NF1* mutations in neurofibromatosis 1 patients with plexiform neurofibromas. Hum Mutat 2002;19:309.
▶94 Krkljus S, Abernathy CR, Johnson JS, Williams CA, Driscoll DJ, et al: Analysis of CpG C-to-T mutations in neurofibromatosis type 1. Mutations in brief no. 129. Online. Hum Mutat 1998; 11:411.
▶95 Messiaen LM, Callens T, Mortier G, Beysen D, Vandenbroucke I, et al: Exhaustive mutation analysis of the *NF1* gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. Hum Mutat 2000;15:541–555.
▶96 Purandare SM, Lanyon WG, Connor JM: Characterisation of inherited and sporadic mutations in neurofibromatosis type-1. Hum Mol Genet 1994;3:1109–1115.
▶97 Upadhyaya M, Ruggieri M, Maynard J, Osborn M, Hartog C, et al: Gross deletions of the neurofibromatosis type 1 *(NF1)* gene are predominantly of maternal origin and commonly associated with a learning disability, dysmorphic features and developmental delay. Hum Genet 1998;102: 591–597.
▶98 Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR, et al: cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the NF1 gene product. Genomics 1991;11:931–940.
▶99 Vandenbroucke I, Van Oostveldt P, Coene E, De Paepe A, Messiaen L: Neurofibromin is actively transported to the nucleus. FEBS Lett 2004;560:98–102.
▶100 Li C, Cheng Y, Gutmann DA, Mangoura D: Differential localization of the neurofibromatosis 1 *(NF1)* gene product, neurofibromin, with the F-actin or microtubule cytoskeleton during differentiation of telencephalic neurons. Brain Res Dev Brain Res 2001;130:231–248.
▶101 Feng L, Yunoue S, Tokuo H, Ozawa T, Yhanq D, et al: PKA phosphorylation and 14–3–3 interaction regulate the function of neurofibromatosis type 1 tumor suppressor neurofibromin. FEBS Lett 2004;557:275–282.
▶102 Hakimi MA, Speicher DW, Shiekhattar R: The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway of neurofibromatosis. J Biol Chem 2002;277:36909–36912.

Dr. Klaus Scheffzek
European Molecular Biology Laboratory (EMBL) Heidelberg
Meyerhofstr. 1
DE–69117 Heidelberg (Germany)
Tel. +49 6221 387 8401, Fax +49 6221 387 519, E-Mail scheffzek@embl.de

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 129–142

Composition of Neurofibromas, *NF1* Expression, and Comparison of Normal and *NF1* Haploinsufficient Cells

J. Peltonen[a], *S. Peltonen*[b]

[a]Institute of Biomedicine, Department of Anatomy and [b]Department of Dermatology, University of Turku, Turku, Finland

Abstract

Neurofibromas are benign hamartomatous, unencapsulated tumors arising from the connective tissue cells of peripheral nerves. Neurofibromas contain all the elements of peripheral nerves but in a disorganized manner. Schwann cells are the crucial pathogenic cell type in neurofibroma formation. It is apparent that the changes in $NF1^{-/-}$ Schwann cells are not sufficient for the development of neurofibromas. Instead, the presence of other cell types of peripheral nerves, such as perineurial cells, fibroblasts, and mast cells with an $NF1^{+/-}$ genotype is needed for the formation of neurofibromas. *NF1* expression is regulated at multiple levels resulting in highly varying levels of *NF1* mRNA and protein, even within minutes. This review summarizes the present knowledge how *NF1* gene expression is regulated at various levels such as transcription, RNA processing, mRNA transport, mRNA degradation, translation, posttranslational modifications, protein targeting and degradation. *NF1* haploinsufficiency at a cellular level leads to down-regulated levels of neurofibromin. The end result seems to be altered cell shape, size, migration, proliferation and intercellular communication. Consequences of *NF1* haploinsufficiency are discussed from the point of view of what symptoms and signs in neurofibromatosis type 1 (NF1) are caused by *NF1* haploinsufficiency compared to the loss of both *NF1* alleles.

Types of Neurofibromas

Neurofibromas are benign hamartomatous, unencapsulated tumors arising from the connective tissue cells of peripheral nerves [1]. Neurofibromas can be classified according to their anatomical location into cutaneous, subcutaneous, and intraneural tumors. Plexiform neurofibromas refer to poorly demarcated tumor masses infiltrating surrounding tissue.

Cutaneous neurofibromas arise from the cells of the connective tissue sheath of dermal nerve tributaries. They are nodular or polypoid and vary in size from millimetres to 2–3 centimetres. In some patients, cutaneous neurofibromas reside within the skin and are visible as slightly purplish macules that may feel softer than the surrounding skin. Subcutaneous neurofibromas are single nodules situated underneath the skin. They may be connected to a larger nerve branch of cutaneous nerve plexus. Intraneural neurofibromas grow within the nerve transforming it into a fusiform, or beaded mass. They affect spinal, cranial, or autonomic nerves, or peripheral nerve trunks. Such neurofibromas infrequently undergo malignant change.

Plexiform neurofibromas grow along sizable peripheral nerves and extend to smaller nerve branches. Involvement of branching nerves often forms a 'worm-like' tangle. The cutaneous part of a plexiform neurofibroma may manifest itself as a tumor mass, or thickened skin which shows hyperpigmentation, surrounded by a hypopigmented halo. About one third of NF1 patients have plexiform neurofibromas which may eventually form large and disfiguring tumor masses in the craniofacial region, trunk, or extremities.

Components of Neurofibromas

Neurofibromas contain all elements of peripheral nerves but organized in a haphazard manner. The development of a neurofibroma tumor is associated with disruption of the intimate communication between axons and Schwann cells of a peripheral nerve. This results in a tumor composed of Schwann cells, fibroblasts, perineurial cells, mast cells, axonal processes and blood vessels embedded in abundant collagenous extracellular matrix [2]. Regardless of the apparent disorganization in the tumor, cutaneous neurofibromas never undergo malignant transformation. Thus, the neoplastic cells of cutaneous neurofibromas have an amazing tendency to retain their benign phenotype.

Schwann Cells

Schwann cells are the crucial pathogenic cell type in neurofibroma formation. Schwann cells account for 60–80% of the cells in neurofibromas, as defined by staining for the S100beta protein [2]. According to the 'two-hit hypothesis', neurofibromas develop when a somatic mutation in Schwann cell lineages disrupts the remaining functional copy of the *NF1* allele leading to biallelic inactivation of the gene [3, 4]. Thus, at least a subpopulation of neoplastic Schwann cells of neurofibromas carries two mutations in the *NF1* gene: the germline mutation and a new somatic mutation [4]. Neoplastic Schwann

cells retain certain differentiated characteristics. They express S100beta, although at a lower level compared to nerve-associated Schwann cells [2]. At the ultrastructural level, neoplastic Schwann cells have complex, branched cytoplasmic processes which are covered by a continuous basement membrane [5, 6]. Thus, Schwann cells can be recognized in the tumor by their positive immunosignal for basement membrane components such as type IV collagen and laminin chains. In contrast to normal Schwann cells, neurofibroma Schwann cells express nerve growth factor (NGF) receptors [7].

There is increasing evidence that tumor suppressor mutations alone do not explain tumorigenesis. Additionally, there are multiple aberrantly expressed growth factors and/or growth factor receptors which result in deregulated growth factor signaling to promote the proliferation, survival, and migration of neoplastic Schwann cells. These potential growth factors include at least neuregulin-1 (NRG-1) and its erbB receptors, the EGF receptor (EGFR; also known as erbB1), hepatocyte growth factor (HGF; also known as scatter factor), PDGF and its receptors and midkine [8]. The changes in *NF1*$^{-/-}$ Schwann cells are, however, not sufficient for the development of neurofibromas. *NF1*$^{-/-}$ Schwann cells form tumors in an *NF1* haploinsufficient background only, which consists of perineurial cells, fibroblasts, and mast cells.

Perineurial Cells

In normal nerve, perineurial cell layers form a tight sheath around groups of axon-Schwann cell units that form nerve fascicles. The role of perineurial cells in the development of neurofibromas has been considered as permissive, since in neurofibromas the neoplastic Schwann cells somehow manage to penetrate the perineurial barrier and proliferate outside of disrupted nerve fascicles. The evaluation of the identity and role of perineurial cells in neurofibromas has been challenging because of the lack of a single characteristic marker protein. At the ultrastructural level, perineurial cells surrounding rudimentary nerve fascicles have long slender cell processes, a continuous basement membrane and numerous intracellular vesicles [6]. Solitary cells in neurofibromas may have a fragmented basement membrane and the pinocytotic vesicles may be less numerous compared to normal perineurial cells [6, 9]. Thus, the presence of basement membranes on perineurial cells makes a clear distinction between perineurial cells and fibroblasts. The latter, by definition, do not have a basement membrane. A panel of molecular markers such as epithelial membrane antigen (EMA), Glut-1 and type IV collagen has been used to identify perineurial cells in neurofibromas [2, 10, 11]. The most promising marker for perineurial cells is the tight junction protein, claudin-1 [12]. The expression of tight junction proteins by perineurial cells is in line with previous ultrastructural observations showing that overlapping processes

of adjacent perineurial cells are occasionally joined by tight junction-like cell contacts [13].

Based on the studies with combinations of antigenic markers, it can be stated that perineurial cells exist in all neurofibromas while the number of them varies between individuals and tumor areas [2, 12]. All neurofibromas show localized areas with perineurial cell differentiation. About 10% of neurofibromas show a major subpopulation of perineurial cells, as estimated by lack of immunoreaction for S100beta protein and positive reactions to EMA and claudin-1 [2, 12].

Fibroblasts

Neurofibroma fibroblasts are believed to originate from endoneurial fibroblasts. They contribute to the accumulation of fibrillar collagens and fibronectin in the tumor stroma. Fibroblasts have abundant cytoplasm with numerous cell organelles, but lack the basement membrane [6]. Immunohistochemically, fibroblasts can be detected by the expression of CD-34 [11].

Mast Cells

In normal skin, mast cells are located in close proximity of sensory nerves and hair follicles. Indeed, mast cells are known to have a functional association with both sensory nerves and hair follicles by various mediators that activated mast cells are able to selectively secrete. Mast cells can also be seen within nerve trunks. In addition, there is a specific Kit-ligand mediated mechanism for mast cell migration to neurofibromas [14]. Not surprisingly, mast cells are numerous in neurofibromas and other peripheral nerve sheath tumors. Mast cells can be recognized by their typical cytoplasmic granules, and can be visualized by Alcian blue staining at pH 1, toluidine blue staining, or by immunodetection of factor VIII-related antigen [2]. The molecular mechanisms explaining how *NF1* haploinsufficient mast cells may contribute in neurofibroma growth are discussed below.

Extracellular Matrix

The extracellular matrix forms a marked proportion of the volume of neurofibromas. Abundant components of this matrix include collagens I, III, IV, V and VI; fibronectin and basement membrane components type IV collagen, laminin, and nidogen [6, 15]. Perineurial cells, Schwann cells and fibroblasts all contribute to the synthesis of the extracellular matrix [16]. Schwann cells contribute to the accumulation of the tumor stroma by producing fibrillar collagen type I, basement membrane components type IV collagen and laminin, and type VI collagen [15, 16].

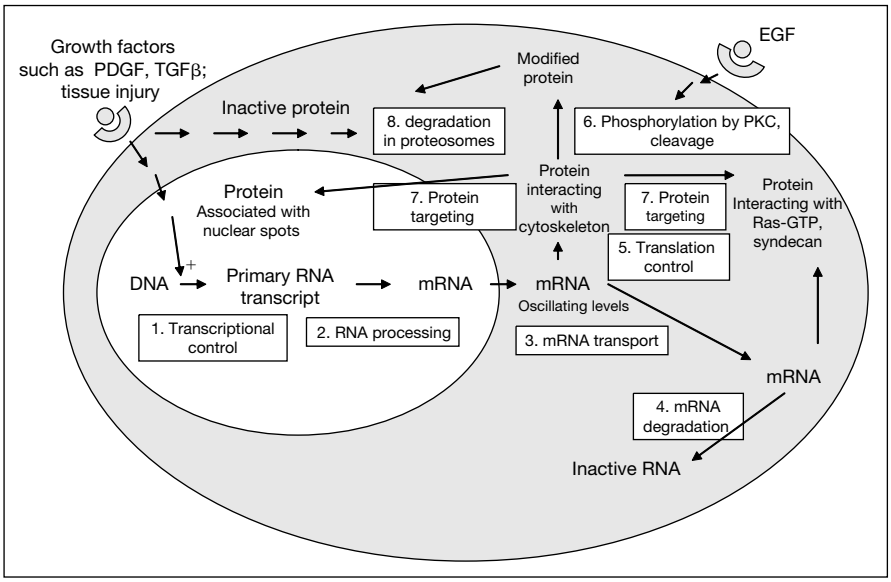


Fig. 1. Life cycle of neurofibromin. *NF1* expression is regulated at multiple steps resulting in highly varying levels of *NF1* mRNA and protein even within minutes. Steps 1–8 in *NF1* regulation are explained in more detail in the text.

NF1 Gene Expression

Life Cycle of Neurofibromin

The fact that all patients are heterozygous for an *NF1* mutation, and that homozygous $Nf1^{-/-}$ experimental mice die in utero, reveals that the *NF1* gene product – neurofibromin – is essential for life. The *NF1* gene is all but a house keeping gene, and *NF1* expression is regulated at multiple levels resulting in highly varying levels of *NF1* mRNA and protein even within minutes (fig. 1). *1. Transcriptional control*. In silico analysis of the 5′ upstream region and intron 1 of the *NF1* gene from human, mouse, rat, and pufferfish has probed potential transcriptional regulatory regions [17]. Because a sequence 310–333 bp upstream of the translation initiation site is identical in human, mouse, and rat and differs by only 1 bp in pufferfish, it may contain the core promoter element for *NF1* transcription. In vivo, *NF1* expression is up-regulated in tissue repair of peripheral nerve, skin and bone [18–21], and during development of peripheral nerve and bone [18, 22, 23]. Molecules leading to up-regulated transcription of

the *NF1* gene include bFGF, PDGF and EGF [19, 24, 25]. Interestingly, the same growth factors are operative in the degradation of neurofibromin (see below). *2. RNA processing*. In northern transfer analysis, *NF1* mRNA is detected as 11–13-kb band/bands. RNA processing results in alternative splicing of exons 9a, 10a-2, 23a, and 48a. Exon 10a-2 is spliced in between exons 10a and 10b [26–29]. Other *NF1* RNA splicing variants may exist. Some of the mutations causing NF1 affect RNA processing creating de novo splice sites which eventually may cause deletions, or insertion of extranumerary exon sequences [30]. *3. mRNA transport*. The 3′ UTR of *NF1* mRNA contains five potential protein binding sites, one of which binds to tumor antigen HuR [31]. HuR is involved in posttranscriptional mRNA stabilization, and transport from nucleus. In the cytoplasm, a part of *NF1* mRNA can be targeted to cell periphery via actin microfilaments in migrating and cell junction forming cells [32]. *4. mRNA levels and degradation*. The mRNA levels may oscillate even within minutes, and the half-life may vary under different conditions [12, 32, 33]. The decay of *NF1* mRNA is poorly understood. Disruption of actin microfilaments facilitates *NF1* mRNA degradation [32, 34, 35], and terminal differentiation of epithelial cells results in down regulation of *NF1* mRNA levels [36]. *5. Translational control*. Neurofibromin I has been used to refer to the isoform not containing sequences encoded by alternatively spliced exons; type II isoform containing 23a; type III containing 48a; and type IV containing 23a and 48a [27]. It is not clear if all *NF1* RNA splice variants translate to protein. Since no alteration in *NF1* mRNA level was detected but neurofibromin levels increased in Schwann cells following nerve injury, it has been suggested that neurofibromin expression in Schwann cells is post-transcriptionally induced [18]. This means increased translational efficiency or protein stabilization. *6. Posttranslational modifications*. In western transfer analysis, neurofibromin is detected as single or double band/bands with an apparent molecular mass of 220–250 kDa [22]. This is less than expected for a 2818 amino acid sequence (estimated ~327 kDa). Compact protein folding may be the reason for this. Phosphorylation, cleavage: Neurofibromin is constitutively phosphorylated at the cysteine/serine-rich domains of the N-terminus and in the C-terminal region [37]. Neurofibromin is also a substrate for protein kinase C. In response to EGF, PKCα phosphorylates neurofibromin on serine residues within cysteine/serine rich domains [35]. This phosphorylation results in increased association of neurofibromin with actin cytoskeleton. The protein bands of ~250 kDa, specific for neurofibromin, are likely to represent differentially phosphorylated forms of neurofibromin. Even though not extensively discussed, additional bands of lower molecular mass are often seen in immunoprecipitation/western analyses. The fact that the sizes of these extra bands seem similar in independent studies suggests that some of them may represent cleavage

products of neurofibromin, or proteins interacting and coprecipitating with neurofibromin [22, 38]. *7. Protein targeting*. For the well established interaction with Ras-GTP [39], a part of the functional neurofibromin pool must be located near the plasma membrane since Ras-GTP is attached to the membrane through a farnesyl residue. The same location allows interaction between neurofibromin and syndecan, a transmembrane proteoglycan [40]. Neurofibromin may in fact be attached to the plasma membrane since the N-terminal splice product *NF1−10a-2* of the *NF1* gene codes for a transmembrane segment [28]. Targeting of neurofibromin to the cytoskeleton takes place through multiple associations. Biochemical evidence has demonstrated interaction of neurofibromin with actin, tubulin and intermediate filaments [41–44]. Tubulin inhibits Ras-GAP activity [41]. The best visualization of interaction with the cytoskeleton comes from double immunodetection of neurofibromin with cytokeratin containing intermediate filaments in epithelial cells. In melanocytes, neurofibromin takes part in melanin transport along microtubules [38]. Peptide sequence KRQEMESGITTPPKMRR encoded by a part of exon 43 directs neurofibromin to the nucleus [45] where its subcellular distribution is sometimes speckled [43, 45]. More specifically, neurofibromin has been suggested to be targeted to nucleoli [45]. *8. Protein degradation*. Neurofibromin is dynamically regulated by the ubiquitin-proteasome pathway. Degradation is rapidly triggered in response to PDGF, EGF and lysophosphatidic acid (LPA) and requires sequences adjacent to the catalytic GAP-related domain of neurofibromin. However, whereas degradation is rapid, neurofibromin levels are re-elevated shortly after growth factor treatment [25].

Tissue Distribution of Neurofibromin

The *NF1* gene is ubiquitously expressed in almost all tissues but most intensely in central and peripheral nervous systems [46]. Several studies implicate that NF1 protein plays a crucial role during development, and the expression level and pattern of NF1 protein have been noted to change markedly and rapidly [22, 46]. These rapid changes have been considered to be related to some of the major morphological changes occurring in tissues such as brain and heart [47]. Mice carrying a homozygous null mutation at the *Nf1* locus die in utero, apparently due to the severe heart malformations [48, 49]. There are clear differences in the expression of the *NF1* gene between species. For example, *Nf1* mRNA was present in developing rat sciatic nerve throughout the period of active Schwann cell proliferation and myelination [18], while in human the neurofibromin immunosignal was barely detectable in peripheral nerves during the first trimester of gestation [22]. The expression level increased dramatically in Schwann cells, perineurial cells, and axons during the second and third trimesters of pregnancy [22].

The pattern of *NF1* gene expression changes markedly also in developing human epidermis, where neurofibromin is associated with cytokeratin filaments terminating on desmosomes and hemidesmosomes [49]. These results suggest that the NF1 tumor suppressor may function in the regulation of epidermal histogenesis via controlling the organization of the keratin cytoskeleton during the assembly of desmosomes and hemidesmosomes.

Haploinsufficiency in NF1

Haploinsufficiency refers to a state where an individual has one functional copy of a gene. The single functional copy of the gene may not produce enough of a gene product, or protein, to result in normal development and cell growth regulation, leading to an abnormal or diseased state.

An important question is what symptoms and signs in NF1 are caused by haploinsufficiency as such, and what is the result and role of loss of both *NF1* alleles. Another aspect and question is: how does the inactivation of one allele or both alleles change the reading of the whole genome.

It now seems that some distinct lesions characteristic to NF1 are associated with loss of both functional alleles in different cell types. Specifically, a subset of Schwann cells in neurofibromas has an $NF1^{-/-}$ genotype [4]. In analogy, melanocytes in café au lait spots have lost both *NF1* alleles [38]. Studies utilizing transgenic mice with cell type specific inactivation of both *Nf1* alleles are described in detail elsewhere in this book. A summary of results on man and mouse suggests that the development of neurofibromas requires the 'second hit Schwann cells', but the tumors develop only if the other cell types in these tumors are *NF1* haploinsufficient.

Haploinsufficiency in *NF1* has been approached in studies utilizing different types of cell cultures derived from patients with NF1, versus control persons. In general, *NF1* deficient cells, e.g. fibroblasts, melanocytes, keratinocytes, osteoblasts, astrocytes and Schwann cells may display more variable sizes and shapes than their $NF1^{+/+}$ counterparts (fig. 2). These findings represent predicted alterations in a situation where cytoskeletal organization is disturbed. In fact, visualization of individual cytoskeletal components has revealed irregular organization of the whole cytoskeletal system. Neurofibromin may thus, in part, exert its effects by controlling the organization of the cytoskeleton and the formation/maintenance of cellular contacts. Both cytoskeletal and cell junctional impairment may contribute to the fact that also calcium-mediated cell signaling is impaired in *NF1* haploinsufficient cells [50]. Other phenomena associated with *NF1* haploinsufficiency include increased astrocyte proliferation, augmentation in angiogenesis, and altered skeletal development [51, 52].

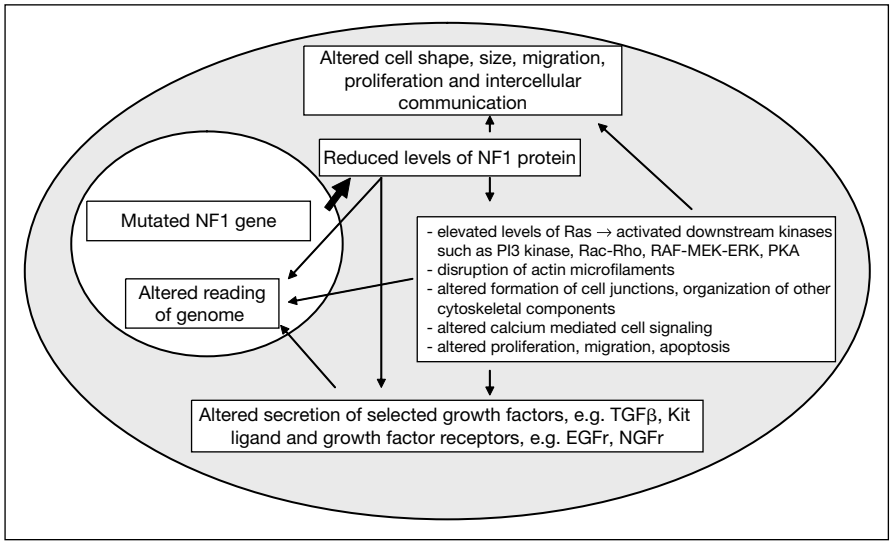


Fig. 2. *NF1* haploinsufficiency at cellular level leads to down-regulated levels of neurofibromin. The end result includes altered cell shape, size, migration, proliferation and intercellular communication. The underlying molecular mechanism may include insufficient intermolecular contacts between neurofibromin and other proteins, and down-stream effects of any of these interactions. The best known down-stream event of *NF1* haploinsufficiency is up-regulation of Ras-GTP signal transduction through the MAPK pathway.

NF1 haploinsufficiency of mast cells has gained attention. Cross talk between mast cells and Schwann cells may contribute to neurofibroma growth. It has been speculated that mast cells would create a cytokine-rich microenvironment that could be permissive for neurofibroma growth [3]. Mast cell migration is mediated by the Ras/PI3K/Rac2 signal transduction pathway, which in turn is enhanced in $NF1^{+/-}$ mast cells. Kit ligand serves as a chemoattractant for mast cells expressing c-Kit, while $NF1^{-/-}$ Schwann cells in the tumor express Kit ligand at a high level. Recent findings on transgenic mice have shown that the K-isoform of Ras is required for c-kit-mediated mast cell proliferation, survival, migration, and degranulation in vitro and in vivo. These cellular functions are hyperactivated in $NF1^{+/-}$ mast cells. Indeed, neurofibromin has been identified as a GAP for K-ras in mast cells [53]. In addition, there are other potential players in the mast cell–Schwann cell interaction, such as α4β1 integrin and various other mast cell mediators. $NF1^{+/-}$ mast cells have

been shown to induce neurofibroma like phenotypes through secreted TGF-beta signaling [54]. However, it is yet to be determined if and how the presence of mast cells induces tumor progression.

Haploinsufficiency of Endothelial Cells in Neurofibromas

Interaction of the major tumor-initiating cell type with other haploinsufficient cell types, such as endothelial cells, is necessary for tumor progression. Tumor vascularization occurs by neoangiogenesis from existing nearby vessels, and the attraction and seeding of circulating endothelial stem cells which give rise to new blood vessels within tumors [55]. Neurofibromas, especially plexiform tumors, are highly vascularized. Studies on vascularization are relevant for searching potential ways to slow or inhibit tumor growth. Angiogenesis can be promoted in two ways: *NF1* haploinsufficiency in endothelial cells leads to altered response to angiogenetic factors, or *NF1* haploinsufficiency in Schwann cells augments angiogenesis. Indeed, there is evidence for both mechanisms. *NF1* haploinsufficient endothelial cells of neurofibromas show morphological alterations [56]. Using the NF1 mouse model it has been shown that angiogenesis is accelerated in NF1 due to hyperproliferation of pericytes and endothelial cells [57]. *NF1* heterozygous endothelial cell cultures showed an exaggerated proliferative response to angiogenic factors, particularly to bFGF [51]. Recent studies have also revealed a discrete Ras effector pathway, which alters the proliferation and migration of *NF1* haploinsufficient endothelial cells in response to neurofibroma-derived growth factors both in vitro and in vivo [58]. These findings support the conclusion that *NF1* heterozygosity in endothelial cells and perhaps inflammatory cells augments angiogenesis, which may promote neurofibroma formation in NF1. Neurofibroma Schwann cells unlike normal Schwann cells promote angiogenesis [59]. Basic fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF) and midkine (MK) have been found to be induced by loss of neurofibromin in $NF1^{+/-}$ Schwann cells in the NF1 mouse model [60].

In summary, the pathomechanism of neurofibroma tumors is a puzzle, the pieces of which are being revealed step by step. Although genetics, animal models, biochemistry and cell biology have brought in vast amount of details, the sequence of events leading to the development of neurofibromas is still mostly unknown. Even the exact structure and relationship of various histological components within the tumor have not been fully characterized. Various different classifications of neurofibroma tumors according to the initiating nerve, tissue morphology, and growth properties have been proposed and reviewed [61], even to the extent that the classification would be an issue for a consensus meeting. This review does not attempt to favor one classification over another or propose a new one. However, the cellular and molecular

components of neurofibromas as described here are applicable to different types of neurofibromas. At present, it seems clear that the combination of double inactivation of *NF1* in a 'permissive' haploinsufficient background explains the full spectrum of the NF1 phenotype at the cellular level.

References

1 Evans GR, Scheithauer B, Komminoth P, Peltonen J: Neurofibromatosis 1; in: DeLellis RA, Lloyd RV, Heitz PU, Eng C (eds): WHO Classification of Tumours. Pathology & Genetics: Tumours of Endocrine Organs, Chapter 5: Inherited Tumour Syndromes. Lyon IARC Press, 2004, pp 243–248.

▶2 Peltonen J, Jaakkola S, Lebwohl M, Renvall S, Risteli L, et al: Cellular differentiation and expression of matrix genes in type 1 neurofibromatosis. Lab Invest 1988;59:760–771.

▶3 Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF: Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 2002;296:920–922.

▶4 Maertens O, Brems H, Vandesompele J, De Raedt T, Heyns I, et al: Comprehensive NF1 screening on cultured Schwann cells from neurofibromas. Hum Mutat 2006;10:1030–1040.

▶5 Hirose T, Sano T, Hizawa K: Ultrastructural localization of S-100 protein in neurofibroma. Acta Neuropathol (Berl) 1986;69:103–110.

▶6 Lassmann H, Jurecka W, Gebhart W: Some electron microscopic and autoradiographic results concerning cutaneous neurofibromas in von Recklinghausen's disease. Arch Dermatol Res 1976; 255:69–81.

▶7 Ross AH, Herlyn M, Maul GG, Koprowski H, Bothwell M, et al: The nerve growth factor receptor in normal and transformed neural crest cells. Ann N Y Acad Sci 1986;486:115–123.

▶8 Carroll SL, Stonecypher MS: Tumor suppressor mutations and growth factor signaling in the pathogenesis of NF1-associated peripheral nerve sheath tumors: II. The role of dysregulated growth factor signaling. J Neuropathol Exp Neurol 2005;64:1–9.

▶9 Erlandson RA, Woodruff JM: Peripheral nerve sheath tumors: an electron microscopic study of 43 cases. Cancer 1982;49:273–287.

▶10 Perentes E, Nakagawa Y, Ross GW, Stanton C, Rubinstein LJ: Expression of epithelial membrane antigen in perineurial cells and their derivatives. An immunohistochemical study with multiple markers. Acta Neuropathol (Berl) 1987;75:160–165.

▶11 Hirose T, Tani T, Shimada T, Ishizawa K, Shimada S, Sano T: Immunohistochemical demonstration of EMA/Glut1-positive perineurial cells and CD34-positive fibroblastic cells in peripheral nerve sheath tumors. Mod Pathol 2003;16:293–298.

▶12 Pummi K, Yla-Outinen H, Peltonen J: Oscillation and rapid changes of NF1 mRNA steady-state levels in cultured human keratinocytes. Arch Dermatol Res 2000;8:422–424.

▶13 Erlandson RA: Peripheral nerve sheath tumors. Ultrastruct Pathol 1985;9:113–122.

▶14 Yang FC, Ingram DA, Chen S, Higgins CM, Ratner N, et al: Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for *Nf1*+/– mast cells. J Clin Invest 2003;112: 1851–1861.

▶15 Peltonen J, Penttinen R, Larjava H, Aho HJ: Collagens in neurofibromas and neurofibroma cell cultures. Ann N Y Acad Sci 1986;486:260–270.

▶16 Jaakkola S, Peltonen J, Riccardi V, Chu ML, Uitto J: Type 1 neurofibromatosis: selective expression of extracellular matrix genes by Schwann cells, perineurial cells, and fibroblasts in mixed cultures. J Clin Invest 1989;84:253–261.

17 Lee TK, Friedman JM: Analysis of *NF1* transcriptional regulatory elements. Am J Med Genet A 2005;137:130–135.

▶18 Wrabetz L, Feltri ML, Kim H, Daston M, Kamholz J, et al: Regulation of neurofibromin expression in rat sciatic nerve and cultured Schwann cells. Glia 1995;15:22–32.

19 Ylä-Outinen H, Aaltonen V, Björkstrand A-S, Hirvonen O, Lakkakorpi J, et al: Upregulation of tumor suppressor protein neurofibromin in normal wound healing and in vitro evidence for platelet derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) elicited increase in neurofibromin mRNA steady-state levels in dermal fibroblasts. J Invest Dermatol 1998;110:232–237.
20 Atit RP, Crowe MJ, Greenhalgh DG, Wenstrup RJ, Ratner N: The *Nf1* tumor suppressor regulates mouse skin wound healing, fibroblast proliferation, and collagen deposited by fibroblasts. J Invest Dermatol 1999;112:835–842.
21 Kuorilehto T, Ekholm E, Nissinen M, Hietaniemi K, Hiltunen A, et al: NF1 gene expression in mouse fracture healing and in experimental rat pseudarthrosis. J Histochem Cytochem 2006;54: 363–370.
22 Hirvonen O, Lakkakorpi J, Aaltonen V, Hirvonen H, Rossi M, et al: Developmental regulation of *NF1* tumor suppressor gene in human peripheral nerve. J Neurocytol 1998;27:939–952.
23 Kuorilehto T, Nissinen M, Koivunen J, Benson MD, Peltonen J: NF1 tumor suppressor protein and mRNA in skeletal tissues of developing and adult normal mouse and NF1-deficient embryos. J Bone Miner Res 2004;19:983–989.
24 Griesser J, Kaufmann D, Maier B, Mailhammer R, Kuehl P, Krone W: Post-transcriptional regulation of neurofibromin level in cultured human melanocytes in response to growth factors. J Invest Dermatol 1997;108:275–280.
25 Cichowski K, Santiago S, Jardim M, Johnson BW, Jacks T: Dynamic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. Genes Dev 2003;17:449–454.
26 Nishi T, Lee PS, Oka K, Levin VA, Tanase S, et al: Differential expression of two types of the neurofibromatosis type 1 (NF1) gene transcripts related to neuronal differentiation. Oncogene 1991;6: 1555–1559.
27 Gutmann DH, Geist RT, Rose K, Wright DE: Expression of two new protein isoforms of the neurofibromatosis type 1 gene product, neurofibromin, in muscle tissues. Dev Dyn 1995;202: 302–311.
28 Kaufmann D, Muller R, Kenner O, Leistner W, Hein C, et al: The N-terminal splice product *NF1−10a-2* of the *NF1* gene codes for a transmembrane segment. Biochem Biophys Res Commun 2002;294:496–503.
29 Vandenbroucke I, Vandesompele J, De Paepe A, Messiaen L: Quantification of *NF1* transcripts reveals novel highly expressed splice variants. FEBS Lett 2002;522:71–76.
30 Baralle M, Skoko N, Knezevich A, De Conti L, Motti D, et al: *NF1* mRNA biogenesis: effect of the genomic milieu in splicing regulation of the *NF1* exon 37 region. FEBS Lett 2006;580: 4449–4456.
31 Haeussler J, Haeusler J, Striebel AM, Assum G, Vogel W, et al: Tumor antigen HuR binds specifically to one of five protein-binding segments in the 3′-untranslated region of the neurofibromin messenger RNA. Biochem Biophys Res Commun 2000;267:726–732.
32 Ylä-Outinen H, Koivunen J, Nissinen M, Björkstrand A-S, Paloniemi M, et al: NF1 tumor suppressor mRNA is targeted to cell-cell contact zone in Ca^{2+}-induced keratinocyte differentiation. Lab Invest 2002;82:353–361.
33 Kaufmann D, Bartelt B, Hoffmeyer S, Muller R: Posttranslational regulation of neurofibromin content in melanocytes of neurofibromatosis type 1. Arch Dermatol Res 1999;291:312–317.
34 Korkiamaki T, Yla-Outinen H, Leinonen P, Koivunen J, Peltonen J: The effect of extracellular calcium concentration on calcium-mediated cell signaling in NF1 tumor suppressor-deficient keratinocytes. Arch Dermatol Res 2005;296:465–472.
35 Mangoura D, Sun Y, Li C, Singh D, Gutmann DH, et al: Phosphorylation of neurofibromin by PKC is a possible molecular switch in EGF receptor signaling in neural cells. Oncogene 2006; 25:735–745.
36 Hermonen J, Hirvonen O, Yla-Outinen H, Lakkakorpi J, Bjorkstrand AS, et al: Neurofibromin: expression by normal human keratinocytes in vivo and in vitro and in epidermal malignancies. Lab Invest 1995;73:221–228.
37 Izawa I, Tamaki N, Saya H: Phosphorylation of neurofibromatosis type 1 gene product (neurofibromin) by cAMP-dependent protein kinase. FEBS Lett 1996;382:53–59.

38 De Schepper S, Boucneau JM, Westbroek W, Mommaas M, Onderwater J, et al: Neurofibromatosis type 1 protein and amyloid precursor protein interact in normal human melanocytes and colocalize with melanosomes. J Invest Dermatol 2006;126:653–659.

39 Xu GF, Lin B, Tanaka K, Dunn D, Wood D, et al: The catalytic domain of neurofibromatosis type I gene product stimulates ras GTPase and complements ira mutants of *S. cerevisae*. Cell 1990;63: 835–841.

40 Hsueh YP, Roberts AM, Volta M, Sheng M, Roberts RG: Bipartite interaction between neurofibromatosis type I protein (neurofibromin) and syndecan transmembrane heparan sulfate proteoglycans. J Neurosci 2001;21:3764–3770.

41 Bollag G, McCormick F, Clark R: Characterization of full-length neurofibromin: tubulin inhibits Ras-GAP activity. EMBO J 1993;12:1923–1927.

42 Gregory PE, Gutmann DH, Mitchell A, Park S, Boguski M, et al: Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. Somat Cell Mol Genet 1993;19:265–274.

43 Koivunen J, Yla-Outinen H, Korkiamaki T, Karvonen SL, Poyhonen M, et al: New function for NF1 tumor suppressor. J Invest Dermatol 2000;114:473–479.

44 Li C, Cheng Y, Gutmann DA, Mangoura D: Differential localization of the neurofibromatosis 1 (NF1) gene product, neurofibromin, with the F-actin or microtubule cytoskeleton during differentiation of telencephalic neurons. Brain Res Dev Brain Res 2001;130:231–248.

45 Vandenbroucke I, Van Oostveldt P, Coene E, De Paepe A, Messiaen L: Neurofibromin is actively transported to the nucleus. FEBS Lett 2004;560:98–102.

46 Daston MM, Ratner N: Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. Dev Dyn 1992;195:216–226.

47 Huynh DP, Nechiporuk T, Pulst SM: Differential expression and tissue distribution of type I and type II neurofibromins during mouse fetal development. Dev Biol 1994;161:538–551.

48 Brannan CI, Perkins AS, Vogel KS, Ratner N, Nordlund ML, et al: Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev 1994;8:1019–1029.

49 Malminen M, Peltonen S, Koivunen J, Peltonen J: Functional expression of NF1 tumor suppressor protein: association with keratin intermediate filaments during the early development of human epidermis. BMC Dermatol 2002;2:10.

50 Korkiamäki T, Ylä-Outinen H, Koivunen J, Karvonen S-L, Peltonen J: Altered calcium mediated cell signaling in keratinocytes cultured from patients with neurofibromatosis type 1. Am J Pathol 2002;160:1981–1990.

51 Wu M, Wallace MR, Muir D: *Nf1* haploinsufficiency augments angiogenesis. Oncogene 2006; 25:2297–2303.

52 Gutmann DH, Loehr A, Zhang Y, Kim J, Henkemeyer M, Cashen A: Haploinsufficiency for the neurofibromatosis 1 (NF1) tumor suppressor results in increased astrocyte proliferation. Oncogene 1999;18:4450–4459.

53 Khalaf WF, Yang FC, Chen S, White H, Bessler W, et al: *K-ras* is critical for modulating multiple c-kit-mediated cellular functions in wild-type and $Nf1^{+/-}$ mast cells. J Immunol 2007;178: 2527–2534.

54 Yang FC, Chen S, Clegg T, Li X, Morgan T, et al: $Nf1^{+/-}$ mast cells induce neurofibroma like phenotypes through secreted TGF-beta signaling. Hum Mol Genet 2006;15:2421–2437.

55 Lyden D, Hattori K, Dias S: Impaired recruitment of bone marrow-derived endothelial and hematopoietic precursor cells block tumor angiogenesis and growth. Nat Med 2001;7:1194–1201.

56 Teixeira F, Martinez-Palomo A, Riccardi VM, Fernandez-Diez J: Vascular changes in cutaneous neurofibromas. Neurofibromatosis 1988;1:5–16.

57 Ozerdem U: Targeting neovascular pericytes in neurofibromatosis type 1. Angiogenesis 2004;7: 307–311.

58 Munchhof AM, Li F, White HA, Mead LE, Krier TR, et al: Neurofibroma-associated growth factors activate a distinct signaling network to alter the function of neurofibromin-deficient endothelial cells. Hum Mol Genet 2006;15:1858–1869.

59 Sheela S, Riccardi VM, Ratner N: Angiogenic and invasive properties of neurofibroma Schwann cells. J Cell Biol 1990;111:645–653.

▶60 Mashour GA, Ratner N, Khan GA, Wang HL, Martuza RL, Kurtz A: The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. Oncogene 2001;20:97–105.
▶61 Riccardi VM: The genetic predisposition to and histogenesis of neurofibromas and neurofibrosarcoma in neurofibromatosis type 1. Neurosurg Focus 2007;22:E3 1–11.

Juha Peltonen
Department of Anatomy, University of Turku
Kiinamyllynkatu 10
FI–20520 Turku (Finland)
Tel. +358 2 333 7226, Fax +358 2 333 7352, E-Mail juha.peltonen@utu.fi

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 143–153

Somatic *NF1* Mutations in Tumors and Other Tissues

T. De Raedt[a], *O. Maertens*[b], *E. Serra*[c], *E. Legius*[a]

[a]Center of Human Genetics, Catholic University of Leuven, Leuven, [b]Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium; [c]Centre de Genètica Médica i Molecular-IDIBELL, L'Hospitalet de Llobregat, Llobregat, Spain

Abstract

Recently the amount of knowledge about *NF1* somatic mutations has increased considerably. This is in part due to the availability of more comprehensive *NF1* mutation screening methods, but also because cell type specific analyses have been performed. NF1 lesions are usually composed of several cell types and for each lesion where a somatic second hit was found, it was only present in one specific cell type. Further examination of somatic *NF1* mutations in homogeneous cell populations of specific cell types can provide a world of information on the pathogenesis of NF1 lesions and on the interaction between different cell types in these lesions.

Neurofibromatosis type 1 (NF1) is characterized by a high variability in the severity of the disease. Neurofibromas and pigmentary abnormalities (café-au-lait macules, Lisch nodules and skin-fold freckling) are the major characteristic disease features, but plexiform neurofibromas, learning problems, orthopedic problems (scoliosis, pseudarthrosis of tibia) and epilepsy are also common [1]. In addition, several tumors are associated with the disease, like optic pathway glioma, pheochromocytoma or malignant peripheral nerve sheath tumor [1].

The cellular and molecular pathophysiology of the different NF1 disease features are not completely understood. For some features we know that a somatic inactivation of the wild type *NF1* allele is required and we know the specific cell types involved (table 1). For other NF1 features these data are not yet available.

Somatic mutations in the *NF1* gene could be the first hit occurring during embryonic development, generating NF1 mosaic patients. Alternatively somatic

Table 1. Overview of NF1 associated features with known somatic *NF1* mutations

Tissue type	*NF1* somatic mutation	Cell type involved	Tissue of origin	Embryological origin	Reference
Dermal neurofibromas	point mutations, LOH	Schwann cell	PNS	neural crest	4–6, 8–24
Plexiform neurofibromas	LOH, intragenic point mutation	Schwann cell	PNS	neural crest	13, 20, 22, 23
MPNST	LOH	Schwann cell	PNS	neural crest	13, 20, 26–28
GIST	LOH, intragenic point mutations	interstitial cells of Cajal	gastro-intestinal tract	neural crest	35–36
JMML	LOH	myeloid precursor cell	bone marrow	mesoderm	39–41
Astrocytomas	LOH	astrocytes	CNS	ectoderm	43–47
Pheochromocytoma	LOH	chromaffin cell	adrenal glands	neural crest	53–55
Tibial pseudarthrosis	LOH	osteoblast or osteoclast	bone	mesoderm (neural crest?)	61
Café-au-lait spot	point mutations	melanocyte	skin	neural crest	79

LOH: loss of heterozygosity; PNS: peripheral nervous system.

NF1 mutations could be second hits inactivating the wild type *NF1* allele in *NF1* heterozygous patients and they can be responsible for NF1 associated neoplastic features. For space limitations, this review will mainly focus on somatic mutations in the *NF1* gene in the typical NF1 associated neoplasias and we will not review mutations in the *NF1* gene in sporadic neoplasias.

Somatic *NF1* Mutations as a 'Second Hit' in NF1 Associated Neoplasias

Peripheral Nerve Sheath Tumors

The most common NF1 associated tumor is the benign peripheral nerve sheath tumor or neurofibroma. Neurofibromas have been subdivided into discrete neurofibromas, which are associated with a single peripheral nerve, and diffuse (plexiform) neurofibromas, which involve multiple nerve fascicles. In a small percentage of NF1 patients plexiform neurofibromas might progress to malignant peripheral nerve sheath tumors (MPNSTs).

Neurofibroma

Neurofibromas contain increased numbers of all cell types present in the normal peripheral nerve [2, 3]. Schwann cells are neural crest derived glial cells that form a protective sheath around single peripheral nerve fibers. Molecular analyses of neurofibromas have shown that a subpopulation of Schwann cells ($SC^{NF1-/-}$) in these tumors exhibit biallelic inactivation of the *NF1* gene [4–6]. Combined analysis of NF1 associated neurofibromas and the derived Schwann cells and fibroblasts, revealed loss of heterozygosity (LOH) in both the original tumor and the Schwann cells, but not in the tumor derived fibroblasts [4]. It was further demonstrated that fibroblasts isolated from neurofibromas carried at least one normal *NF1* allele and expressed *NF1* mRNA and protein, whereas the Schwann cells typically lacked the *NF1* transcript [5]. Selective expansion of human Schwann cells with specific cell culture systems results in pure populations of Schwann cells and fibroblasts from neurofibromas. This cell culture system confirmed that Schwann cells, but not fibroblasts, harbored the somatic *NF1* mutation in all studied tumors and showed that two genetically distinct ($NF1^{+/-}$ and $NF1^{-/-}$) Schwann cell subpopulations are present in neurofibromas [6]. Based on Knudson's two-hit hypothesis [7] Schwann cells can be considered as the initiating cell type in neurofibroma formation. The high mutation detection rate (76%) of *NF1* [8] in selectively grown Schwann cells emphasizes that genetic alterations and not epigenetic events are the major mechanism for the inactivation of *NF1* in neurofibromas [9–13]. It has been shown that LOH in neurofibromas can be the result of mitotic recombination, a deletion on the long

arm of chromosome 17, or loss of one chromosome 17 with reduplication of the other chromosome [14, 15]. While LOH is responsible for the somatic inactivation of *NF1* in ~20% of neurofibromas from *NF1* non-microdeletion patients (51/256), [8, 13, 14, 16–24] LOH was not observed in neurofibromas from *NF1* microdeletion patients (0/53) [8, 13, 14, 18, 19]. Why the wild type *NF1* allele in microdeletion patients, typically presenting with more neurofibromas at a younger age, is more vulnerable to other types of somatic inactivation events remains unclear.

MPNST

Approximately 10% of individuals with NF1 will develop an MPNST [25]. Some MPNSTs arise in pre-existing plexiform neurofibromas implying a model of multi-step tumor progression. Besides *NF1* inactivation [13, 20, 26–28], additional genetic changes have been identified in MPNSTs but not in benign neurofibromas. Alterations associated with malignant progression include homozygous deletion of *CDKN2A* which encodes $p16^{INK4A}$ and $p14^{ARF}$ [29, 30] and *TP53* loss [31, 32]. In most MPNSTs the karyotypes are complex [33].

Gastrointestinal Stromal Tumors

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and are associated with NF1 [34]. In contrast to sporadic GIST NF1 associated GISTs do not have *KIT* and *PDGFRA* activating mutations but complete inactivation of the wild type *NF1* allele in the interstitial cells of Cajal [35]. Interstitial cells of Cajal are the autonomic pacemaker cells regulating peristalsis in the digestive tract. Both *NF1* intragenic mutations, an *NF1* deletion and LOH due to mitotic recombination have been described in NF1 related GIST [35, 36].

Stewart et al. [37] reported an *NF1* somatic mutation in a gastric carcinoid tumor arising in an NF1 patient indicating that this specific type of tumor also results from a complete *NF1* inactivation.

JMML

Children with NF1 have an increased risk of developing juvenile myelomonocytic leukemia (JMML) [38]. Loss of the normal *NF1* allele and homozygous inactivation of the *NF1* gene in bone marrow of NF1 children with JMML have been identified [39, 40]. Consistently, two mechanisms underlying LOH in myeloid malignancies from these NF1 children have been recently described [41]. Among the 10 bone marrow samples analyzed eight showed LOH for almost the entire chromosome 17q arm generated by mitotic recombination. In the remaining two samples a deleted region of less than 9 Mb containing the *NF1* gene was observed [41].

Astrocytomas

About 15–20% of the children with NF1 have optic pathway tumors [42]. Many of the brain tumors are pilocytic astrocytomas typically involving the optic nerve, optic chiasm, hypothalamus and brainstem. Astrocytomas are derived from astrocytes, the glial cells in the central nervous system. Loss of neurofibromin expression has been found in NF1 associated astrocytomas [43–45]. A high percentage of NF1 associated astrocytomas show LOH of the *NF1* region (16/19 = 84%) [43, 46, 47]. Moreover, in half of the 14 tumors with informative markers the LOH also involves 17p [46, 47] indicating that besides *NF1* inactivation, inactivation of additional genes on chromosome 17, possibly *TP53*, seems to be important in the development of pilocytic astrocytomas needing surgery. The frequency of LOH for *NF1* is significantly different from what has been observed in neurofibromas (16/19 versus 51/256; Fisher's exact test: $p < 0.0001$).

Pheochromocytomas

Pheochromocytomas are rare catecholamine-producing neuroendocrine tumors arising from the neural crest derived chromaffin cells of the adrenal medulla [48] and 1–6 cases are observed per million people per year [49, 50]. Pheochromocytomas also occur as part of several syndromes including von Hippel-Lindau syndrome, multiple endocrine neoplasia types IIA and IIB, paraganglioma syndromes types 3 and 4, and NF1 (~1% [51], 0.1–5.7% [52]). LOH for the *NF1* region has been reported in 9/16 pheochromocytomas analyzed [53–55]. In 3 tumors it could be shown that the wild type *NF1* allele was lost [55]. In 7 tumors LOH involved the 17p and 17q arms, suggesting either a complete loss of a chromosome 17 copy, or loss and reduplication of chromosome 17 [53–55]. There is however not enough consistent data in the literature to assess the percentage of pheochromocytomas with LOH as a second hit.

Somatic *NF1* Mutations as a 'Second Hit' in Other NF1 Related Traits

Bone Abnormalities

Osseous abnormalities such as scoliosis, sphenoid wing dysplasia and pseudarthrosis of long bones are associated with NF1. Pseudarthrosis most commonly affects the tibia, occurs in about 2–5% of NF1 patients, and is most often unilateral, what is consistent with a random molecular event [1, 56–60].

Stevenson et al. (2006) [61] have recently reported evidence of double inactivation of the *NF1* gene in tibial pseudarthrosis. The authors identified in two cases LOH for intragenic and flanking *NF1* markers, and in one of the cases they demonstrated that the wild type *NF1* allele was lost. The authors hypothesized that loss of *NF1* function with subsequent Ras deregulation may lead to altered osteoblastic/osteoprogenitor differentiation and proliferation, impaired bony callus formation, and overgrowth of cellular tissue due to preferred fibroblast/myofibroblast differentiation of multipotential mesenchymal cells in stroma and periosteal cells.

Café-au-lait macules

Café-au-lait macules (CALMs) are hyperpigmented patches of the skin that usually occur shortly after birth. Although the etiopathogenesis of NF1 related pigmentary lesions remains largely unknown, a significant increase in the density of melanocytes, the neural crest derived pigment producing cells, was demonstrated in NF1 CALM skin compared with NF1 normal skin, control normal skin and control CALM skin [62]. These NF1 CALM melanocytes also display a higher melanin content and melanogenesis [63]. Molecular analysis of 5 CALMs derived from different *NF1* patients revealed bi-allelic inactivation of the *NF1* gene in melanocytes, while keratinocytes and fibroblasts were heterozygous for the germline NF1 mutation [64]. It is very likely that the bi-allelic *NF1* inactivation in melanocytes is the underlying molecular mechanism in *NF1* related pigmentary lesions. Moreover a similar bi-allelic inactivation of *SPRED1* has been shown in CALM in the NF1-like syndrome caused by *SPRED1* mutations [65].

Somatic *NF1* Mutation as a First Hit Resulting in *NF1* Mosaicism

In some NF1 patients an *NF1* mutation is not found during routine mutation analysis of peripheral blood leukocytes. If *NF1* mutation analysis in different tissues of these individuals is performed mosaicism for an *NF1* mutation is frequently found (Maertens O and Messiaen L, personal communication). In these patients the first hit in *NF1* occurred somatically (post zygotic), and is at least present in the cell types responsible for the observed NF1 features. These mosaic patients often present with a milder form of NF1 (generalized mosaic NF1) or with NF1 limited to one or more segments of the body (segmental NF1).

Generalized Mosaic NF1

Generalized mosaic NF1 patients present usually with mild manifestations of classical NF1. Hence the somatic mutation of *NF1* must have occurred in an early stage of embryonic development.

Up to now all patients identified with a generalized mosaic form of NF1 carried a (micro)deletion of *NF1* as first hit mutation [66–74]. This is probably due to the technical difficulties of identifying mosaic intragenic *NF1* mutations. A recurrent somatic deletion (the type II *NF1* microdeletion) has been described in mosaic NF1 [68, 69].

Segmental NF1

Segmental neurofibromatosis is defined as neurofibromatosis limited to one or more parts of the body. As a limited part of the body is affected with NF1, it is thought that the mutation occurred in a later stage during embryonic development. Like in classic NF1 patients both first and second hit mutations are found in the cell types giving rise to the specific NF1 feature in question (in melanocytes for the CALM, in Schwann cells for the neurofibromas) [75]. Somatic first hit mutations in three other segmental patients have been described [76–78].

Gonadal Mosaicism

A final form of mosaic neurofibromatosis is gonadal mosaicism. Individuals with gonadal mosaicism do not present any clinical features of NF1 because the somatic mutation is only present in the gonads. Gonadal mosaicism can be recognized if NF1 features are absent in the parents and 2 or more of their children present with NF1. Lazaro et al. [79] described 2 NF1 siblings with a 12-kb deletion in the *NF1* gene. This deletion was present in 10% of the sperm cells of the father but not in his blood cells and the father did not show any clinical features of NF1.

Conclusions

Due to the availability of more comprehensive *NF1* mutation screening methods and cell type specific cultures our understanding of the importance of somatic *NF1* mutations increased substantially. Analysis of affected cell types in patients with generalized mild NF1 but without *NF1* mutation in peripheral blood cells will be instrumental to determine the frequency of mosaic NF1. Further examining somatic *NF1* mutations in homogeneous cell populations of specific cell types can provide additional information on the pathogenesis of NF1 lesions such as glomus tumors of the finger tips, dysplastic scoliosis and non-ossifying fibromas of the bone. To promote standardization and facilitate further investigation of the somatic mutation spectrum in NF1, the somatic *NF1* mutation database (http://medgen.ugent.be/LOVD2) was created [80].

References

1 Ferner RE, Huson SM, Thomas N, Moss C, Willshaw H, et al: Guidelines for the diagnosis and management of individuals with Neurofibromatosis 1 (NF1). J Med Genet 2007;44:81–88.
2 Peltonen J, Jaakkola S, Lebwohl M, Renvall S, Risteli L, Virtanen I, Uitto J: Cellular differentiation and expression of matrix genes in type 1 neurofibromatosis. Lab Invest 1988;59: 760–771.
3 Rosenbaum TP, Patrie KM, Ratner N: Neurofibromatosis Type 1: Genetic and cellular mechanisms of peripheral nerve tumor formation. The Neuroscientist 1997;3:412–420.
4 Kluwe L, Friedrich R, Mautner VF: Loss of NF1 allele in Schwann cells but not in fibroblasts derived from an NF1-associated neurofibroma. Genes Chromosomes Cancer 1999;24: 283–285.
5 Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E: Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. Hum Mol Genet 2000;9:1059–1066.
6 Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, et al: Schwann cells harbor the somatic *NF1* mutation in neurofibromas: evidence of two different Schwann cell subpopulations. Hum Mol Genet 2000;9:3055–3064.
7 Knudson AG Jr: Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 1971;68:820–823.
8 Maertens O, Brems H, Vandesompele J, De Raedt T, Heyns I, et al: Comprehensive NF1 screening on cultured Schwann cells from neurofibromas. Hum Mut 2006;27:1030–1040.
9 Horan MP, Cooper DN, Upadhyaya M: Hypermethylation of the neurofibromatosis type 1 (NF1) gene promoter is not a common event in the inactivation of the *NF1* gene in NF1-specific tumours. Hum Genet 2000;107:33–39.
10 Harder A, Rosche M, Reuss DE, Holtkamp N, Uhlmann K, et al: Methylation analysis of the neurofibromatosis type 1 (NF1) promoter in peripheral nerve sheath tumours. Eur J Cancer 2004;40: 2820–2828.
11 Luijten M, Redeker S, van Noesel MM, Troost D, Westerveld A, Hulsebos TJ: Microsatellite instability and promoter methylation as possible causes of *NF1* gene inactivation in neurofibromas. Eur J Hum Genet 2000;8:939–945.
12 Fishbein L, Eady B, Sanek N, Muir D, Wallace MR: Analysis of somatic *NF1* promoter methylation in plexiform neurofibromas and Schwann cells. Cancer Genet Cytogenet 2005;157: 181–186.
13 Upadhyaya M, Han S, Consoli C, Majounie E, Horan M, et al: Characterization of the somatic mutational spectrum of the neurofibromatosis type 1 (NF1) gene in neurofibromatosis patients with benign and malignant tumors. Hum Mutat 2004;23:134–146.
14 De Raedt T, Maertens O, Chmara M, Brems H, Heyns I, et al: Somatic loss of wild type NF1 allele in neurofibromas: Comparison of NF1 microdeletion and non-microdeletion patients. Genes Chromosomes Cancer 2006;45:893–904.
15 Serra E, Rosenbaum T, Nadal M, Winner U, Ars E, Estivill X, Lazaro C: Mitotic recombination effects homozygosity for *NF1* germline mutations in neurofibromas. Nat Genet 2001;28:294–296.
16 Wiest V, Eisenbarth I, Schmegner C, Krone W, Assum G: Somatic *NF1* mutation spectra in a family with neurofibromatosis type 1: toward a theory of genetic modifiers. Hum Mutat 2003;22: 423–427.
17 Serra E, Puig S, Otero D, Gaona A, Kruyer H, et al: Confirmation of a double-hit model for the NF1 gene in benign neurofibromas. Am J Hum Genet 1997;61:512–519.
18 Serra E, Ars E, Ravella A, Sanchez A, Puig S, et al: Somatic *NF1* mutational spectrum in benign neurofibromas: mRNA splice defects are common among point mutations. Hum Genet 2001;108: 416–429.
19 Sawada S, Florell S, Purandare SM, Ota M, Stephens K, Viskochil D: Identification of *NF1* mutations in both alleles of a dermal neurofibroma. Nat Genet 1996;14:110–112.
20 Rasmussen SA, Overman J, Thomson SA, Colman SD, Abernathy CR, et al: Chromosome 17 loss-of-heterozygosity studies in benign and malignant tumors in neurofibromatosis type 1. Genes Chromosomes Cancer 2000;28:425–431.

21 Lothe RA, Slettan A, Saeter G, Brogger A, Borresen AL, Nesland JM: Alterations at chromosome 17 loci in peripheral nerve sheath tumors. J Neuropathol Exp Neurol 1995;54:65–73.
22 Eisenbarth I, Beyer K, Krone W, Assum G: Toward a survey of somatic mutation of the *NF1* gene in benign neurofibromas of patients with neurofibromatosis type 1. Am J Hum Genet 2000;66:393–401.
23 Daschner K, Assum G, Eisenbarth I, Krone W, Hoffmeyer S, et al: Clonal origin of tumor cells in a plexiform neurofibroma with LOH in NF1 intron 38 and in dermal neurofibromas without LOH of the *NF1* gene. Biochem Biophys Res Commun 1997;234:346–350.
24 Colman SD, Williams CA, Wallace MR: Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the *NF1* gene. Nat Genet 1995;11:90–92.
25 Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A: Malignant peripheral nerve sheath tumours in neurofibromatosis 1. J Med Genet 2002;39:311–314.
26 Legius E, Marchuk DA, Collins FS, Glover TW: Somatic deletion of the neurofibromatosis type 1 gene in a neurofibrosarcoma supports a tumour suppressor gene hypothesis. Nat Genet 1993;3:122–126.
27 Lothe RA, Slettan A, Saeter G, Brogger A, Borresen AL, Nesland JM: Alterations at chromosome 17 loci in peripheral nerve sheath tumors. J Neuropathol Exp Neurol 1995;54:65–73.
28 Lothe RA, Saeter G, Danielsen HE, Stenwig AE, Hoyheim B, O'Connell P, Borresen AL: Genetic alterations in a malignant schwannoma from a patient with neurofibromatosis (NF1). Pathol Res Pract 1993;189:465–471; discussion 464–471.
29 Nielsen GP, Stemmer-Rachamimov AO, Ino Y, Moller MB, Rosenberg AE, Louis DN: Malignant transformation of neurofibromas in neurofibromatosis 1 is associated with CDKN2A/p16 inactivation. Am J Pathol 1999;155:1879–1884.
30 Kourea HP, Orlow I, Scheithauer BW, Cordon-Cardo C, Woodruff JM: Deletions of the *INK4A* gene occur in malignant peripheral nerve sheath tumors but not in neurofibromas. Am J Pathol 1999;155:1855–1860.
31 Menon AG, Anderson KM, Riccardi VM, Chung RY, Whaley JM, et al: Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proc Natl Acad Sci USA 1990;87:5435–5439.
32 Legius E, Dierick H, Wu R, Hall BK, Marynen P, Cassiman JJ, Glover TW: *TP53* mutations are frequent in malignant NF1 tumors. Genes Chromosomes Cancer 1994;10:250–255.
33 Mertens F, Dal Cin P, De Wever I, Fletcher CD, Mandahl N, et al: Cytogenetic characterization of peripheral nerve sheath tumours: a report of the CHAMP study group. J Pathol 2000;190:31–38.
34 Zoller ME, Rembeck B, Oden A, Samuelsson M, Angervall L: Malignant and benign tumors in patients with neurofibromatosis type 1 in a defined Swedish population. Cancer 1997;79:2125–2131.
35 Maertens O, Prenen H, Debiec-Rychter M, Wozniak A, Sciot R, et al: Molecular pathogenesis of multiple gastrointestinal stromal tumors in NF1 patients. Hum Mol Genet 2006;15:1015–1023.
36 Stewart DR, Corless CL, Rubin BP, Heinrich MC, Messiaen LM, et al: Mitotic recombination as evidence of alternative pathogenesis of gastrointestinal stromal tumours in neurofibromatosis type 1. Journal of medical genetics 2007;44:e61.
37 Stewart W, Traynor JP, Cooke A, Griffiths S, Onen NF, et al: Gastric carcinoid: germline and somatic mutation of the neurofibromatosis type 1 gene. Fam Cancer 2007;6:147–152.
38 Stiller CA, Chessells JM, Fitchett M: Neurofibromatosis and childhood leukaemia/lymphoma: a population-based UKCCSG study. Br J Cancer 1994;70:969–972.
39 Miles DK, Freedman MH, Stephens K, Pallavicini M, Sievers EL, et al: Patterns of hematopoietic lineage involvement in children with neurofibromatosis type 1 and malignant myeloid disorders. Blood 1996;88:4314–4320.
40 Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P, McCormick F: Loss of the normal *NF1* allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. N Engl J Med 1994;330:597–601.
41 Stephens K, Weaver M, Leppig KA, Maruyama K, Emanuel PD, Le Beau MM: Shannon KM: Interstitial uniparental isodisomy at clustered breakpoint intervals is a frequent mechanism of NF1 inactivation in myeloid malignancies. Blood 2006;108:1684–1689.

42 Listernick R, Charrow J, Greenwald M, Mets M: Natural history of optic pathway tumors in children with neurofibromatosis type 1: a longitudinal study. J Pediatr 1994;125:63–66.
43 Gutmann DH, Donahoe J, Brown T, James CD, Perry A: Loss of neurofibromatosis 1 (NF1) gene expression in NF1-associated pilocytic astrocytomas. Neuropathol Appl Neurobiol 2000;26: 361–367.
44 Gutmann DH, James CD, Poyhonen M, Louis DN, Ferner R, et al: Molecular analysis of astrocytomas presenting after age 10 in individuals with NF1. Neurology 2003;61:1397–1400.
45 Li J, Perry A, James CD, Gutmann DH: Cancer-related gene expression profiles in NF1-associated pilocytic astrocytomas. Neurology 2001;56:885–890.
46 Kluwe L, Hagel C, Tatagiba M, Thomas S, Stavrou D, et al: Loss of *NF1* alleles distinguish sporadic from NF1-associated pilocytic astrocytomas. J Neuropathol Exp Neurol 2001;60: 917–920.
47 Tada K, Kochi M, Saya H, Kuratsu J, Shiraishi S, et al: Preliminary observations on genetic alterations in pilocytic astrocytomas associated with neurofibromatosis 1. Neuro Oncol 2003;5:228–234.
48 Lenders JW, Eisenhofer G, Mannelli M, Pacak K: Phaeochromocytoma. Lancet 2005;366: 665–675.
49 Beard CM, Sheps SG, Kurland LT, Carney JA, Lie JT: Occurrence of pheochromocytoma in Rochester, Minnesota, 1950 through 1979. Mayo Clin Proc 1983;58:802–804.
50 Stenstrom G, Svardsudd K: Pheochromocytoma in Sweden 1958–1981. An analysis of the National Cancer Registry Data. Acta Med Scand 1986;220:225–232.
51 Riccardi VM: Von Recklinghausen neurofibromatosis. N Engl J Med 1981;305:1617–1627.
52 Walther MM, Herring J, Enquist E, Keiser HR, Linehan WM: von Recklinghausen's disease and pheochromocytomas. J Urol 1999;162:1582–1586.
53 Gutmann DH, Cole JL, Stone WJ, Ponder BA, Collins FS: Loss of neurofibromin in adrenal gland tumors from patients with neurofibromatosis type I. Genes Chromosomes Cancer 1994;10:55–58.
54 Hering A, Guratowska M, Bucsky P, Claussen U, Decker J, et al: Loncarevic I: Characteristic genomic imbalances in pediatric pheochromocytoma. Genes Chromosomes Cancer 2006;45: 602–607.
55 Xu W, Mulligan LM, Ponder MA, Liu L, Smith BA, Mathew CG, Ponder BA: Loss of NF1 alleles in phaeochromocytomas from patients with type I neurofibromatosis. Genes Chromosomes Cancer 1992;4:337–342.
56 Friedman JM, Birch PH: Type 1 neurofibromatosis: a descriptive analysis of the disorder in 1,728 patients. Am J Med Genet 1997;70:138–143.
57 Huson SM, Harper PS, Compston DA: Von Recklinghausen neurofibromatosis. A clinical and population study in south-east Wales. Brain 1988;111:1355–1381.
58 Korf BR: Diagnostic outcome in children with multiple cafe au lait spots. Pediatrics 1992;90: 924–927.
59 Stevenson DA, Birch PH, Friedman JM, Viskochil DH, Balestrazzi P, et al: Carey JC: Descriptive analysis of tibial pseudarthrosis in patients with neurofibromatosis 1. Am J Med Genet 1999;84:413–419.
60 Riccardi VM: Neurofibromatosis: Phenotype, Natural History and Pathogenesis. Baltimore and London: The John Hopkins University Press; 1992.
61 Stevenson DA, Zhou H, Ashrafi S, Messiaen LM, Carey JC, et al: Double inactivation of *NF1* in tibial pseudarthrosis. Am J Hum Genet 2006;79:143–148.
62 De Schepper S, Boucneau J, Vander Haeghen Y, Messiaen L, Naeyaert JM, Lambert J: Café-au-lait spots in neurofibromatosis type 1 and in healthy control individuals: hyperpigmentation of a different kind? Arch Dermatol Res 2006;297:439–449.
63 Kaufmann D, Wiandt S, Veser J, Krone W: Increased melanogenesis in cultured epidermal melanocytes from patients with neurofibromatosis 1 (NF1). Hum Genet 1991;871:144–150.
64 De Schepper S, Maertens O, Callens T, Naeyaert J-M, Lambert J, Messiaen L: Somatic mutation analysis in NF1 café-au-lait spots reveals two *NF1* hits in the melanocytes. J Invest Dermatol 2008;128:1050–1053.
65 Brems H, Chmara M, Sahbatou M, Denayer E, Taniguchi K, Kato R, Somers R, Messiaen L, De Schepper S, Fryns JP, Cools J, Marynen P, Thomas G, Yoshimura A, Legius E: Germline loss-of-function mutations in *SPRED1* cause a neurofibromatosis 1-like phenotype. Nat Genet 2007;39: 1120–1126.

66 Ainsworth PJ, Chakraborty PK, Weksberg R: Example of somatic mosaicism in a series of de novo neurofibromatosis type 1 cases due to a maternally derived deletion. Hum Mutat 1997;9:452–457.
67 Colman SD, Rasmussen SA, Ho VT, Abernathy CR, Wallace MR: Somatic mosaicism in a patient with neurofibromatosis type 1. Am J Hum Genet 1996;58:484–490.
68 Kehrer-Sawatzki H, Kluwe L, Sandig C, Kohn M, Wimmer K, et al: High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the *JJAZ1* gene. Am J Hum Genet 2004;75:410–423.
69 Petek E, Jenne DE, Smolle J, Binder B, Lasinger W, et al: Mitotic recombination mediated by the *JJAZF1* (KIAA0160) gene causing somatic mosaicism and a new type of constitutional *NF1* microdeletion in two children of a mosaic female with only few manifestations. J Med Genet 2003;40:520–525.
70 Rasmussen SA, Colman SD, Ho VT, Abernathy CR, Arn PH, et al: Constitutional and mosaic large *NF1* gene deletions in neurofibromatosis type 1. J Med Genet 1998;35:468–471.
71 Riva P, Corrado L, Natacci F, Castorina P, Wu BL, et al: *NF1* microdeletion syndrome: refined FISH characterization of sporadic and familial deletions with locus-specific probes. Am J Hum Genet 2000;66:100–109.
72 Streubel B, Latta E, Kehrer-Sawatzki H, Hoffmann GF, Fonatsch C, Rehder H: Somatic mosaicism of a greater than 1.7-Mb deletion of genomic DNA involving the entire *NF1* gene as verified by FISH: further evidence for a contiguous gene syndrome in 17q11.2. Am J Med Genet 1999;87:12–16.
73 Tonsgard JH, Yelavarthi KK, Cushner S, Short MP, Lindgren V: Do *NF1* gene deletions result in a characteristic phenotype? Am J Med Genet 1997;73:80–86.
74 Wu BL, Boles RG, Yaari H, Weremowicz S, Schneider GH, Korf BR: Somatic mosaicism for deletion of the entire NF1 gene identified by FISH. Hum Genet 1997;99:209–213.
75 Maertens O, De Schepper S, Vandesompele J, Heyns I, Janssens S, Speleman F, Legius E, Messiaen L: Molecular dissection of isolated disease features in neurofibromatosis type 1. Am J Hum Genet 2007;81:243–251.
76 Tinschert S, Naumann I, Stegmann E, Buske A, Kaufmann D, Thiel G, Jenne DE: Segmental neurofibromatosis is caused by somatic mutation of the neurofibromatosis type 1 (NF1) gene. Eur J Hum Genet 2000;8:455–459.
77 Vandenbroucke I, van Doorn R, Callens T, Cobben JM, Starink TM, Messiaen L: Genetic and clinical mosaicism in a patient with neurofibromatosis type 1. Hum Genet 2004;114:284–290.
78 Consoli C, Moss C, Green S, Balderson D, Cooper DN, Upadhyaya M: Gonosomal mosaicism for a nonsense mutation (R1947X) in the *NF1* gene in segmental neurofibromatosis type 1. J Invest Dermatol 2005;125:463–466.
79 Lazaro C, Gaona A, Lynch M, Kruyer H, Ravella A, Estivill X: Molecular characterization of the breakpoints of a 12-kb deletion in the *NF1* gene in a family showing germ line mosaicism. Am J Hum Genet 1995;57:1044–1049.
80 Maertens O, Speleman F, Legius E, Messiaen L: The somatic *NF1* mutation database. (submitted)

Eric Legius, MD, PhD
Department of Human Genetics, Catholic University Leuven
Herestraat 49
BE–3000 Leuven (Belgium)
Tel. +32 16 345 903, Fax +32 16 346 051, E-Mail Eric.Legius@uz.kuleuven.ac.be

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 154–166

NF2: Mutations and Management of Disease

D.G.R. Evans, A. Wallace

Academic Unit of Medical Genetics Regional Genetics Service and National Molecular Genetics Reference Laboratory, St Mary's Hospital, Manchester, UK

Abstract

NF2 is a dominantly inherited tumour predisposition syndrome caused by mutations in the *NF2* gene on chromosome 22. Affected individuals inevitably develop schwannomas and to a decreasing frequency meningiomas and ependymomas with the classical disease feature being bilateral vestibular nerve schwannomas. Most patients currently become completely deaf, although rehabilitation with brain stem implants is improving this outcome. In excess of 50% of patients represent de novo mutations and as many as one third are mosaic for the causative mutation. Although truncating mutations (nonsense and frameshifts) are the most frequent germline event and cause the most severe disease, single and multiple exon deletions are common and a strategy for detection of these is vital for a sensitive analysis. NF2 represents a difficult management problem with most patients facing reduced life expectancy historically. Surgery remains the focus of current management although watchful waiting and occasionally radiation treatment have a role. In the future the development of tailored drug therapies aimed at the genetic level may herald a targeted systemic treatment for this devastating, life limiting condition.

Neurofibromatosis type 2 (NF2) in contrast to NF1 is characterised by the development of schwannomas, meningiomas and ependymomas, with the great majority of patients having bilateral involvement of the eighth cranial nerve [1]. Although the disease still bears the name 'neurofibromatosis', neurofibromas are relatively infrequent. NF2 was first described in 1822 by the Scottish surgeon, Wishart [2]. NF1 was fully delineated in the late nineteenth century by von Recklinghausen. However, it was Harvey Cushing, who bracketed them together in 1916 [3], and is largely responsible for the continued confusion between the two conditions. Indeed, the neurofibromatosis literature prior to

1985 has many NF2 cases being described as part of von Recklinghausen disease, with bilateral vestibular schwannomas (VS) even being included in a major patient series [4]. The conditions were eventually recognised as separate entities with the localisation of the respective genes to chromosome 17 and 22 [5, 6]. This was followed by the formal clinical delineation at a U.S. National Institutes of Health (NIH) consensus meeting in 1987 [7]. It is now known that NF2 is a genetically homogeneous condition, with no evidence for heterogeneity of classical NF2 (bilateral VS).

NF2 is an autosomal dominant disease with usually a 50% risk of transmission from an affected individual to their offspring, which was first confirmed in a large family reported by Gardner and Frazier in 1930. Over 50% of patients have no family history and represent de novo mutations in the NF2 gene [1, 8]. Individuals who inherit a pathogenic mutation in the *NF2* gene will almost always develop symptoms by 60 years of age [8]; very occasionally, patients will have apparent non-penetrance. Although the transmission rate is 50% in the second generation and beyond, the risk of transmission in an apparently sporadic case of NF2 is less than 50% due to mosaicism (see below) [9].

Molecular Genetics

Seizinger et al. were the first to show loss of constitutional heterozygosity of chromosome 22, with DNA markers lost in tumours from a patient with NF2 [10]. Linkage studies then confirmed NF2 families were linked to chromosome 22. The *NF2* gene was then isolated by the simultaneous discovery of constitutional and tumour deletions in a cell membrane-related gene which has been called merlin or schwannomin by the two groups who isolated it [11, 12].

Germline and Mosaic Mutations

Standard mutation techniques, such as Single Strand Conformational Polymorphism (SSCP) analysis or Denaturing Gradient Gel Electrophoresis, detect between 35 and 66% of pathogenic mutations [13–16]. The majority of these mutations are truncating mutations, leading to a smaller and probably non-functional protein product. Early studies suggested that missense mutations (which result in a complete protein product) and large deletions (which result in no protein product) both caused predominantly mild phenotypes. Larger studies of detailed genotype/phenotype correlations in multiple families have confirmed this finding [14–17]. Phenotype is more variable in patients with splice-site mutations, with milder disease in patients with mutations in exons 9–15 [18, 19]. Definitive evidence from effect on survival has been established with missense mutation patients having statistically better survival

Table 1. MLPA abnormalities in 54 unrelated families

MLPA abnormality	Number of occasions (mosaic)
Exon 1-intron1 (deletes intronic CA repeat)	17 (3)
Whole gene (exons 1–17) deletion	15 (2)
Exons 5–17 deletion	3 (2)
Exons 2–3 deletion	2
Exons 2–10 deletion	2
Exons 13–15 deletion	1
Exons 1–16 deletion	2 (1)
Exons 1–10 deletion	2
Exons 1–3 deletion	1
Exons 1–4 deletion	1 (1)
Exons 1–2 deletion	1 (1)
Exons 15–17 deletion	1
Exons 8–17 deletion	1
Exons 8–15 deletion	1
Exon 3 deletion	1
Exon 7 deletion	1
Exon 5 deletion	1
Exons 12–14 duplication	1

than nonsense/frameshift mutations [20]. Similarly to other tumour prone disorders the frequency of large genomic rearrangements did not become apparent until some time after the gene was cloned, due to the poor availability of methods to allow routine clinical screening for their presence. About half of these are detectable with standard cytogenetic analysis with FISH [21]. A small number of constitutional abnormalities such as chromosome translocations and ring 22 would still need a cytogenetic analysis to confirm their presence. The majority of large scale rearrangements are now routinely detected by Multiplex Ligation-dependent Probe Amplification (MLPA) and are now known to make up around 15% of NF2 germline aberrations [21, 22]. The commonest of this class of mutations is a deletion of the *NF2* promoter, exon 1 and most of intron 1 and deletions of the whole gene, indeed combined these two account for over half of all MLPA abnormalities (table 1). The differing frequency of the various types of *NF2* mutation between familial, sporadic and mosaic cases can be seen in table 2. This represents the output from one centre dedicated to whole gene analysis with the great majority of patients having been screened by a technique capable of the detection of large deletions or duplications. More extensive lists

Table 2. Mutations identified in 473 families with NF2 in the Manchester (UK) genetics laboratory

Type of mutation	Detection in 2nd generation (n=93)	Detection in sporadic non-mosaic patients (% non-mosaic)	Mosaic mutations (% of mosaic)	Total (%)
Splice site	28 (30%)	38 (22%)	3 (6%)	69 (15%)
MLPA positive	18 (18%)	26 (14%)	10 (15%)	54 (12%)
FSD	15 (15%)	30 (18%)	18 (24%)	63 (13%)
Nonsense	14 (14%)	58 (35%)	24 (36%)	96 (20%)
Missense	6 (6%)	5 (3%)	1 (1.5%)	12 (2.5%)
FSI	3 (3%)	11 (7%)	3 (5%)	17 (4%)
IFD	1 (1%)	1	3 (5%)	5 (1%)
Ring 22	0	0	3 (5%)	3
Not found	8 (9%)	212 (56%)	146	146/473 (31%)
Total	93	168/380 (44%)	66	473

of *NF2* mutations described worldwide are available online and in recent reviews [23–25], but these are less likely to reflect the true ratios of different classes of mutations, as a variety of different techniques have been used. The sensitivity of genetic testing using sequence analysis and MLPA can be derived from table 2. 91% of mutations have been identified in the second generation of NF2 families. Of the missing 8 families 4 have been subjected to more detailed analysis including RNA analysis. Two putative splicing variants deep in the introns have been identified (Messaien L, personal communication), but no other mutations have been found.

A considerable proportion of NF2 patients, particularly milder cases, have mosaic disease, in which only a proportion of cells contain the mutated *NF2* gene. The initiating mutation occurs after conception, leading to two separate cell lineages. The proportion of cells affected depends on how early in development the mutation occurs. Recent evidence suggests that up to 20–30% of NF2 cases without a family history of the disease are mosaic, carrying the mutation in too small a proportion of their cells to be detected from a blood sample [10, 26, 27]. This accounts for the milder disease course in many individuals with unfound mutations, and since only a subset of germ cells will carry the mutation, there is less than a 50% risk of transmitting the disease to their offspring. However, if an offspring has inherited the mutation, they will usually be more severely affected than their parent, since the offspring will carry the mutation in all of its cells. One of the features that suggested that mosaicism existed in NF2

was that *NF2* mutations were harder to find in blood in sporadic cases than in patients who had inherited the disease from an affected parent. Mosaicism may be particularly likely in NF2 if the tumours are predominantly on one side of the body. The mosaic mutation can be detected by analysing tumour material from an affected individual. If an identical mutation is found in two tumours from that individual, their offspring can be tested for the presence of the mutation.

C>T transitions leading to a nonsense mutation are the most common mutations in the *NF2* gene [24, 25]. These account for the only significant variation in mutation frequency across the *NF2* gene apart from the lack of mutations in exons 16 and 17 and a relatively low frequency in exon 9 [24].

Somatic Mutations

There are notable similarities, but also differences between the spectra of germline and somatic *NF2* mutations. Among germline mutations in classic NF2, nonsense mutations are more common than frameshift mutations by a ratio of 1.3:1, but this ratio is reversed for somatic mutations [24]. In both situations *NF2* nonsense mutations, C>T transitions in CGA codons and non-CGA codons are the most common single base-pair transitions. However there is a marked absence of mutations in exons 14 and 15 in sporadic meningioma. One recently reported phenomenon is the increasing drift from nonsense mutations in the germline and early somatic mutations to a predominance of frameshift mutations in the tumours of older patients with vestibular schwannoma [28]. This is likely to be due to a deficiency in certain DNA repair pathways in older patients [28].

Although it is thought that effectively all schwannomas require inactivation of the *NF2* gene, no reports apart from those studying NF2 protein have confirmed a 100% knock out of both copies of the *NF2* gene. This is likely due to diversity of mechanisms to inactivate the gene. The standard approach of mutational analysis and loss of constitutional heterozygosity (LOH) on tumours will detect involvement of NF2 in about 80–90% of schwannomas, but both copies can only be confirmed as affected in about 50–60% of cases. The method of LOH is also not straightforward, whilst most cases involve loss of chromosome 22 or at least the long arm, a proportion is now known to be due to mitotic recombination with essentially two identical copies of a mutated *NF2* gene and distal 22q [29]. This mechanism is now thought to be a primary cause of *NF2* inactivation in schwannomatosis (see chapter on molecular data of schwannomatosis). It is now known that between 20–40% of sporadic schwannomas are inactivated by *NF2* methylation [30, 31] and in a similar fashion to TP16 this could involve both copies of *NF2* and explain why some tumours do not harbour identifiable point mutations or LOH. Approximately 60% of sporadic meningiomas have *NF2* gene involvement and promoter methylation again plays a significant role [32].

NF2 Management

NF2 presents many difficult management dilemmas. The mainstay of management of NF2 is surgical removal of symptomatic cranial and spinal tumours, but also the timing of removal of vestibular schwannomas. Surgical results are certainly far better when managed by an experienced team [33–35]. There is clear evidence of a reduction in mortality with a significantly increased life expectancy for NF2 patients managed at 3 specialty centres in the UK (OR 0.34) [36]. It is important to balance the use of microsurgery and radiation treatment, which can have a role in patients who have particularly aggressive tumours, or who are poor surgical risks, or who refuse surgery. Teams experienced in the positioning of brainstem implants can offer partial auditory rehabilitation to those who are deaf, although results are still behind those achievable for cochlear implants. Although the cochlear nerve may be left initially intact after surgery its blood supply may be damaged, nonetheless a few patients can be rehabilitated successfully with a cochlear implant.

Outcomes

Even with improvements in microsurgery and with use of radiation therapy, the great majority of individuals with NF2 become completely deaf. The tumours in NF2 are more difficult to treat than those of sporadic unilateral VS, as NF2 VS are often multifocal, appearing 'like a bunch of grapes' around the vestibular nerve in particular. There is evidence for a histological difference, with NF2 VS being more lobular and less vascular then their sporadic counterparts [37]. This leads to a greater risk of facial nerve damage in NF2. Loss of facial nerve function is one of the most feared aspects of the condition for many sufferers, although in good surgical hands this complication is now much less common [33, 34]. Patients may also be severely disabled by a combination of poor balance, visual problems and weakness due to spinal tumours. Indeed, many NF2 patients become wheelchair-bound in early adulthood. Many patients with multi-tumour disease die in their twenties and thirties. In view of the multiplicity of problems affecting many patients it is strongly recommended that NF2 patients are managed by a multidisciplinary team in specialist centres [35].

Specialty Centres

A typical NF2 specialist centre will require involvement of a number of key staff members.

Permanent Clinic Staff: Neurosurgeon, Otolaryngologist, physician (neurologist/geneticist), clinic nurse/patient link worker and a dedicated clinic secretary. Clinic equipment: lightwriter/pallantype.

In order to minimise patient visits morning scanning with lunchtime radiological review and an afternoon clinic is preferable.

Each Clinic Should Have Access to a Named: Neuroradiologist, ophthalmologist, peripheral nerve surgeon, plastic surgeon, neurologist/geneticist (if not involved in main clinic), hearing therapist, physiotherapist, paediatrician.

Patients should be given the option of a radiation therapy opinion, which will only be available at a few centres.

Genetic Testing and Counselling

Because detection of tumours at an early stage is effective in improving the clinical management of NF2, pre-symptomatic genetic testing is an integral part of the management of NF2 families. Once a mutation has been identified in an affected individual, a 100-% specific test is available for the family. However, mutation screening may not reveal the causative mutation. Predictive diagnosis by linkage analysis using intragenic markers or markers flanking the *NF2* gene is also possible in the great majority of families with two or more living affected individuals. In most families with more than one affected individual, linkage analysis may still remain the test of choice since it will give >99% certainty of affected status. By combining this with a cumulative age at onset curve [38], the risk to an unaffected 30-year-old with a normal scan and favourable DNA result is extremely small, although 100-% confidence can still only be attained with the identification of family-specific mutations. Age at onset curves aid genetic counselling; for example, the risk of having inherited NF2 for an asymptomatic at-risk individual 25 years of age, prior to screening, drops to 25%. Tumour analysis plays a vital role in providing genetic testing for the offspring of sporadic patients. Indeed analysis should if possible first be carried out on tumour so that a targeted approach can be used on the blood sample. If both mutational events are identified in the *NF2* gene and neither is present in the blood the patient must be mosaic for one of these mutations [26, 27, 39, 40]. Even if only LOH is identified this still allows exclusion of NF2 in 50% of offspring if they can be shown to have inherited the allele 'lost' in the tumour [40]. At-risk individuals who are shown not to have inherited the mutated *NF2* gene do not need further follow-up. Even if tumour is not available the risks of NF2 to offspring can be substantially reduced in an isolated patient with negative genetic testing in blood [41].

Screening Protocol

Children of affected patients should be considered to be at 50% risk of NF2 and screening for NF2 can start at birth. Cataracts can affect vision in early life and other tumour implications are present in the first ten years of life, particularly cranial meningiomas. Formal screening for VS should start at ten years, as it is rare for tumours to become symptomatic before that time even in severely affected families. Annual audiological tests including auditory brainstem response are still a useful adjunct to MRI [38]. Surgery is unlikely to be more successful for tumours <6 mm than for tumours sized 6 mm, but VS growth is higher in younger patients, so for asymptomatic at-risk individuals without tumours, MRI screening every two years for those <20 years old and every 3–5 years for those aged >20 years should be sufficient. The initial MRI scan could be at around 12 years of age, or 10 years in severely affected families. Once tumours are present, MRI screening should probably be at least annual. Spinal tumours are in 60–80% of NF2 patients on MRI [42–44]. While only 25–30% of patients with spinal tumours require a spinal operation from a symptomatic tumour, a full annual neurological examination is probably a wise precaution with Spinal MRI only every 3 years or if there are new symptoms. If no tumours are present on the initial scan a further scan 5–10 years later may be reasonable.

In most families it is now possible to develop a genetic test so that screening can be targeted to affected individuals only. Identifying the affected patient's mutation not only allows testing of at risk relatives, but may also give important indicators as to the patient's own prognosis. As 25–30% of NF2 patients are mosaic frozen tumour should be taken at operation (with patient consent) for genetic tests.

Managing Affected Children

NF2 is being recognised more and more frequently in childhood often before VS have developed. Recognition of the more severe disease course with early presentation and the more atypical features such as mononeuropathy are important.

Surgery

VS in NF2 are more difficult to treat than those of sporadic unilateral VS because NF2-related VS are often multifocal in the eighth nerve complex, and

because of the potential for associated facial nerve schwannomas. Surgery to remove VS in NF2 almost always leads to total deafness with loss of the cochlear nerve. Despite the great improvement in VS surgery over the last three decades, facial nerve damage and other adverse outcomes remain a real possibility during tumour removal, especially in the hands of less experienced VS surgeons. Facial weakness may threaten the health of the eye as a result of loss of the protective blink reflex and as the lacrimal gland is also supplied by the facial nerve the loss of tear production will increase this risk. If facial nerve damage coexists with loss of corneal sensation from damage to the trigeminal nerve then the eye becomes exceptionally vulnerable to corneal ulceration and blindness.

The cornerstone of modern NF2 management is conservation of function, and the maintenance of 'quality of life'. The mere presence of a tumour is not an indication for its removal. Serious thought must be given to the benefits that are sought and the risks and complications of the surgery, and the treatment must be tailored to the needs of the individual patient [35]. Attempts at hearing preservation surgery should be limited to experienced centres who can offer a realistic chance of maintaining both the cochlear nerve, but also cochlea function. If hearing is lost after apparent cochlear nerve preservation the patient may still be suitable for a cochlear implant. In many if not most instances the best policy will be to observe VS to decide on the best time to balance surgical morbidity against the almost inevitable loss of hearing.

The principle of minimal interference for VS applies equally to schwannomas on other cranial nerves, to intracranial meningiomas, and to spinal tumours. It is very uncommon to have to remove a schwannoma growing on a cranial nerve other than the eighth because these tumours appear to have a much slower growth pattern than NF2 VS. Spinal tumours are only considered for excision if they are clearly producing symptoms or physical signs. In the absence of any dramatic growth of tumours, the head should be scanned every year and the spine every 3 years.

Radiation Therapy

It is important to balance the use of microsurgery and radiation treatment, which has a role in patients who are poor surgical candidates, or who refuse or wish to avoid surgery because of its associated risks. Radiation therapy should be *mentioned* as a management option, even if the tumour is larger than the size criterion for treatment. The upper limit of size for radiotherapy is generally a maximum intracranial diameter of 3 cm [45]. The patient should be aware of the management options even if the tumour is not suitable for a particular treatment

modality. In NF2 cases selected for radiosurgery tumour control rates are of the order of 50%, with 40% retaining pre-treatment hearing for at least 3 years [46]. This is nonetheless substantially worse than for sporadic vestibular schwannomas.

Surgeons should use clinical judgement as to when to recommend radiation therapy [35]. Follow-up for life with interval scanning is necessary, although this would be required for NF2 anyway. Patients should be made aware of the variable reported outcomes of the treatment and the risk of the radiation-induced malignant change, which has been reported disproportionately more in NF2 than sporadic patients [47]. The tumour may also be more difficult to excise after radiotherapy, and that reported facial nerve outcomes after surgery following stereotactic radiation therapy are frequently poor [48].

New Therapies

The progress being made in cellular research (see next chapter) especially with regard to pathways in which the *NF2* gene product interacts raises the hopes of a targeted therapy such as a herceptin for breast cancer and Glivec for GIS tumours.

Differential Diagnosis

The main differential diagnosis of NF2 is schwannomatosis and some patients with multiple non cranial schwannomas turn out to have mosaic NF2 [27]. However, patients fulfilling the most sensitive Manchester criteria are unlikely to be misclassified [49].

References

1 Evans DGR, Huson S, Donnai D, Neary W, Blair V, Newton V, Harris R: A clinical study of type 2 neurofibromatosis. Q J Med 1992;84:603–618.

2 Wishart JH: Case of tumours in the skull, dura mater, and brain. Edinburgh Med Surg J 1822;18:393–397.

3 Cushing H: Tumors of the Nervus Acusticus and the Syndrome of the Cerebello-Pontine Angle. WB Saunders, Philadelphia, 1917.

4 Crowe FW, Schull WJ, Neal JV: A clinical pathological and genetic study of multiple neurofibromatosis. Charles C. Thomas, Springfield Ill, 1956.

5 Seizinger BR, Rouleau GA, Ozelius LG, Ozelius LJ, Faryniarz AG, et al: Genetic linkage of von Recklinghausen neurofibromatosis to the nerve growth factor receptor gene. Cell 1987;49: 589–594.

6 Rouleau G, Seizinger BR, Ozelius LG, Hobbs WJ, Trofatter JA, et al: Genetic linkage analysis of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. Nature 1987;329: 246–248.

7 National Institutes of Health Consensus Development Conference Statement on Neurofibromatosis. Arch Neurol 1987;45:575–579.

8 Evans DGR, Huson SM, Donnai D, Neary W, Blair V, et al: A genetic study of type 2 neurofibromatosis in the north west of England and the UK: I. Prevalence, mutation rate, fitness and confirmation of maternal transmission effect on severity. J Med Genet 1992;29:841–846.

9 Evans DGR, Wallace A, Trueman L, Strachan T: Mosaicism in classical neurofibromatosis type 2: a common mechanism for sporadic disease in tumor prone syndromes? Am J Hum Genet 1998; 63:727–736.

10 Seizinger BR, Martuza RL, Gusella JF: Loss of genes on chromosome 22 in tumorigenesis of human acoustic neuroma. Nature 1986;322:644–647.

11 Troffater JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, et al: A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 1993;72: 791–800.

12 Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, et al: Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 1993;363: 515–521.

13 MacCollin M, Ramesh V, Jacoby LB, Louis DN, Rubio MP, et al: Mutational analysis of patients with neurofibromatosis 2. Am J Hum Genet 1994;55:314–320.

14 Parry DM, MacCollin M, Kaiser-Kupfer MI, Pulaski K, Nicholson HS, et al: Germ-line mutations in the neurofibromatosis 2 gene: correlations with disease severity and retinal abnormalities. Am J Hum Genet 1996;59:529–539.

15 Ruttledge MH, Andermann AA, Phelan CM, Claudio JO, Han F-Y, et al: Type of mutation in the neurofibromatosis type 2 gene (NF2) frequently determines severity of disease. Am J Hum Genet 1996;59:331–342.

16 Kluwe L, Bayer S, Baser ME, Hazim W, Haase W, Funsterer C, Mautner: VF Identification of NF2 germ-line mutations and comparison with neurofibromatosis 2 phenotypes. Hum Genet 1996;98:534–538 [published erratum in Hum Genet 1997;99:292].

17 Kluwe L, MacCollin M, Tatagiba M, Thomas S, Hazim W, Haase W, Mautner VF: Phenotypic variability associated with 14 splice-site mutations in the *NF2* gene. Am J Med Genet 1998;77: 228–233.

18 Evans DGR, Trueman L, Wallace A, Mason S, Strachan T: Genotype/phenotype correlations in type 2 neurofibromatosis: evidence for more severe disease with truncating mutations. J Med Genet 1998;35:450–455.

19 Baser ME, Kuramoto L, Joe H, Friedman JM, Wallace AJ, et al: Genotype-Phenotype Correlations for nervous system tumors in neurofibromatosis 2: A population-based study. Am J Hum Genet 2004;75:231–239.

20 Baser ME, Kuramoto L, Woods RH, Friedman JM, Wallace AJ, et al: The location of constitutional neurofibromatosis 2 (NF2) splice-site mutations is associated with the severity of NF2. J Med Genet 2005;42:540–546.

21 Tsilchorozidou T, Menko, F, Lalloo F, Kidd A, Da Silva R, et al: Constitutional rearrangements of chromosome 22 as a cause of neurofibromatosis type 2. J Med Genet 2004;41:529–534.

22 Kluwe L, Nygren AO, Errami A, Heinrich B, Matthies C, Tatagiba M, Mautner V: Screening for large mutations of the *NF2* gene. Genes Chromosomes Cancer 2005;42:384–391.

23 Baser ME: Neurofibromatosis 2 (NF2) mutation databases. http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html

24 Baser ME, and the contributors to the International NF2 Mutation Database: The distribution of constitutional and somatic mutations in the neurofibromatosis 2 gene. Hum Mutat 2006;27: 297–306.

25 Ahronowitz I, Xin W, Kiely R, Sims K, Maccollin M, Nunes FP: Mutational spectrum of the NF2 gene: a meta-analysis of 12 years of research and diagnostic laboratory findings. Hum Mutat 2006;28:1–12.

26 Kluwe L, Mautner VF, Heinrich B, Dezube R, Jacoby LB, Friedrich RE, MacCollin M: Molecular study of frequency of mosaicism in neurofibromatosis 2 patients with bilateral vestibular schwannomas. J Med Genet 2003;40:109–114.

27 Moyhuddin A, Baser ME, Watson C, Purcell S, Ramsden RT, et al: Somatic mosaicism in neurofibromatosis 2: prevalence and risk of disease transmission to offspring. J Med Genet 2003;40:459–463.
28 Evans DGR, Maher ER, Baser ME: Age-related shift in the mutation spectra of germline and somatic *NF2* mutations: hypothetical role of DNA repair mechanisms. J Med Genet 2005;42: 630–632.
29 Warren C, James LA, Varley JM, Ramsden RT, Evans DG: Identifying recurrent regions of chromosome loss and gain in 76 vestibular schwannomas using comparative genomic hybridisation (CGH). J Med Genet 2003;40:802–806.
30 Kino T, Takeshima H, Nakao M, Nishi T, Yamamoto K, et al: Identification of the cis-acting region in the *NF2* gene promoter as a potential target for mutation and methylation-dependent silencing in schwannoma. Genes Cells 2001;6:441–454.
31 Gonzalez-Gomez P, Bello MJ, Alonso ME, Lomas J, Lopez-Marin I, et al: CpG island methylation in sporadic and neurofibromatosis type 2-associated schwannomas. Clin Cancer Res 2003;9: 5601–5606.
32 Lomas J, Bello MJ, Arjona D, Alonso ME, Martinez-Glez V, et al: Genetic and epigenetic alteration of the *NF2* gene in sporadic meningiomas. Genes Chromosomes Cancer 2005;42:314–319.
33 Evans DGR, Ramsden R, Huson SM, Harris R, Lye R, King TT: Type 2 neurofibromatosis: the need for supraregional care? J Laryngol Otol 1993;107:401–406.
34 Slattery WH, Brackmann DE, Hitselberger W: Hearing preservation in neurofibromatosis type 2. Am J Otol 1998;19:638–643.
35 Evans DGR, Baser ME, O'Reilly B, Rowe J, Gleeson M, et al: Management of the patient and family with Neurofibromatosis 2: A consensus conference statement. Brit J Neurosurg 2005;19: 5–12.
36 Baser ME, Friedman JM, Aeschilman D, Joe H, Wallace AJ, Ramsden RT, Evans DGR: Predictors of the risk of mortality in neurofibromatosis 2. Am J Hum Genet 2002;71:715–723.
37 Sobel RA, Wang Y: Vestibular (acoustic) schwannomas: histological features in neurofibromatosis 2 and in unilateral cases. J Neuropathol Exp Neurol 1993;52:106–113.
38 Evans DGR, Newton V, Neary W, Baser ME, Wallace A, et al: Use of MRI and audiological tests in pre-symptomatic diagnosis of type 2 neurofibromatosis (NF2). J Med Genet 2000;37:944–947.
39 Mohyuddin A, Neary WJ, Wallace AJ, Purcell S, Wu C-L, et al: Molecular genetic exclusion of NF2 in young patients diagnosed with a unilateral vestibular schwannoma. J Med Genet 2002;39: 315–322.
40 Kluwe L, Friedrich RE, Tatagiba M, Mautner V: Presymptomatic diagnosis for children of sporadic neurofibromatosis 2 patients, a method based on tumor analysis. Genet Med 2002;4: 27–30.
41 Evans DGR, Ramsden RT, Shenton A, Gokhale C, Bowers NL, Huson SM, Wallace A: Mosaicism in NF2 an update of risk based on uni/bilaterality of vestibular schwannoma at presentation and sensitive mutation analysis including MLPA. J Med Genet 2007;44:424–428.
42 Mautner VF, Lindenau M, Baser ME, Tatagiba M, Haase W, et al: The neuroimaging and clinical spectrum of neurofibromatosis 2. Neurosurg 1996;38:880–885.
43 King A, Biggs N, Ramsden RT, Wallace A, Gillespie J, Evans DGR: Spinal tumors in neurofibromatosis type 2: is emerging knowledge of genotype predictive of natural history? J Neurosurg Spine 2005;2:574–579.
44 Patronas NJ, Courcoutsakis N, Bromley CM, Katzman GL, MacCollin M, Parry DM: Intramedullary and spinal canal tumors in patients with neurofibromatosis 2: MR imaging findings and correlation with genotype. Radiology 2001;218:434–442.
45 Rowe JG, Radatz M, Walton L, Kemeny AA: Stereotactic radiosurgery for type 2 neurofibromatosis acoustic neuromas: patient selection and tumour size. Stereotact Funct Neurosurg 2002;79: 107–116.
46 Rowe JG, Radatz MW, Walton L, Soanes T, Rodgers J, Kemeny AA: Clinical experience with gamma knife stereotactic radiosurgery in the management of vestibular schwannomas secondary to type 2 neurofibromatosis. J Neurol Neurosurg Psychiatry 2003;74:1288–1293.
47 Baser ME, Evans DGR, Jackler RK, Sujansky E, Rubenstein A: Malignant peripheral nerve sheath tumors, radiotherapy, and neurofibromatosis 2. Brit J Cancer 2000;82:998.

48 Friedmann RA, Slattery WH, Brackmann DE, Shwartz MS, Hitselberger WE: Resection of Acoustic Neuroma following Failed Stereotactic Radiosurgical Treatment: Facial nerve outcome. Fourth International Conference on Vestibular Schwannoma and Other CPA Lesions. Conference Proceedings, in Baguley DM, Ramsden RT, Moffat DA (eds): Pubs: Immediate Proceedings, Bungay, Norfolk 2002, pp 29–30.

▶49 Baser ME, Friedman JM, Wallace AJ, Ramsden RT, Joe H, Evans DGR: Evaluation of diagnostic criteria for neurofibromatosis 2. Neurology 2002;59:1759–1765.

D. Gareth R. Evans
Department of Medical Genetics, St Mary's Hospital
Hathersage Road
Manchester M13 0JH (UK)
Tel. +44 161 276 6228, Fax +44 161 276 6145, E-Mail gareth.evans@cmmc.nhs.uk

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 167–176

Function of Merlin in Genesis of Tumours and Other Symptoms of NF2

C.O. Hanemann

Clinical Neurobiology, Peninsula College of Medicine and Dentistry,
The John Bull Building, Tamar Science Park, Research Way,
Plymouth, UK

Abstract

Mutations in both alleles of *merlin*, likely resulting in loss of function, are found in all Neurofibromatosis 2 (NF2) associated tumours and in all spontaneous schwannomas as well as in the majority of spontaneous meningiomas. In this article expression patterns, binding partners of merlin, phenotypes of disease models and based on that hypothesis on how *merlin* mutations lead to tumour formation are discussed.

Merlin (**M**oesin-**e**zrin-**r**adixin-**l**ike prote**in**), also occasionally called schwannomin, is a 69 kDa protein encoded by the *NF2* gene [1, 2]. It shows close homology to the family of ERM (ezrin, radixin, moesin) proteins, which act as membrane cytoskeletal linkers. Highest homology is found in the N-terminal globular FERM domain. Merlin also acts as a tumour suppressor. As alluded to in the chapter by Gareth Evans all NF2 tumours are caused by mutations in the gene coding for merlin. In addition *merlin* mutations cause spontaneous brain tumours i.e. schwannomas and meningiomas. Tumours caused by mutations in *merlin* are thus genetically well defined and frequent [3]. To understand the role of *merlin* mutations in tumorigenesis the expression pattern and interaction partners of merlin will first be described. This will be followed by a short discussion of phenotypes associated with *merlin* mutations. The chapter will finish with a short paragraph hypothesising on possible mechanisms of tumorigenesis.

Expression

Merlin is expressed in a variety of tissues in embryonic development [4]. In adulthood it is expressed in lens [5], retina [6], testis, ovary, adrenal gland and neuronal tissue [7]. There are conflicting data on expression in other tissues. Expression in the neuronal tissue, however, includes both expression in Schwann cells and expression in neurons [8]. Thus *merlin*, although expressed in tissues affected in NF2, is also expressed in tissues not affected in NF2.

On a subcellular level merlin is primarily found in actin rich cellular protrusions like lamellipodia and membrane ruffles [9]. It can however also be found in focal adhesions co-localising with focal adhesion and focal complex proteins [10]. Merlin levels increase under high cell density and under withdrawal of serum. Also merlin shuttles to the nucleus in a cell cycle dependent manner [11].

There are different splice forms of merlin, however the by far most prominent are isoform 1 and 2 differing only in the very C-terminal end. Isoform 1 consisting of exons 1–15 and 17 coding for 595 AA while isoform 2 comprises exons 1–16 resulting in a 590-AA protein. It is thought that isoform 1 can adopt a closed conformation on dephosphorylation and act then as a tumour suppressor although this is not finally settled yet [12].

Merlin Interaction Partners

Many merlin interaction partners have been identified in the last couple of years. These include transmembrane proteins, scaffolding/adapter proteins, signalling molecules/kinases, cytoskeletal proteins, various other proteins and proteins of yet unknown function (table 1). Until now only some of those merlin interaction partners have been examined in cells/tissues affected by *merlin* mutations in humans, nevertheless these interactions provide important insight into merlin's function.

Merlin's C-terminal domain can bind its N-terminal FERM domain and the FERM domain of the ERM proteins especially ezrin [13]. This binding, as with the binding to binding partners such as CD44, has been shown to be regulated by phosphorylation at serine 518. P21-activated kinase (PAK), downstream of the small GTPases Rac and Cdc42 and cAMP dependent protein kinase (PKA), have been shown to phosphorylate merlin [12, 14–16]. In a negative feedback-loop merlin inhibits Rac activation [17, 18].

For some of the interaction partners it has been shown or speculated that merlin exerts its role as a tumour suppressor through the interaction with the

Table 1. Merlin interaction partners

Transmembrane	Scaffolding/ Adaptor	Signalling molecules/kinases	Cytoskeletal	Various	Unknown function
β1 integrin [52]	NHERF (EBP50) [51, 53]	Mixed lineage kinase (MLK3) [47]	Microtubules [54]	ERM-proteins [55, 56]	Schip-1 [57]
CD44 [44]	Grb2 [46]	P12-activated kinase (PAK) [14, 58]	Actin [54]	Transactivation-responsive RNA-binding protein (TRBP) [59]	MAP [60]
Layilin [35]	Syntenin [61]	Rho-GTP dissociation inhibitor (Rho-GDI) [62]	N-WASP [37]	Translation initiating factor eIF3c [63]	
erbB2	Paxillin [10]	PIKE-L [64]		Cell cycle regulator Hei10 [65]	
Paranodin [66]	Magicin [67]	Ral guanine nucleotide dissociation stimulator (RalGDS) [68]	βII-spectrin [69]		
		Hepatocyte growth factor regulated tyrosine kinase substrate (HRS) [70]			
		Protein kinase A subunit RIβ (PKA-RIβ) [71]			

binding partner. Importantly some interaction partners are components of multimolecular signalling pathways and thus part of a bigger picture, which I will discuss in more detail in the final chapter.

Phenotypes of Merlin Mutation

The phenotypes of patients harbouring merlin mutation have been superbly described in the previous chapter by Gareth Evans. The different mouse and in vitro models have, however, been instrumental in understanding how merlin loss leads to tumorigenesis and thus are briefly described here.

Showing that merlin can indeed reduce proliferation and alter abnormal cellular behaviour in a rat schwannoma cell line caused by *Neu* mutation demonstrated that merlin acts as a tumour suppressor in these cells. This has

also been demonstrated in other cell lines [12, 19]. As these and other cell lines used, have, however, different underlying mutations, one has to be careful to transfer results one to one to explain phenotypes of cells where the primary mutation is merlin loss.

In mice the loss of both *NF2* alleles due to classical knock out is embryonically lethal [20]. Heterozygous knock out mice do not develop schwannomas nor meningiomas nor ependymomas, the tumours found in NF2, but a variety of malignant tumours mainly sarcomas and liver carcinomas [21] indicating a more general role of merlin in mouse embryogenesis and tumorigenesis.

Mice with *merlin* conditionality knocked out using a Schwann cell specific promoter developed Schwann cell hyperplasia and also schwannomas at a later age [22]. This nicely confirms the role of merlin in development of schwannomas and allows different research questions to be addressed.

Using an elegant approach to knock out *merlin* specifically in meningeal cells Kalamarides et al. [23] showed that this is sufficient to develop meningiomas. To the author's knowledge there are no animal models for ependymoma or retinal hamartomas which can be found in NF2 patients.

Some interaction partners mentioned in table 1 are not found in human Schwann cells which might be one explanation why mouse models have a slightly different phenotype than the human one. Obviously the timing of the mutation in the human disease and in the animal models is also different; this might be another reason for the differing phenotypes between mice and men.

Comparing primary human schwannoma cells with normal human Schwann cells as an in vitro model, it was shown that these *merlin* deficient human cells show slightly increased proliferation and increased cell spreading [24, 25] reversible on the reintroduction of *merlin* [26], increased adhesion to extracellular matrix [27], slightly decreased apoptosis [28] and altered cytoskeleton [25, 29].

An interesting finding is that in patients with NF2 associated polyneuropathy only mutation in one allele could be found in peripheral nerves raising the possibility that merlin haploinsufficiency is enough to cause disease, though not enough for schwannoma formation [30].

Possible Disease Mechanism

It is impossible to mention all hypotheses on how merlin loss could lead to tumorigenesis, thus only those which have accumulated evidence through publications including publications on merlin interaction partners will be discussed.

Although *merlin* is expressed in all tissues affected, it is also expressed in tissues not affected in NF2. This is, however, a frequently encountered problem in human genetics, and may be caused by other proteins taking up merlin's role in tissue not affected by *merlin* loss or by differential expression of merlin binding partners.

Most data on merlin's role in tumorigenesis are from different in vitro models. As it has been shown on many occasions that there is no merlin expression in tumours caused by *merlin* mutations, it is generally assumed that *merlin* loss/inactivation is the starting point. There has been the question of third hit derived from mathematical modelling [31] and the fact that conditional knockout mice develop schwannomas late in life. However, so far no additional hits have been found in NF2 patients respectively the tumours caused by merlin loss.

Interestingly Schwann cell hyperplasia and Schwann cell tumours in transgenic mice expressing *merlin* mutation (which can also be found in patients) lacking amino acids 39–121 suggest that there can be a dominant negative effect [32]. However, in humans evidence for a dominant negative effect is missing as merlin has not been detected in tumours and the mice showed quite high expression of the mutant merlin.

Before discussing individual theories one also has to consider that most theories are based on comparison of $NF2^{-/-}$ and $NF2^{+/+}$ cells in confluent as well as subconfluent conditions. In confluent cultures different cell types e.g. $NF2^{-/-}$ mouse fibroblasts as well as $NF2^{-/-}$ mouse schwannoma cells show loss of contact inhibition [33]. In subconfluent cultures $NF2^{-/-}$ cells, including primary human schwannoma cells, show slightly increased proliferation and increased adhesion to the extracellular matrix [24, 25, 27].

Regarding adhesion it is interesting to note that merlin binds to adhesion molecules such as β1 integrin and layilin and to molecules which are part of the focal adhesion complexes such as paxillin and focal adhesion kinase [10, 34, 35]. Thus it makes sense that *merlin* deficient cells show deregulated adhesion as shown in schwannoma cells [27], which also might explain why schwannoma cells seem not to properly myelinate axons but instead build pseudomesaxon around the extracellular matrix [36]. Additionally, merlin seems to be directly involved in cytoskelatal organisation. Not only does it bind actin and tubulin but it inhibits the actin nucleation promotion factor N-Wasp, thereby regulating actin polymerisation [37]. This implies that merlin loss would lead to more actin rich cellular protrusions, which has been clearly demonstrated in human schwannoma cells [38]. Of interest is also that merlin inhibits the Rac effector Pak in a negative feedback loop [14, 39], and inhibits Rac recruitment to the membrane [40]. Thus when merlin is lost Rac is activated and recruited to the membrane where it is possibly further activated by integrins, which are

overexpressed in schwannomas [27]. Supporting this hypothesis merlin loss leads to activated Rac at the membrane in human Schwannoma cells [18]. Of note is that this GTPase activation is non-localised and long-lasting in schwannoma cells [38, 41]. The randomly activated GTPases are also relevant in the regulation of adhesion, and actin rich polymerisation of merlin deficient cells. One could therefore postulate that *NF2*$^{-/-}$ schwannoma cells are nonpolarised, in contrast to the highly polarised normal Schwann cells. It has also been shown that Paxillin, part of the complex in focal adhesion, is also important in the recruitment of merlin to the membrane where merlin can then be phosphorylated by Cdc42 via Pak [42].

Looking at contact inhibition, *NF2*$^{-/-}$ fibroblasts, keratinoycytes and human schwannoma cells show impaired cell-cell contact due to destabilised adherens junctions [33; Flaiz et al., in press]. Merlin's role in stabilising adherens junctions might involve the PDZ protein erbin, found to bind indirectly to merlin [43]. In confluent cells dephosphorylated merlin binds the transmembrane hyaluronan receptor CD44 and acts as a growth inhibitor [44]. Merlin also inhibits contact inhibition by suppressing Rac recruitment to the membrane [40]. CD44 and integrins are also believed to act as coreceptors for tyrosine kinase receptors, prompting the study of the signalling pathways downstream of these receptors especially the Ras-Raf-Mek-Erk pathway and its role in cell growth in merlin deficient cells. Merlin was shown to inhibit the Ras-Raf-Mek-Erk pathway at different levels [45–48]. In these experiments the Erk pathway was stimulated with a number of growth factors including PDGF. Merlin's ability to inhibit growth factor induced stimulation of the Erk pathway seemed to be more pronounced in confluent cultures. Interestingly it could be shown that merlin inhibits Ras and Rac activation upstream of the Raf-Mek-Erk after growth factor stimulation [48]. Thus there is accumulating evidence that merlin inhibits growth factor induced mitogenic signalling pathways. This interestingly includes inhibiting Ras pointing out a parallel to NF1, where the Ras-Neurofibromin is mutated.

Another interesting finding is that merlin interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS). HRS is a regulator of tyrosine kinase trafficking to the degradation pathway and an inhibitor of the STAT pathway. Merlin requires HRS to inhibit the STAT pathway and acts as a growth suppressor via HRS [49, 50].

An attractive, although yet unproven, hypothesis is that merlin is involved in a degradation of growth factors. This is supported by the fact that merlin binds ebp50 [51] and erbin [43] both shown to be relevant in growth factor distribution.

In summary there is accumulating evidence that merlin has an important role in the coordination of two relevant and interdependent processes which are cellular adhesion and growth factor receptor response.

References

1 Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, et al: A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor (published erratum appears in Cell 1993;75:826). Cell 1993;72:791–800.

2 Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, et al: Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 1993;363:515–521.

3 Hanemann CO, Bartelt-Kirbach B, Diebold R, Kampchen K, Langmesser S, Utermark T: Differential gene expression between human schwannoma and control Schwann cells. Neuropathol Appl Neurobiol 2006;32:605–614.

4 Akhmametyeva EM, Mihaylova MM, Luo H, Kharzai S, Welling DB, Chang LS: Regulation of the neurofibromatosis 2 gene promoter expression during embryonic development. Dev Dyn 2006;235:2771–2785.

5 Claudio JO, Veneziale RW, Menko AS, Rouleau GA: Expression of schwannomin in lens and Schwann cells. Neuroreport 1997;8:2025–2030.

6 Chan CC, Koch CA, Kaiser-Kupfer MI, Parry DM, Gutmann DH, Zhuang Z, Vortmeyer AO: Loss of heterozygosity for the *NF2* gene in retinal and optic nerve lesions of patients with neurofibromatosis 2. J Pathol 2002;198:14–20.

7 Gutmann DH, Wright DE, Geist RT, Snider WD: Expression of the neurofibromatosis 2 (NF2) gene isoforms during rat embryonic development. Hum Mol Genet 1995;4:471–478.

8 Gronholm M, Teesalu T, Tyynela J, Piltti K, Bohling T, et al: Characterization of the NF2 protein merlin and the ERM protein ezrin in human, rat, and mouse central nervous system. Mol Cell Neurosci 2005;28:683–693.

9 Gonzalez-Agosti C, Xu L, Pinney D, Beauchamp R, Hobbs W, Gusella J, Ramesh V: The merlin tumor suppressor localizes preferentially in membrane ruffles. Oncogene 1996;13:1239–1247.

10 Fernandez-Valle C, Tang Y, Ricard J, Rodenas-Ruano A, Taylor A, et al: Paxillin binds schwannomin and regulates its density-dependent localization and effect on cell morphology. Nat Genet 2002;31:354–362.

11 Muranen T, Gronholm M, Renkema GH, Carpen O: Cell cycle-dependent nucleocytoplasmic shuttling of the neurofibromatosis 2 tumour suppressor merlin. Oncogene 2005;24:1150–1158.

12 Sherman L, Xu HM, Geist RT, Saporito-Irwin S, Howells N, et al: Interdomain binding mediates tumor growth suppression by the NF2 gene product. Oncogene 1997;15:2505–2509.

13 Gutmann DH, Sherman L, Seftor L, Haipek C, Hoang LK, Hendrix M: Increased expression of the NF2 tumor suppressor gene product, merlin, impairs cell motility, adhesion and spreading. Hum Mol Genet 1999;8:267–275.

14 Kissil JL, Wilker EW, Johnson KC, Eckman MS, Yaffe MB, Jacks T: Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1. Mol Cell 2003;12:841–849.

15 Xiao GH, Beeser A, Chernoff J, Testa JR: p21-activated kinase links rac/Cdc42 signaling to merlin. J Biol Chem 2002;277:883–886.

16 Alfthan K, Heiska L, Gronholm M, Renkema GH, Carpen O: Cyclic AMP-dependent protein kinase phosphorylates Merlin at serine 518 independently of P21-activated kinase and promotes Merlin-Ezrin heterodimerization. J Biol Chem 2004;279:18559–18566.

17 Shaw RJ, Paez JG, Curto M, Yaktine A, Pruitt WM, et al: The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. Dev Cell 2001;1:63–72.

18 Kaempchen K, Mielke K, Utermark T, Langmesser S, Hanemann CO: Upregulation of the Rac1/JNK signaling pathway in primary human schwannoma cells. Hum Mol Genet 2003;12:1211–1221.

19 Huynh MD, Pulst SM: Neurofibromatosis 2 antisense oligonucleotides induce reversible inhibition of schwannomin synthesis and cell adhesion in STS26T and T98G cells. Oncogene 1996;13: 73–84.

20 McClatchey AI, Saotome I, Ramesh V, Gusella JF, Jacks T: The Nf2 tumor suppressor gene product is essential for extraembryonic development immediately prior to gastrulation. Genes Dev 1997;11:1253–1265.

21 McClatchey AI, Saotome I, Mercer K, Crowley D, Gusella JF, Bronson RT, Jacks T: Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors. Genes Dev 1998;12:1121–1133.
22 Giovannini M, Robanus-Maandag E, van der Valk M, Niwa-Kawakita M, Abramowski V, et al: Conditional biallelic *Nf2* mutation in the mouse promotes manifestations of human neurofibromatosis type 2. Genes Dev 2000;14:1617–1630.
23 Kalamarides M, Niwa-Kawakita M, Leblois H, Abramowski V, Perricaudet M, et al: *Nf2* gene inactivation in arachnoidal cells is rate-limiting for meningioma development in the mouse. Genes Dev 2002;16:1060–1065.
24 Rosenbaum C, Kluwe L, Mautner VF, Friedrich RE, Mueller HW, Hanemann CO: Isolation and characterization of Schwann cells from neurofibromatosis type 2 patients. Neurobiol Dis 1998;5:55–64.
25 Pelton PD, Sherman LS, Rizvi TA, Marchionni MA, Wood P, Friedman RA, Ratner N: Ruffling membrane, stress fiber, cell spreading and proliferation abnormalities in human Schwannoma cells. Oncogene 1998;17:2195–2209.
26 Schulze KM, Hanemann CO, Muller HW, Hanenberg H: Transduction of wild-type merlin into human schwannoma cells decreases schwannoma cell growth and induces apoptosis. Hum Mol Genet 2002;11:69–76.
27 Utermark T, Kaempchen K, Hanemann CO: Pathological adhesion of primary human schwannoma cells is dependent on altered expression of integrins. Brain Pathol 2003;13:352–363.
28 Utermark T, Kaempchen K, Antoniadis G, Hanemann CO: Reduced apoptosis rates in human schwannomas. Brain Pathol 2005;15:17–22.
29 Utermark T, Schubert SJ, Hanemann CO: Rearrangements of the intermediate filament GFAP in primary human schwannoma cells. Neurobiol Dis 2005;19:1–9.
30 Hanemann CO, Diebold R, Kaufmann D: Role of NF2 haploinsufficiency in NF2-associated Polyneuropathy. Brain Pathol 2007;17:371–376.
31 Woods R, Friedman JM, Evans DG, Baser ME, Joe H: Exploring the 'two-hit hypothesis' in NF2: tests of two-hit and three-hit models of vestibular schwannoma development. Genet Epidemiol 2003;24:265–272.
32 Giovannini M, Robanus-Maandag E, Niwa-Kawakita M, van der Valk M, Woodruff JM, et al: Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. Genes Dev 1999;13:978–986.
33 Lallemand D, Curto M, Saotome I, Giovannini M, McClatchey AI: *NF2* deficiency promotes tumorigenesis and metastasis by destabilizing adherens junctions. Genes Dev 2003;17: 1090–1100.
34 James MF, Beauchamp RL, Manchanda N, Kazlauskas A, Ramesh V: A NHERF binding site links the betaPDGFR to the cytoskeleton and regulates cell spreading and migration. J Cell Sci 2004;117:2951–2961.
35 Bono P, Cordero E, Johnson K, Borowsky M, Ramesh V, Jacks T, Hynes RO: Layilin, a cell surface hyaluronan receptor, interacts with merlin and radixin. Exp Cell Res 2005;308:177–187.
36 Dickersin GR: The electron microscopic spectrum of nerve sheath tumors. Ultrastruct Pathol 1987;11:103–146.
37 Manchanda N, Lyubimova A, Ho HY, James MF, Gusella JF, et al: The NF2 tumor suppressor Merlin and the ERM proteins interact with N-WASP and regulate its actin polymerization function. J Biol Chem 2005;280:12517–12522.
38 Flaiz C, Kaempchen K, Matthies C, Hanemann CO: Actin rich protusions and non localized GTPase activation in Merlin deficient Schwannoma cells. J Neuropathol Exp Neurol 2007;66:608–616.
39 Hirokawa Y, Tikoo A, Huynh J, Utermark T, Hanemann CO, et al: A clue to the therapy of neurofibromatosis type 2: NF2/merlin is a PAK1 inhibitor. Cancer J 2004;10:20–26.
40 Okada T, Lopez-Lago M, Giancotti FG: Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. J Cell Biol 2005;171:361–371.
41 Nakai Y, Zheng Y, MacCollin M, Ratner N: Temporal control of Rac in Schwann cell-axon interaction is disrupted in NF2-mutant schwannoma cells. J Neurosci 2006;26:3390–3395.
42 Thaxton C, Lopera J, Bott M, Baldwin ME, Kalidas P, Fernandez-Valle C: Phosphorylation of the NF2 tumor suppressor in Schwann cells is mediated by Cdc42-Pak and requires paxillin binding. Mol Cell Neurosci 2007;34:231–242.

43 Rangwala R, Banine F, Borg JP, Sherman LS: Erbin regulates mitogen-activated protein (MAP) kinase activation and MAP kinase-dependent interactions between Merlin and adherens junction protein complexes in Schwann cells. J Biol Chem 2005;280:11790–11797.
44 Morrison H, Sherman LS, Legg J, Banine F, Isacke C, et al: The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev 2001;15:968–980.
45 Lim JY, Kim H, Kim YH, Kim SW, Huh PW, et al: Merlin suppresses the SRE-dependent transcription by inhibiting the activation of Ras-ERK pathway. Biochem Biophys Res Commun 2003;302:238–245.
46 Lim JY, Kim H, Jeun SS, Kang SG, Lee KJ: Merlin inhibits growth hormone-regulated Raf-ERKs pathways by binding to Grb2 protein. Biochem Biophys Res Commun 2006;340:1151–1157.
47 Chadee DN, Xu D, Hung G, Andalibi A, Lim DJ, et al: Mixed-lineage kinase 3 regulates B-Raf through maintenance of the B-Raf/Raf-1 complex and inhibition by the NF2 tumor suppressor protein. Proc Natl Acad Sci USA 2006;103:4463–4468.
48 Morrison H, Sperka T, Manent J, Giovannini M, Ponta H, Herrlich P: Merlin/neurofibromatosis type 2 suppresses growth by inhibiting the activation of Ras and Rac. Cancer Res 2007;67:520–527.
49 Scoles DR, Nguyen VD, Qin Y, Sun CX, Morrison H, Gutmann DH, Pulst SM: Neurofibromatosis 2 (NF2) tumor suppressor schwannomin and its interacting protein HRS regulate STAT signaling. Hum Mol Genet 2002;11:3179–3189.
50 Scoles DR, Qin Y, Nguyen V, Gutmann DH, Pulst SM: HRS inhibits EGF receptor signaling in the RT4 rat schwannoma cell line. Biochem Biophys Res Commun 2005;335:385–392.
51 Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, et al: NHE-RF, a regulatory cofactor for Na(+)-H+ exchange, is a common interactor for merlin and ERM (MERM) proteins. J Biol Chem 1998;273:1273–1276.
52 Obremski VJ, Hall AM, Fernandez-Valle C: Merlin, the neurofibromatosis type 2 gene product, and beta1 integrin associate in isolated and differentiating Schwann cells. J Neurobiol 1998;37:487–501.
53 Bretscher A, Chambers D, Nguyen R, Reczek D: ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu Rev Cell Dev Biol 2000;16:113–143.
54 Xu HM, Gutmann DH: Merlin differentially associates with the microtubule and actin cytoskeleton. J Neurosci Res 1998;51:403–415.
55 Sainio M, Zhao F, Heiska L, Turunen O, den Bakker M, et al: Neurofibromatosis 2 tumor suppressor protein colocalizes with ezrin and CD44 and associates with actin-containing cytoskeleton. J Cell Sci 1997;110:2249–2260.
56 Heiska L, Alfthan K, Gronholm M, Vilja P, Vaheri A, Carpen O: Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5- bisphosphate. J Biol Chem 1998;273:21893–21900.
57 Goutebroze L, Brault E, Muchardt C, Camonis J, Thomas G: Cloning and characterization of SCHIP-1, a novel protein interacting specifically with spliced isoforms and naturally occurring mutant NF2 proteins. Mol Cell Biol 2000;20:1699–1712.
58 Kissil JL, Johnson KC, Eckman MS, Jacks T: Merlin Phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. J Biol Chem 2002;277:10394–10399.
59 Lee JY, Moon HJ, Lee WK, Chun HJ, Han CW, et al: Merlin facilitates ubiquitination and degradation of transactivation-responsive RNA-binding protein. Oncogene 2006;25:1143–1152.
60 Lee IK, Kim KS, Kim H, Lee JY, Ryu CH, et al: MAP, a protein interacting with a tumor suppressor, merlin, through the run domain. Biochem Biophys Res Commun 2004;325:774–783.
61 Jannatipour M, Dion P, Khan S, Jindal H, Fan X, Laganiere J, Chishti AH, Rouleau GA: Schwannomin isoform-1 interacts with syntenin via PDZ domains. J Biol Chem 2001;276:33093–33100.
62 Maeda M, Matsui T, Imamura M, Tsukita S, Tsukita S: Expression level, subcellular distribution and rho-GDI binding affinity of merlin in comparison with Ezrin/Radixin/Moesin proteins. Oncogene 1999;18:4788–4797.
63 Scoles DR, Yong WH, Qin Y, Wawrowsky K, Pulst SM: Schwannomin inhibits tumorigenesis through direct interaction with the eukaryotic initiation factor subunit c (eIF3c). Hum Mol Genet 2006;15:1059–1070.

64 Rong R, Tang X, Gutmann DH, Ye K: Neurofibromatosis 2 (NF2) tumor suppressor merlin inhibits phosphatidylinositol 3-kinase through binding to PIKE-L. Proc Natl Acad Sci USA 2004; 101:18200–18205.
65 Gronholm M, Muranen T, Toby GG, Utermark T, Hanemann CO, Golemis EA, Carpen O: A functional association between merlin and HEI10, a cell cycle regulator. Oncogene 2006;25: 4389–4398.
66 Denisenko-Nehrbass N, Goutebroze L, Galvez T, Bonnon C, Stankoff B, et al: Association of Caspr/paranodin with tumour suppressor schwannomin/merlin and beta1 integrin in the central nervous system. J Neurochem 2003;84:209–221.
67 Wiederhold T, Lee MF, James M, Neujahr R, Smith N, et al: Magicin, a novel cytoskeletal protein associates with the NF2 tumor suppressor merlin and Grb2. Oncogene 2004;23:8815–8825.
68 Ryu CH, Kim SW, Lee KH, Lee JY, Kim H, et al: The merlin tumor suppressor interacts with Ral guanine nucleotide dissociation stimulator and inhibits its activity. Oncogene 2005;24:5355–5364.
69 Scoles DR, Huynh DP, Morcos PA, Coulsell ER, Robinson NG, Tamanoi F, Pulst SM: Neurofibromatosis 2 tumour suppressor schwannomin interacts with betaII-spectrin. Nat Genet 1998;18:354–359.
70 Gutmann DH, Haipek CA, Burke SP, Sun CX, Scoles DR, Pulst SM: The NF2 interactor, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), associates with merlin in the 'open' conformation and suppresses cell growth and motility. Hum Mol Genet 2001;10:825–834.
71 Gronholm M, Vossebein L, Carlson CR, Kuja-Panula J, Teesalu T, et al: Merlin links to the cAMP neuronal signaling pathway by anchoring the RIbeta subunit of protein kinase A. J Biol Chem 2003;278:41167–41172.

C. Oliver Hanemann
Chair of Clinical Neurobiology, Peninsula College of Medicine and Dentistry
The John Bull Building, Tamar Science Park
Research Way, Plymouth PL6 8BU (UK)
Tel. +44 175 243 7418, Fax +44 175 251 7846, E-Mail Oliver.Hanemann@pms.ac.uk

Kaufmann D (ed): Neurofibromatoses.
Monogr Hum Genet. Basel, Karger, 2008, vol 16, pp 177–188

Molecular Studies on Schwannomatosis

L. Kluwe

Laboratory for Phakomatosis and Malformation, Department of Maxillofacial Surgery, University Hospital Hamburg-Eppendorf, Hamburg, Germany

Abstract

Schwannomatosis is a genetic disease characterized by multiple non-vestibular schwannomas with an unknown genetic cause. One recent study suggested that schwannomatosis may be as common as neurofibromatosis type 2. Schwannomatosis and NF2 share common clinical and molecular features, but at the same time differ fundamentally from one another. The majority of schwannomatosis-associated tumors carry typical truncating mutations of the *NF2* gene. However, in some sporadic and all familial schwannomatosis cases tumors from one patient do not share a common *NF2* mutation. Furthermore, the genetic alterations found in tumors cannot be found in non-tumor tissues of the patient. Loss of heterozygosity of the *NF2* locus is found at a high frequency for familial schwannomatosis tumors (86%). Linkage studies using families have clearly excluded the *NF2* locus for schwannomatosis and located a candidate region proximal to the *NF2* gene. This 5.6 Mb candidate region contains the immunoglobulin super locus, approximately 60 known genes and multiple low copy repeats. Current studies are focused on comparative genome hybridization with very densely packed oligomer probes covering the candidate region, investigating candidate genes there and direct sequencing of the 5.6 Mb interval.

Clinical Aspect of Schwannomatosis

Schwannomatosis is a third form of neurofibromatosis characterized by multiple schwannomas. The lack of vestibular schwannomas distinguishes schwannomatosis clearly from NF2. Furthermore, unlike NF2 patients, schwannomatosis patients do not develop ependymomas and ocular abnormalities [1–4]. Schwannomatosis-associated schwannomas often cause pain, while NF2-schwannomas mostly lead to neurological deficits [5]. Though some NF1 patients also develop multiple spinal tumors, these are mostly neurofibromas, and thus pathologically different from spinal schwannomas of schwannomatosis patients.

One recent study revealed that schwannomatosis may be as common as NF2 [6]. Surgically, schwannomatosis patients represent 2.4% to 5% of patients undergoing resection of their schwannomas. More than 90% of schwannomatosis are isolated cases, thus without family history [2, 7]. Approximately one third of schwannomatosis patients develop tumors in a segmental distribution, a single limb or spinal segment [8].

The following diagnostic criteria for schwannomatosis have recently been defined by a working consensus group [7]:

Definite: Age >30 years AND two or more non-intradermal schwannomas, at least 1 with histologic confirmation AND no evidence of vestibular tumor on high-quality MRI scan AND no known constitutional *NF2* mutation

Or

One pathologically confirmed non-vestibular schwannoma plus a first-degree relative who meets above criteria

Possible: Age <30 years AND two or more non-intradermal schwannomas, at least 1 with histologic confirmation AND no evidence of vestibular tumor on high-quality MRI scan AND no known constitutional *NF2* mutation

Or

Age>45 years AND two or more non-intradermal schwannomas, at least 1 with histologic confirmation AND no symptoms of 8th nerve dysfunction AND no known constitutional *NF2* mutation

Or

Radiographic evidence of a non-vestibular schwannoma and first degree relative meeting criteria for definite schwannomatosis

Segmental: meets criteria for either definite or possible schwannomatosis but limited to one limb or five fewer contiguous segments of the spine.

Genetics of NF2 and Sporadic Vestibular Schwannomas

Schwannomatosis shares common features with NF2 and sporadic schwannomas. The *NF2* gene is a typical tumor suppressor gene, whereby bi-allelic inactivation leads to the development of NF2-associated tumors. While the 1st (constitutional) inactivation events are often small mutations, such as point mutations and deletion/insertion of single and several bp, the 2nd (somatic) mutation may also be another small mutation or the loss of the remaining intact allele of the gene [9–11]. Tumors from one NF2 patient share the same constitutional mutation, but have distinctive somatic mutations. The constitutional mutation also exists in non-tumor tissues such as peripheral leukocytes of the patient and is shared by other affected family members.

Sporadic, single vestibular schwannomas develop in non-NF2 patients. Mutations and loss of heterozygosity (LOH) of the *NF2* gene have been found in these tumors. However, unlike in the case of NF2 patients, *NF2* mutations do not exist in non-tumor tissues of patients with sporadic schwannomas.

Somatic Alterations of the NF2 Gene in Schwannomatosis-Associated Tumors

Like NF2-associated and sporadic schwannomas, schwannomatosis-associated schwannomas also bear alterations in the *NF2* gene [12–14]. The majority of such *NF2* mutations are typical truncating mutations and distributed in exons 1 through 12, similar to those found in NF2-associated and sporadic schwannomas.

However, in some sporadic and all familial schwannomatosis patients, each tumor has a distinct *NF2* mutation. Unlike NF2, the mutations found in tumors of a schwannomatosis patient cannot be found in non-tumor tissues, such as peripheral leukocytes. Finally, no common *NF2* mutation is shared by patients from one schwannomatosis family. This pattern of the *NF2* gene inactivation is completely different from that of NF2, where all tumors from one patient share a common constitutional mutation, which is present in non-tumor tissues and shared by other affected family members.

As with NF2, LOH of the *NF2* locus is found in schwannomatosis associated tumors. The allele retained in different tumors from one schwannomatosis patient is always the same. This retained allele is the same allele shared by affected family members. Very high frequency (86%) of *NF2*-LOH was found in 28 schwannomas from 8 schwannomatosis families [MacCollin, unpublished data]. In comparison, *NF2*-LOH was found in 47% of 50 sporadic and 8 NF2-related schwannomas (53 vestibular schwannomas) [10], and 47% of 40 NF2-associated skin schwannomas [15, 16].

Regarding genetic alterations of the *NF2* gene, schwannomatosis tumors can be devided into 4 groups: (1) tumors in which no alteration is found; (2) tumors with mutation and without LOH; (3) tumors with LOH and without mutation, and (4) tumors with mutation and LOH. Interestingly, two intragenic *NF2* mutations have only been found in each of two schwannomatosis tumors [12, Evans, unpublished data]. In all other schwannomatosis tumors with two genetic alterations, one of them is always loss of the *NF2*-allele.

These results demonstrate common molecular features shared by schwannomatosis and NF2, while also highlighting the fundamental differences between the two conditions. Schwannomatosis is clearly related to NF2, but cannot be categorized as a subgroup of NF2. Additional events are apparently necessary for this disease.

Mechanism of *NF2*-LOH in Schwannomatosis Tumors

Studies using microsatellite markers on the long arm of chromosome 22 (22q) revealed that tumors from sporadic schwannomatosis patients have either retention or loss of most markers. LOH in these tumors cover the entire long arm of chromosome 22 [13, 14]. The majority of tumors from familial schwannomatosis patients also exhibit the same pattern of LOH, that is, either retention or loss of the entire chromosome arm. However, in three tumors from two unrelated families heterozygosity was lost in the *NF2* locus but retained in the proximal region. In another tumor, the *NF2* locus was heterozygous, but LOH was found for the proximal region.

Dual color fluorescence in situ hybridization (FISH) was performed in 12 tumors exhibiting 22q LOH in order to determine the mechanism of LOH in schwannomatosis-related tumors. Seven of the 12 schwannomatosis tumors (58%) exhibited two FISH signals for the *NF2* probe, indicating isodisomy corresponding to mitotic recombination or nondisjunction [14].

A study using comparative genomic hybridization (CGH) reported isodisomy in 6 (38%) out of 16 sporadic and NF2-associated schwannomas with LOH of the *NF2* locus [17]. Therefore, isodisomy is not specific for schwannomatosis-associated schwannomas, but also accounts for LOH in some sporadic and NF2-associated schwannomas. Due to the small sample size, it is not clear if isodisomy is more frequent in schwannomatosis-associated schwannomas.

Linkage Studies

Since schwannomatosis is clearly related to NF2, linkage analysis for the *NF2* locus was conducted in schwannomatosis families. Analysis of families number 4 and 5 (fig. 1), in the 2.5-Mb region from D22S193 to D22S430 was consistent with passage of a single allele to all affected family members and to obligate but non-expressing carriers (maximum two point LOD score of 1.611 at theta = 0 [12]). Analysis of additional affected members from family 5 and analysis of families 1 and 11 increased this two point LOD score for the intronic marker NF2tet to 2.5 at theta = 0. In these four families and in families number 3 and 8 a total of 17 tumors with LOH at the *NF2* locus all retained the allele derived from the affected parent.

In two families affected members are found not to share an *NF2* region haplotype (two point LOD scores, family 9 at NF2tet = –1.41, family 10 at po53/54 = –1.76). This result is of critical importance, as it clearly excluded the *NF2* gene itself for having inheritable alterations leading to schwannomatosis.

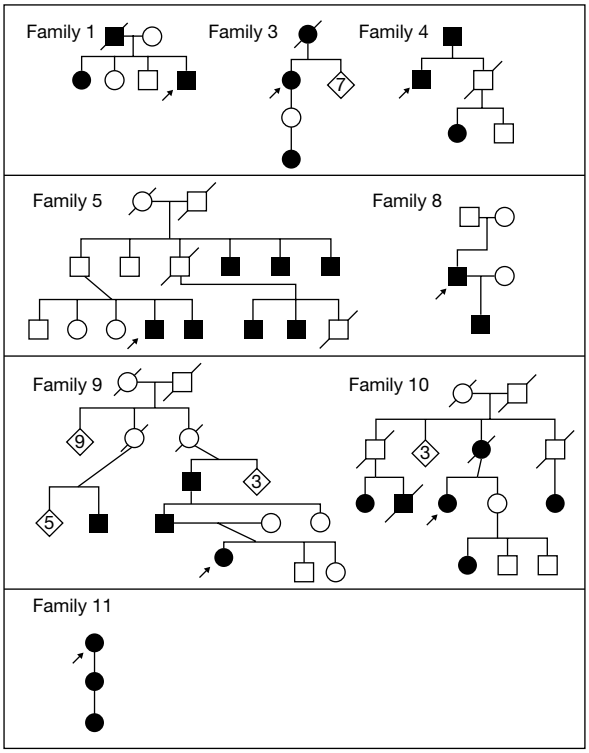


Fig. 1. Families used for linkage analysis. All families except family 9 had at least two tumors studied which show different *NF2* mutations against LOH, excluding the possibility of typical germline *NF2* mutation. In all families except for 9 and 10 all obligate carriers and retained tumor alleles carry identical *NF2* region haplotypes. All affected individuals have only schwannomas, except for a single individual in family 10 who has two meningiomas.

Haplotyping with markers proximal and distal to *NF2* in affected members of 6 families (1, 4, 5, 9, 10, and 11 in fig. 1), led to the discovery of a candidate region. Multipoint analysis revealed a maximum LOD score of 6.60 near marker D22S1174 in the proximal portion of chromosome 22 (fig. 2). Because family 9 displays unusual patterns of LOH raising the issue of locus heterogeneity, analysis was repeated without family 9, yielding a multipoint LOD score of 5.04 spanning the region D22S420 to D22S1148 [14].

With the addition of more families and development of additional markers a candidate region is now defined on a 5.6 Mb interval proximal to the *NF2* gene, with each end ascertained in two families (fig. 3). Markers not shared by affected members and thus defining the proximal and distal ends are D22S264 at 19,097 kb and D22S1148 at 24,670 (NCBI build 35.1), respectively. The

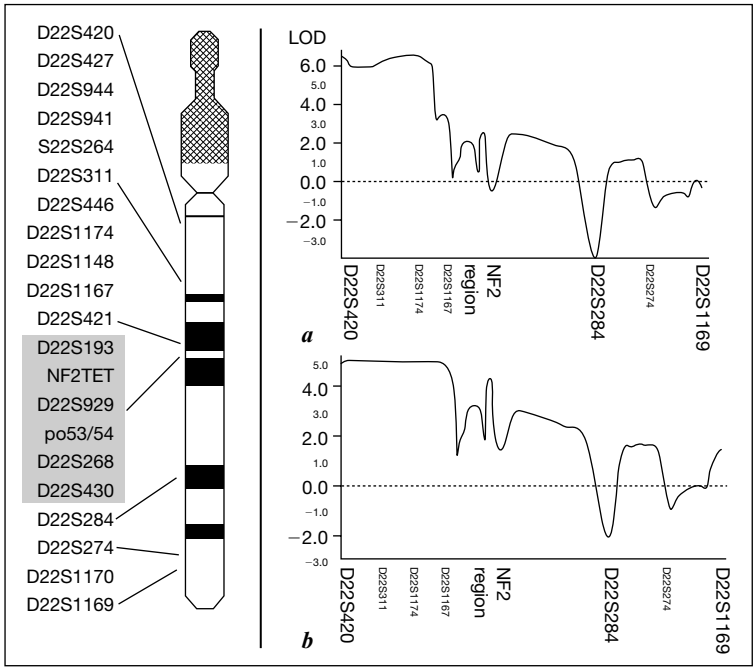


Fig. 2. Results of linkage analysis using markers over the long arm of chromosome 22. Multipoint LOD scores with (***a***) and without (***b***) family 9. Markers in the *NF2* region are shaded. LOD score around 6 was obtained for the region from marker D22S430 to D22S1148, defining a candidate region for alterations leading to schwannomatosis.

5.6 Mb candidate region contains the immunoglobulin super locus, approximately 60 known genes and several low copy repeats (LCRs). No gaps are known in the current build. If we reduce the stringency and define each end with only one family, the candidate region can be further narrowed down to a 3.1 Mb interval between markers LK502 and LK509, corresponding to 20,885 kb and 24,029 kb of the NCBI build 35.1 (fig. 3).

Initial Sequencing of the Candidate Region

One focus of the current studies for finding alterations for schwannomatosis is direct sequencing of the 5.6 Mb candidate region. For PCR-based resequencing, hemizygous material containing only the affected allele is required.

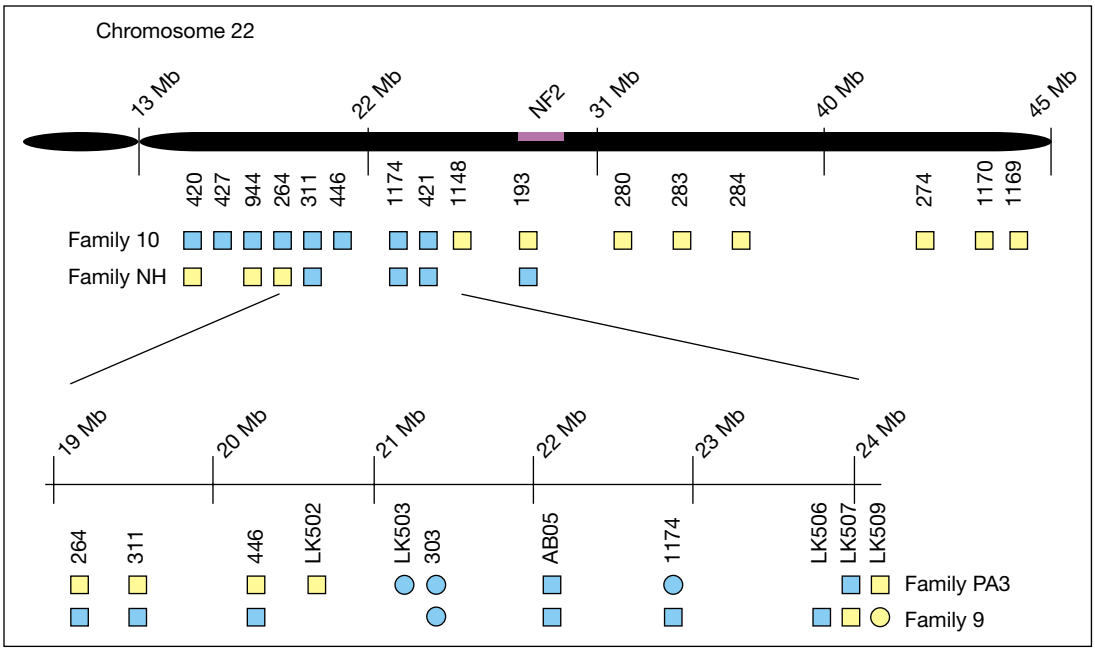


Fig. 3. Refining the candidate region using additional families and markers on the long arm of chromosome 22, 13 to 45 Mb according to the NCBI build 35.1. Each line represents data from one family. Microsatellite polymorphisms are boxed in blue when shared by all affected family members and in yellow when not shared. The candidate region of 5.6 Mb from the marker D22S264 (19.097 Mb) to the marker D22S1148 (24.670 Mb) is defined by a total of four families, two for each end. A smaller core candidate region encompassing 3.1 Mb between markers LK502 and LK509 was defined by family 9 and family PA3. Markers represented as squares are highly polymorphic and thus conclusive as for shared or unshared at those positions and are highly reliable. Markers in circle are less polymorphic and could be coincidently shared by affected family members.

To create such resource, lymphoblastoid cell lines of non-founder schwannomatosis patients were fused with a mouse or a hamster background. Obtained clones were genotyped using microsatellite markers in the candidate region and those with the affected alleles were identified. To date, three such clones from three unrelated non-founders have been established (fig. 4). The clone containing the affected allele of the candidate region derived from an affected non-founder in family 1 (fig. 4), C37 was the first obtained. DNA extracted from this clone was therefore used for the following initial sequencing.

Interval 22,300 kb – 22,400 kb (NCBI 35.1) was chosen for the initial pilot sequencing. In a 140 bp segment around 22,307 kb, hemizygous changes were

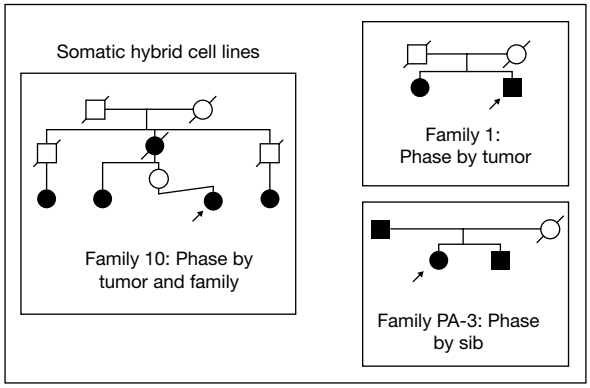


Fig. 4. Resources used for somatic cell hybrid construction. Non-founders from three schwannomatosis families (1, 10 and PA-3) were used. The first clone (clone 37) containing the affected allele of the candidate region is derived from a non-founder in family 1 and phased by tumor. Later, two additional clones containing the affected allele were obtained from two other families.

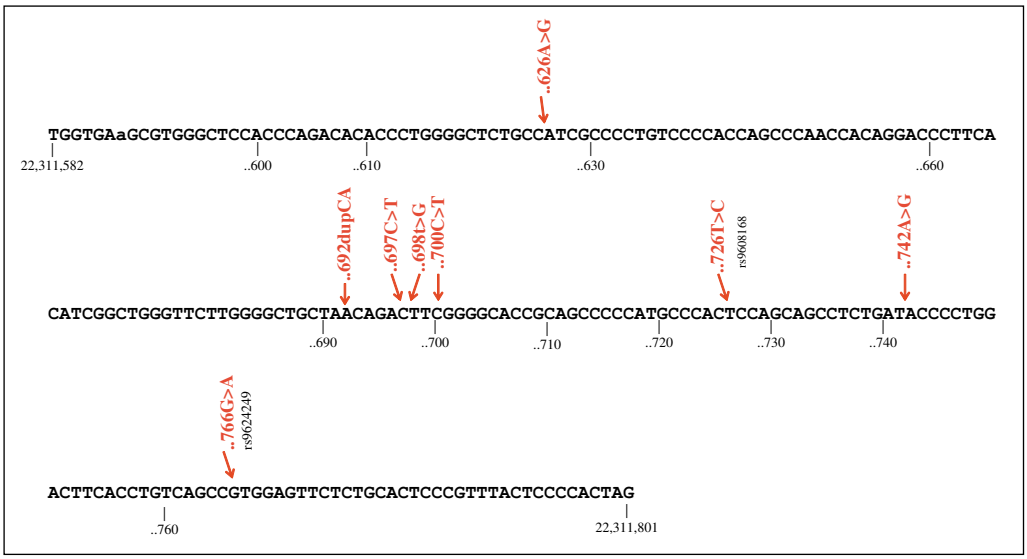


Fig. 5. Segment amplified from clone 37 containing multiple hemizygous changes. This segment corresponds to 22,311,582–22,311,801 of chromosome 22, according to the NCBI assembly build 35.1. Alterations in red were only found in the clone DNA, in DNA from the patient from whom the clone was derived and in DNA from the affected sibling of the patient. Two alterations 22,311,726T>C and 22,311,766G>A are known SNPs (rs9608168 and rs9624249).

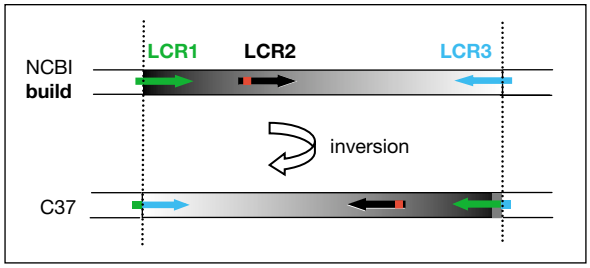


Fig. 6. Three LCRs and a rare inversion polymorphism in the candidate region. A segment between LCR1 and LCR3 of about 2 Mb is inverted in the clone with hemizygous affected allele of the candidate region. The same inversion was found in other samples at a frequency of 4%. Segment with multiple alterations in LCR2 is marked in red.

found at 8 positions in the DNA from C37 (fig. 5). Two changes are known single nucleotide polymorphisms while the other 6 are novel. The same changes were found in heterozygous DNA of the patient from whom clone C37 was derived as well as in the DNA of his affected sister. However, these changes were not found in the control panel of 50 non-schwannomatosis patients, nor in the panel of 17 non-founder schwannomatosis patients. These changes may be either family 1-specific or related to schwannomatosis. Unfortunately no DNA sample is available from unaffected members of this family. We thus cannot examine whether or not the changes also exist in asymptomatic parents.

Interestingly, the segment with these multiple alterations is in one of three LCRs in the candidate region (fig. 6). LCR1 and LCR2 are in the same orientation while LCR3 at the distal end of the candidate region is in an inverted orientation. The 6 family 1-specific changes are located in LCR2 (indicated by red bar in fig. 6). The altered sequence of LCR2 does not match its counterpart in LCR1 or LCR3, indicating that the alterations are not the result of any simple conversion between LCRs.

Nevertheless, this finding raised the question of possible rearrangement between the LCRs. To further explore this issue, PCR was performed using various combination of primers in the three LCRs. Indeed, an inversion between LCR1 and LCR3 involving a segment of approximately 2 Mb was detected (fig. 6) in the C37 DNA. However, analyzing additional samples revealed this inversion as a rare polymorphism with a frequency of approximately 4%.

The preliminary results of the pilot sequencing showed the complexity of the candidate region but also demonstrated that using hemizygous material, PCR-based sequencing has the potential of detecting small lesions as well as large rearrangements.

CGH Studies

A recent CGH study reported rearrangements of the immunoglobulin lambda locus in several lymphoblast cell lines derived from NF2 and schwannomatosis patients. However, this rearrangement was also found in lymphoblast cells derived from normal controls, though not in a pooled sample of DNA directly from leukocytes of 10 other normal controls. It seems to be an alteration associated with lymphoblast. From one familial schwannomatosis patient, DNA from leukocytes was available for the analysis which exhibited the same rearrangement [18]. The same study further reported missense mutations in the *CABIN1* gene located in the candidate region. However, the pathogenic value of those missense mutations have not yet been proven.

CGH using a 12,814 clone Agilent Technologies chip has failed to detect any copy number change on blood samples from four unrelated affected non-founders (unpublished data of Shen & Gusella at MGH, Boston). Neither did a CGH with Affymetrix 100k chip on DNA samples of lymphoblastic origin from several non-founder schwannomatosis patients detect any alteration (Shaikh at CHOP). This chip however does not have probes within the LCRs and the quality of some of the datapoints was not satisfactory. Furthermore, the limited resolution left the possibility open for smaller deletion or duplication.

In an ongoing study, a fine-tiled CGH array containing more than 300,000 probes covering the 5.6 Mb candidate region has been designed. Two somatic hybrid clones with affected allele, one clone with unaffected allele (as reference), and DNA from lymphoblastoid cell lines derived from several non-founder schwannomatosis patients are being analyzed using this array. The probes are very densely packed with a mean interval of 6 bp for two adjacent probes. This array CGH of high resolution may detect copy number changes of small fragments in the candidate region.

A Mutation in the *INI1/SMARCB1* Gene

The *INI1/SMARCB1* gene is a tumor suppressor gene in the candidate region, near marker D22S1174. *INI1* = integrase interactor 1, or *SMARCB1* = SWI-SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily B, member 1, has been reported to be involved in chromatin remodeling and regulations of cell cycle, growth and differentiation [19]. A recent study screened the 9 exons of this gene in a schwannomatosis family and found a heterozygous nonsense mutation of C>T in exon 1 in blood of the proband and seborrheic keratosis lesion of the affected father, but not in the blood of the unaffected mother. Furthermore, the same mutation was found in a total of

4 tumors from the two affected family members. In addition, a nonsense mutation was found in exon 5 in one tumor of the father. However, no *NF2* mutation was found in any of the tumors from this family [17].

At this stage, it is not yet conclusive if *INI1* gene mutations account for all schwannomatosis cases. Studies are in progress to screen larger numbers of schwannomatosis patients and tumors. It is especially essential to clarify the role of *NF2* mutations in schwannomatosis-associated tumors.

A high rate of LOH of the chromosome 22 long arm (22q) is one of the striking findings of previous studies. Allele loss of this chromosome arm may cause inactivation of both the *INI1* gene and *NF2* gene. In a patient with a germline *INI1* mutation, 22q LOH in a Schwann cell is the 2nd inactivation for the *INI1* gene and the 1st inactivation regarding the *NF2* gene. Supposed that these Schwann cells gain a growth advantage due to bi-allelic inactivation of *INI1*, a large number of such cells would arise, leading to a similar situation as in an NF2 patient in the sense that a large number of cells have only one intact *NF2* allele. In a large size of cell population, the probability is now high, that a spontaneous *NF2* mutation occurs on the remaining allele which is now the 2nd inactivation and thus sufficient for a tumor development.

Closing Remark

Current knowledge suggests that the direct cause for tumor development in schwannomatosis is the inactivation of the *NF2* gene. The pathogenesis of such inactivation is however different from that of NF2. The lack of vestibular schwannomas in schwannomatosis is an especially intriguing feature and thus among the key issues that need to be addressed. The possible explanations may be that (1) inactivation of the *NF2* gene occurs out of the time-window which is required for development of vestibular schwannomas and (2) inactivation of the *NF2* gene occurs only in a certain subpopulation of Schwann cells which do not exist in vestibular nerve.

Identification of the constitutional genetic alterations for schwannomatosis will likely enable us to understand the mysterious pathogenesis of this disease and open new perspectives for diagnosis and clinical treatment for the patients. Such findings may further contribute to a more thorough understanding of the genomic organization, regulation, and instability. Finally, LOH is the 2nd hit in approximately half of NF2 associated tumors. Identification of genetic alterations promoting such somatic events will contribute to a better understanding of pathogenesis of NF2 associated tumors. Strategies against such promoting effects may be used as therapeutic treatments for suppressing development of the tumors.

References

1 Evans DG, Huson SM, Donnai D, Neary W, Blair V, et al: A genetic study of type 2 neurofibromatosis in the United Kingdom. II. Guidelines for genetic counselling. J Med Genet 1992;29:847–852.

2 MacCollin M, Woodfin W, Kronn D, Short MP: Schwannomatosis: a clinical and pathologic study. Neurology 1996;46:1072–1079.

3 Mautner VF, Lindenau M, Baser ME, Hazim W, Tatagiba M, et al: The neuroimaging and clinical spectrum of neurofibromatosis 2. Neurosurgery 1996;38:880–885.

4 Parry DM, Eldridge R, Kaiser-Kupfer MI, Bouzas EA, Pikus A, Patronas N: Neurofibromatosis 2 (NF2): clinical characteristics of 63 affected individuals and clinical evidence for heterogeneity. Am J Med Genet 1994;52:450–461.

5 Mautner VF, Lindenau M, Baser ME, Kluwe L, Gottschalk J: Skin abnormalities in neurofibromatosis 2. Arch Dermatol 1997;133:1539–1543.

6 Antinheimo J, Sankila R, Carpen O, Pukkala E, Sainio M, Jaaskelainen J: Population-based analysis of sporadic and type 2 neurofibromatosis-associated meningiomas and schwannomas. Neurology 2000;54:71–76.

7 MacCollin M, Chiocca EA, Evans DG, Friedman JM, Horvitz R, et al: Diagnostic criteria for schwannomatosis. Neurology 2005;64:1838–1845.

8 Leverkus M, Kluwe L, Roll EM, Becker G, Brocker EB, Mautner VF, Hamm H: Multiple unilateral schwannomas: segmental neurofibromatosis type 2 or schwannomatosis? Br J Dermatol 2003;148:804–809.

9 Jacoby LB, MacCollin M, Louis DN, Mohney T, Rubio MP, et al: Exon scanning for mutation of the *NF2* gene in schwannomas. Hum Mol Genet 1994;3:413–419.

10 Jacoby LB, MacCollin M, Barone R, Ramesh V, Gusella JF: Frequency and distribution of *NF2* mutations in schwannomas. Genes Chromosomes Cancer 1996;17:45–55.

11 Kluwe L, Mautner VF: Mosaicism in sporadic neurofibromatosis 2 patients. Hum Mol Genet 1998;7:2051–2055.

12 Jacoby LB, Jones D, Davis K, Kronn D, Short MP, Gusella J, MacCollin M: Molecular analysis of the *NF2* tumor-suppressor gene in schwannomatosis. Am J Hum Genet 1997;61:1293–1302.

13 Kaufman DL, Heinrich BS, Willett C, Perry A, Finseth F, Sobel RA, MacCollin M: Somatic instability of the *NF2* gene in schwannomatosis. Arch Neurol 2003;60:1317–1320.

14 MacCollin M, Willett C, Heinrich B, Jacoby LB, Acierno JS Jr, Perry A, Louis DN: Familial schwannomatosis: exclusion of the *NF2* locus as the germline event. Neurology 2003;60:1968–1974.

15 Warren C, James LA, Ramsden RT, Wallace A, Baser ME, Varley JM, Evans DG: Identification of recurrent regions of chromosome loss and gain in vestibular schwannomas using comparative genomic hybridisation. J Med Genet 2003;40:802–806.

16 Kluwe L, Friedrich RE, Hagel C, Lindenau M, Mautner V-F: Mutations and allelic loss of the *NF2* gene in neurofibromatosis 2-associated skin tumors. J Invest Dermatol 2000;114:1017–1021.

17 Hulsebos TJM, Plomp AS, Wolterman RA, Robanus-Maandag EC, Baas F, Wesseling P: Germline mutation of *INI1/SMARCB1* in familial schwannomatosis. Am J Hum Genet 2007;80:805–810.

18 Buckley PG, Mantripragada KK, Diaz de Stahl T, Piotrowski A, Hansson CM, et al: Identification of genetic aberrations on chromosome 22 outside the NF2 locus in schwannomatosis and neurofibromatosis type 2. Hum Mutat 2005;26:540–549.

19 Roberts CW, Orkin SH: The SWI/SNF complex–chromatin and cancer. Nat Rev Cancer 2004;4:133–142.

Lan Kluwe
Laboratory for Phakomatosis and Malformation, Department of Maxillofacial Surgery
University Hospital Hamburg-Eppendorf
Martinistr. 52, DE–20246 Hamburg (Germany)
Tel. +49 40 42803 8267, Fax +49 40 42803 9665, E-Mail kluwe@uke.uni-hamburg.de

Author Index

Assum, G. 103

Boltshauser, E. 21

D'Angelo, I. 113
De Raedt, T. 143

Evans, D.G.R. 154

Hanemann, C.O. 167
Huson, S.M. 1

Kaufmann, D. IX
Kehrer-Sawatzki, H. 89
Kluwe, L. 177

Legius, E. 143

Maertens, O. 143
Mautner, V.-F. 21
Messiaen, L.M. 63

Peltonen, J. 129
Peltonen, S. 129

Scheffzek, K. 113
Schmegner, C. 103
Schmid, M. VII
Serra, E. 143
Stevenson, D.A. 32
Swensen, J.J. 32

Tinschert, S. 78

Upadhyaya, M. 46

Viskochil, D.H. 32

Wallace, A. 154
Welti, S. 113
Wimmer, K. 63

Subject Index

AAT deletion 13, 54
Absence of positive selection in humans 111
Active site 115
Astrocytomas 25, 147
Attention deficit hyperactivity disorder (ADHD) 23

Benign variant 71
Bone abnormalities 24, 29, 147

Café-au-lait macules (CALMs) 148
CAL-only phenotype 13
Capillary malformation syndromes 39
Cardio-facio-cutaneous (CFC) syndrome 39
Cardiovascular malformations 83
Catalysis 115
Cognitive function deficits 22
Comparative genomic hybridization (CGH) 186
Connective tissue 129
  abnormalities 84
Conservation of isochore structure 108
Copy number variation (CNV) 97
Costello syndrome 38
Craniofacial dysmorphism 81
Cutaneous neurofibromas 23, 130
Cytoskeleton 135

de novo 155
Deep intronic splice mutation 69
Disease
  mechanism 170
  models 171

Endothelial cells 138
Extracellular matrix 132

Fibroblasts 132
Fixation bias 108
Functional constraint 104

GAP related domain (GRD) 114
Gastrointestinal stromal tumor (GIST) 29, 146
Genetic counselling 1, 15, 160
Genomic rearrangements during mammalian evolution 106
Genotype/phenotype correlations 12, 32, 41, 46, 51, 80
Germline mutations 155
Gonadal mosaicism 149
Gross *NF1* deletions 89
GTPase activation 33, 115

Haploinsuffiency 32, 78, 129, 136
Haplotypes 109
Hypertension 29

INI1/SMARCB1 mutation 186
Intellectual impairment 82
Interspecies comparison 104

Juvenile myelomonocytic leukemia (JMML) 146

LEOPARD syndrome 36
Linkage analysis 180
Lipid binding 119
Loss of heterozygosity (LOH) 145, 179
Low copy repeats (LCR) 91, 96, 185

Malignant peripheral nerve sheath tumor (MPNST) 28, 85, 146
Mast cell 132
Meningioma 154, 161
Merlin 167
  expression 168
  interaction partners 168
  mutation phenotypes 169
Mismatch repair deficiency (MMR-D) 9
Missense mutation 66, 117
Modifying genes 52, 80
Mosaic mutations 155
Mosaicism 148
Multiplex ligation-dependent probe amplification (MLPA) 156
Mutation 186
  bias 108
Mutational spectrum 63

Neurofibroma 84, 145
  composition 129
  treatment 27
  types 129
Neurofibromatosis (NF)
  classification 2
  definition 2
  type 1 (NF1) 1, 6, 21, 32
    clinical features 6, 50
    diagnostic criteria 11
    in adults 26
    in children 22
    subtypes 12
    variants 51
  type 2 (NF2) 154, 167
    genetics 178
    management 159
    mutations 157
    screening 161
Neurofibromatosis-Noonan syndrome (NFNS) 15, 37, 56
Neurofibromin 47, 50, 113, 133
  NF1-PH domain 121
  NF1-Sec domain 119
  tissue distribution 135
NF1 gene
  alternative splicing 47
  coding sequences 104
  evolution 103, 106
  expression 133
  GC-content 107
  large deletion 79
  microdeletion 66, 78, 89, 92
    syndrome 78
    mechanisms 95
  mosaicism 148
  mRNA 133
  mutation 63
    analysis 65
  pseudogenes 49, 70
  segmental duplications 91
  somatic mutation 143
  structure 47, 90
NF2 gene
  LOH 180
  mutations 156
  somatic alterations 179
NF1REPs 91
Non-allelic homologous recombination (NAHR) 90, 95
Noonan syndrome 36

Oncogenesis 171
Optic pathway glioma (OPG) 25
Overgrowth 83

Pathogenic variant 71
Perineurial cells 131
Phenotype 35, 78
Pheochromocytoma 147
Phospholipids 119
PI3K/Akt pathway syndromes 40

Pleckstrin homology domain (PH) 114
Plexiform neurofibromas 23, 27, 130
Population bottleneck 110
Pseudarthrosis 24
Psychological aspects 29

Ras
  GTPase Activating Protein (RasGAP) 33, 114
  pathway 32
  signal transduction 33
Recurrent mutations 72
RNA-based mutation analysis 63, 67

Schwann cells 130
Schwannoma 154, 158, 177
Schwannomatosis 177
  associated tumors 179
  clinical aspects 177
Scoliosis 24
Sec14 domain 114
Segmental (localized) NF1 12, 149
Skeletal anomalies 84
Somatic mutation 158
Spinal NF 15
Splicing 66, 68
SPRED1 14
Strong negative selection 103
Subcutaneous neurofibromas 130

Tumor 145, 167

Vascular complications 25, 29
Vestibular schwannoma 155, 161, 178

Watson syndrome 14, 36