The Pathoetiology of Neurofibromatosis 1

Eeva-Mari Jouhilahti,* Sirkku Peltonen,† Anthony M. Heape,‡ and Juha Peltonen*†

From the Department of Cell Biology and Anatomy,* Institute of Biomedicine, University of Turku, Turku, the Department of Dermatology,† University of Turku and Turku University Hospital, Turku, and the Department of Anatomy and Cell Biology,‡ Institute of Biomedicine, University of Oulu, Oulu, Finland

Although a mutation in the NF1 gene is the only factor required to initiate the neurocutaneous-skeletal neurofibromatosis 1 (NF1) syndrome, the pathoetiology of the multiple manifestations of this disease in different organ systems seems increasingly complex. The wide spectrum of different clinical phenotypes and their development, severity, and prognosis seem to result from the cross talk between numerous cell types, cell signaling networks, and cell–extracellular matrix interactions. The bi-allelic inactivation of the NF1 gene through a “second hit” seems to be of crucial importance to the development of certain manifestations, such as neurofibromas, café-au-lait macules, and glomus tumors. In each case, the second hit involves only one cell type, which is subsequently clonally expanded in a discrete lesion. Neurofibromas, which are emphasized in this review, and cutaneous neurofibromas in particular, are known to contain a subpopulation of NF1-diploinsufficient Schwann cells and a variety of NF1-haploinsufficient cell types. A recent study identified a multipotent precursor cell population with an NF1“/−” genotype that resides in human cutaneous neurofibromas and that has been suggested to play a role in their pathogenesis. (Am J Pathol 2011, 178:1932–1939; DOI: 10.1016/j.ajpath.2010.12.056)

Neurofibromatosis 1 as a Clinical Entity

Neurofibromatosis 1 (NF1; Online Mendelian Inheritance of Man no. 162200; http://www.ncbi.nlm.nih.gov/omim, last accessed March 10, 2011), previously known as von Recklinghausen disease, is an autosomal dominant disorder that affects approximately 1 in 3500 persons worldwide.1,2 Half of the patients with NF1 have inherited their NF1 mutation, and the other half are caused by a de novo NF1 mutation. The findings and the degree of severity of NF1 are highly variable. The hallmarks of NF1 include café-au-lait macules and multiple benign cutaneous neurofibromas, which, typically, are detectable in adulthood by simple visual inspection.

NF1 can affect nearly every organ system, and the complications vary between individuals, even within a single family. The clinical diagnosis is based on the presence of two or more of the following findings: six or more café-au-lait macules with diameters >5 mm in prepubertal patients and >15 mm in postpubertal patients; two or more neurofibromas of any type or one plexiform neurofibroma; axillary or inguinal freckling; optic glioma; two or more Lisch nodules of the iris; a distinctive osseous lesion, such as sphenoid wing dysplasia or pseudarthrosis; or a first-degree relative diagnosed as having NF1 according to the preceding criteria.3 It has been suggested that a pathogenic mutation in the NF1 gene be added to the list of diagnostic criteria.4

The most common complications of NF1 are included in the previously listed diagnostic criteria. Additional features include short stature, scoliosis, headache, speech disorders, attention deficit disorder and attention-deficit/hyperactivity disorder, and learning disabilities. Rare complications, affecting <5% of patients, include epilepsy, hydrocephalus, cardiovascular problems, and dystrophic scoliosis.5 The lifetime risk of malignant tumors arising from peripheral nerves is estimated to be 10% to 13%.6 The pathoetiology of the complications in NF1 is, however, largely unknown.

Genetic Background

NF1 is caused by mutations in the NF1 gene that encodes the tumor suppressor protein neurofibromin.7,8 The NF1 gene is located on chromosome band 17q11.2, spanning approximately 280 kb of genomic DNA, and is composed of 57 constitutive exons and 4 alternatively spliced exons (9a, 10a2, 23a, and 48a).9 However, the large size of the NF1 gene alone does not explain the high frequency of de

Supported by grants from the Academy of Finland, the University of Turku, the Turku University Foundation, Turku Graduate School of Biomedical Sciences, and the Hospital District of Southwest Finland.

Accepted for publication December 10, 2010.

Address reprint requests to Juha Peltonen, M.D., Ph.D., Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku, Kiinanyllynkatu 10, 20520 Turku, Finland. E-mail: juha.peltonen@utu.fi.
novel mutations. The inheritance of NF1 follows an autosomal dominant trait, and all affected individuals are apparently heterozygous for an NF1 mutation because persons with constitutive inactivation of both alleles of the NF1 gene have not been found. Homozygous NF1-/- mice die in utero, indicating that neurofibromin is essential for life. On the other hand, mice with an NF1+/− genotype do not develop neurofibromas and appear otherwise normal during the first year of life. Later, however, NF1+/− mice develop tumor types similar to those typically seen in old wild-type mice but with a higher frequency.

Approximately 50% of human NF1 cases are caused by a new sporadic mutation in the NF1 gene. Sporadic germline NF1 gene mutations exhibit a sex bias, with most (>80%) of new mutations being of paternal origin, whereas most of the so-called microdeletions seem to be of maternal origin. Microdeletions cover the entire NF1 gene and its flanking regions, which contain 17 genes.

Genetic Testing

The penetrance of NF1 is 100%, meaning that all patients with an NF1 mutation have NF1. A comprehensive NF1 mutation screen can detect gene mutations in more than 92% of tested patients fulfilling the NIH diagnostic criteria. Mutation analysis is especially important in very young children with a negative family history and who only partially fulfill the NIH diagnostic criteria. Molecular testing for NF1 is also useful for diagnostic confirmation in the case where an adult patient with café-au-lait macules and axillary freckles has no neurofibromas. These patients with a mild phenotype but fulfilling NF1 diagnostic criteria might have Legius syndrome; the mutation is, in this case, in another gene, SPRED1. Approximately 1% to 2% of patients meeting NIH clinical diagnostic criteria for NF1 in fact harbor SPRED1 mutations.

Functions of Neurofibromin

The NF1 gene is transcribed into an 11- to 13-kb mRNA with an approximately 9-kb open reading frame and is translated into a ubiquitously expressed, 280-kDa protein called neurofibromin. Direct or indirect interactions have been reported between neurofibromin and several other proteins, including the transmembrane proteoglycan syndecan, actin, tubulin, and intermediate filaments, but very little is known about its function, and none of the known interactions alone can explain the different symptoms related to NF1. Only two potentially functional domains of neurofibromin have been described, RasGAP and Sec14, but the biological role of the Sec14 domain is unknown. The RasGAP-related domain (Ras-GRD) accelerates the conversion of active Ras-GTP to inactive Ras-GDP in various cell types and acts as a negative regulator of the p21ras signaling pathway. The Ras GTases interact with multiple pathways, including the Raf/Mek/Erk mitogen-activated protein kinase cascade, which regulates cellular growth and differentiation. NF1 belongs to a class of developmental syndromes called RASopathies, which are caused by germline mutations in genes that encode protein components of the Ras/mitogen-activated protein kinase pathway.

Differential Diagnosis: Other Ras Pathway Syndromes

Several clinical syndromes with disease-causing mutations in genes encoding proteins of the Ras signaling pathway have phenotypes overlapping with those of NF1. These syndromes include Noonan syndrome, LEOPARD syndrome, cardiofaciocutaneous syndrome, Costello syndrome, and Legius syndrome. These conditions all share a variable degree of learning difficulty, cardiac defects, facial dysmorphism, short stature, macrocephaly, and skin abnormalities. The clinical features of the recently described Legius syndrome resemble and partially overlap with those of NF1, including multiple café-au-lait spots, axillary freckling, and macrocephaly. Legius syndrome also seems to be associated with cognitive abnormalities. However, patients with Legius syndrome have neither Lisch nodules in the iris nor neurofibromas or central nervous system tumors. The syndrome is caused by a mutation in the SPRED1 gene at 15q14, and the gene product is part of the Sprouty/SPRED proteins, which are negative regulators of Ras.

Genotype-Phenotype Correlations in NF1

Only two genotype-phenotype correlations have been reported for the NF1 gene, leading to speculations on possible modifiers of the haploinsufficient state of the NF1 gene product. The first correlation relates to patients with NF1 and NF1 microdeletions, which cover the entire NF1 gene and lead to a severe form of NF1. Patients with an NF1 microdeletion have a higher burden of cutaneous neurofibromas, may have dysmorphic features, and often develop learning disabilities. The second association involves a specific, 3-bp, in-frame deletion in exon 17 of the NF1 gene, a condition that is characterized by a mild phenotype in many patients. These patients show typical pigmented features of NF1 and Lisch nodules but do not develop cutaneous or clinically detectable plexiform neurofibromas.

Mild forms of NF1 can be caused by mosaicism, a situation in which the NF1 mutation occurs after fertilization. Somatic mutations occurring early during embryonic development produce generalized mosaicism, which is characterized by a mild disease indistinguishable from classic NF1. Mutations occurring at a later stage of embryonic development result in segmental NF1, where manifestations are restricted to one segment of the body. Gonadal mosaicism is a rare form of mosaicism; the mutation is present only in the gonads, and it can be suspected if two or more children of unaffected parents have NF1. It has been speculated but, to our knowledge, not published that mosaicism can explain at least some of the cases in which a mildly affected patient with no family history of NF1 has a child who develops a severe form of the disease.
**Regulation of NF1 Gene Expression**

The expression of NF1 is regulated at multiple levels, resulting in levels of NF1 mRNA and protein that can vary considerably, even within minutes (Table 1). Proteins interacting with either NF1 mRNA or the protein or causing their degradation can be considered as modifiers of NF1 expression. Only one protein, the tumor antigen HuR, is known to interact with NF1 mRNA, binding specifically to one of the five potential protein-binding sites of its 3′ untranslated region. In general, HuR has been shown to be involved in posttranscriptional mRNA stabilization and transport from the nucleus. HuR has also been associated with the regulation of tumor growth. In the cytoplasm of migrating and cell junction–forming cells, some of the NF1 mRNA is targeted to the cell periphery via actin microfilaments.

Furthermore, the silencing of NF1 mRNA expression and the rapid oscillation of NF1 mRNA and protein levels highlight the complexity of regulation of the NF1 tumor suppressor gene. A recent study has shown that a microRNA, miR-10b, may regulate NF1 tumorigenesis by targeting the NF1 mRNA 3′ untranslated region and modulating the Ras signaling pathway. In general, microRNAs hybridize to the 3′ untranslated region of target mRNAs and can repress mRNA translation or mediate its cleavage.

**NF1 as a Tumor Suppressor Gene**

All individuals with NF1 are heterozygous for an NF1 mutation that causes haploinsufficiency, and, thus, the single functional copy of the gene does not produce enough neurofibromin to ensure normal development and the regulation of cell growth, thereby leading to a diseased state. Note that mutations of the NF1 gene have also been detected in cancers unrelated to NF1.

Some distinct lesions characteristic of NF1 are associated with the loss of both functional NF1 alleles in certain cell types. A somatic “second-hit” mutation has been demonstrated in several benign and malignant tumors in NF1, and it is best characterized in a subset of Schwann cells present in neurofibromas. These facts support the view that the NF1 gene is a tumor suppressor gene that follows the Knudson two-hit hypothesis of tumor formation. In general, somatic inactivation of a tumor suppressor can occur via three principal mechanisms: loss of heterozygosity (LOH), somatic intragenic mutations, and promoter hypermethylation. The mechanisms leading to LOH are mitotic recombination, chromosomal microdeletion, and chromosome loss with reduplication.

**The Spectrum of NF1 Second-Hit Mutations**

The mutational spectrum differs between NF1 germline mutations and somatic or second-hit mutations. A recent study revealed a high occurrence of somatic frameshift mutations and also showed that deletions comprising at least 4 nucleotides are remarkably more common as second-hit mutations, whereas the frequency of somatic missense mutations is lower than that observed in the NF1 germline mutational spectrum. In the same study, two separate mutations were detected in 26 of 29 cutaneous neurofibromas, and the remaining 3 had LOH. In a recent study, NF1 diploinsufficiency was found in all nine neurofibromas, seven of which had a unique second mutation and two LOH. Thus, LOH does not emerge as a leading cause for the inactivation of both NF1 alleles in cutaneous neurofibromas. Collectively, earlier studies have reported LOH to be responsible for somatic inactivation in approximately 20% of neurofibromas sampled from patients without microdeletions. Furthermore, the spectrum of somatic mutations differs between NF1 microdeletion patients and the general NF1 population, revealing an absence of LOH as a second hit in patients with NF1 microdeletions.
Pathoetiology of the Lesions in NF1

NF1 is characterized by the variability of its clinical manifestations, whose pathoetologies remain, in many cases, rather elusive. These manifestations include Lisch nodules, learning disabilities, speech disorders, and a variety of skeletal manifestations, such as short stature, osteopenia/osteoporosis, and lytic bone lesions. A bi-allelic inactivation of the NF1 gene has been demonstrated in neurofibromas, café-au-lait macules, and glomus tumors. In addition, LOH of the NF1 gene has been shown in two patients with NF1 and pseudarthrosis. Bi-allelic inactivation has also been observed in certain NF1-related malignancies, such as malignant peripheral nerve sheath tumors (MPNSTs), pheochromocytomas, astrocytomas, and juvenile myelomonocytic leukemia. These observations raise the important questions as to what symptoms and signs are caused by haploinsufficiency as such and what is the role played by the loss of both NF1 alleles.

NF1 Lesions Caused by LOH or Somatic Second-Hit Mutations

Café-au-lait macules in adults are well circumscribed, uniformly light brown to dark brown macules, averaging 2 to 5 cm in diameter. They can be present at birth, although they usually become detectable during early childhood, growing proportionately to body growth, but they have no tendency toward malignancy. De Schepper et al showed that melanocytes cultured from café-au-lait macules of patients with NF1 carry a somatic or second-hit mutation in the NF1 gene, suggesting that the melanocyte is the primary pathogenic cell in these pigmented lesions. This notion increases the understanding of the pathoetiology of these lesions. Loss of expression of the wild-type allele without a detectable second-hit mutation was shown to affect melanocytes derived from café-au-lait macules of one patient with NF1.

Glomus tumors are benign but painful tumors of the fingertips and toes. Affected individuals present with the triad of severe paroxysmal pain, cold intolerance, and localized tenderness. The tumors arise from the glomus body, a highly innervated thermoregulatory shunt containing concentric layers of contractile α-actin-positive glomus cells, which harbor bi-allelic inactivation of the NF1 gene. Glomus tumors may affect up to 5% of the adult NF1 patient population.

Neurofibromas are lesions characterized by bi-allelic inactivation of the NF1 gene in a subpopulation of Schwann cells. The current understanding of the key role of Schwann cells is based on studies with conditional knockout mice and the genetic analysis of cells cultured from human neurofibromas. These studies have collectively shown that bi-allelic inactivation of the NF1 gene is a prerequisite for neurofibroma formation but that tumorigenesis can occur only in an NF1-/- background. Neurofibromas can be classified into two main categories: cutaneous and plexiform. Both are benign hamartomatous tumors traditionally considered to arise from the supporting and connective tissue cells of peripheral nerves. Neurofibromas contain all elements of peripheral nerves but organized in a haphazard manner: histological analysis, immunohistochemical analysis, and electron microscopy have revealed cellular differentiation characteristic of Schwann cells, which are the most numerous, fibroblasts, perineural cells, mast cells, axonal processes, and blood vessels, all embedded in an abundant collagenous extracellular matrix. However, the cutaneous and plexiform neurofibromas display marked differences: their maximal growth rates follow different timetables, their average size is quite different, and the plexiform neurofibromas have a marked potential for malignant transformation, whereas the cutaneous neurofibromas never turn malignant. The pathogenesis of these different tumor types should, therefore, be considered separately. Several mouse models have been generated to elucidate the pathogenesis of neurofibromas. However, these models only partially reflect the human disease, and most mouse studies have focused exclusively on plexiform neurofibromas.

Pathogenesis of Plexiform Neurofibromas

Approximately one-third of patients with NF1 develop clinically detectable plexiform neurofibromas. These tumors may form large disfiguring masses involving nerve trunks and infiltrating the surrounding tissues. Plexiform neurofibromas are thought to be congenital lesions, and the risk of their malignant transformation highlights their clinical importance. The bi-allelic inactivation leading to the development of plexiform neurofibromas apparently takes place in embryonic cells that have the potential to generate large populations of cells carrying a single NF1 second-hit mutation. The growth of plexiform neurofibromas during early childhood distinguishes these tumors from cutaneous neurofibromas, which are undetectable before puberty.

Mouse studies have demonstrated that Nf1-/- Schwann cells are necessary, but not sufficient, for tumor formation to occur. Parada and coworkers demonstrated that Nf1floxflox;Krox20cre mice, in which 5% to 10% of Schwann cells are nullizygous for Nf1 in an Nf1 heterozygous background, developed plexiform neurofibromas. In contrast, Nf1floxflox/floxflox;Krox20cre mice with both Nf1 alleles inactivated only in Schwann cells (other cells are wild type) failed to develop neurofibromas. Thus, tumor progression apparently requires interactions between Nf1-/- Schwann cells and Nf1+/- cell lineages in the tumor microenvironment. Mouse plexiform neurofibromas fail to develop if the Nf1+/- bone marrow is replaced with bone marrow from an Nf1+/- animal, suggesting that the growth and maintenance of neurofibromas is partially dependent on the presence of Nf1+/- mast cells. Schwann cells with an Nf1-/- genotype secrete soluble kit ligand, which is chemotactic to mast cells, enhancing mast cell migration, proliferation, and survival. On the other hand, mast cells are also components of normal human and mouse peripheral nerves.

Imatinib mesylate (Gleevec/Glivec; Novartis, East Hanover, NJ), a potential inhibitor of c-kit, was used to treat Nf1floxflox;Krox20cre mice. Volumetric analysis per-
formed with 2-[18F]-fluoro-2-deoxy-D-glucose positron emission tomography showed that imatinib mesylate reduced tumor volume and metabolic activity by approximately 50% in treated mice. This led to the first clinical trial in which a 3-year-old girl with a highly vascularized, nonresectable, and progressively growing neurofibroma was treated with imatinib mesylate. After 3 months of treatment, magnetic resonance imaging indicated a 70% reduction in tumor volume. However, it is still unclear whether these results can be replicated in a wider population of patients with NF1 of varying ages and tumor burdens.

Approximately 2% to 5% of plexiform neurofibromas progress to MNSTs. However, because bi-allelic inactivation of the NF1 gene occurs in benign neurofibromas, mutations in the NF1 gene alone are not sufficient for the progression from a plexiform neurofibroma to an MPNST. Additional mutations in other regulatory genes have been described in MNSTs, including the homozygous deletion of CDKN2A, which encodes p16INK4A, and p14ARF and TP53 loss. Furthermore, karyotypes in many MNSTs are complex. It remains to be elucidated whether NF1-related MNSTs are always derived from plexiform neurofibromas or whether they can arise directly from peripheral nerves.

**Pathogenesis of Cutaneous Neurofibromas**

Cutaneous neurofibromas occur in virtually all adults with NF1. They appear at puberty and increase in number and size with age and pregnancy. However, cutaneous neurofibromas have restricted growth potential; the tumor diameter usually varies between a few millimeters and approximately 2 cm, rarely exceeding 3 cm. A subpopulation of cells cultured from human cutaneous neurofibromas displays characteristics typical of Schwann cells and harbor a bi-allelic inactivation of the NF1 gene. The limited growth of cutaneous neurofibromas may reflect the developmentally late occurrence of the NF1 second-hit mutation essential for their growth. The NF1 diploinsufficient Schwann cells may, thus, have a limited potential for clonal cell division. The clonality of NF1−/− Schwann cells in a discrete neurofibroma is supported by the facts that each cutaneous neurofibroma has its own unique second-hit mutation and that only one second-hit mutation is found in each tumor. The NF1−/− genotype obviously provides these cells with a growth advantage, but it is not known what proportion of the Schwann cells in a cutaneous neurofibroma carries an NF1−/− genotype and how much of the tumor growth can be explained by the increase in the number of other cells with an NF1−/− genotype.

The previous understanding has been that the cutaneous neurofibromas may arise from small nerve twigs. However, Schwann cells do not represent a continuously dividing cell population, which makes them unlikely hosts for the second-hit mutation. The question of the cellular origin of cutaneous neurofibromas has been approached using a mouse model with an Nf1−/− background and a tamoxifen-inducible second hit in dermal stem/progenitor cells. These mice developed cutaneous neurofibromas on topical application of tamoxifen and subsequent double Nf1 gene inactivation. Analogous lineage tracing experiments using human tissues are, however, not currently possible. In addition to the dermis, other potential sources of multipotent cells with high mitotic potentials include subcutaneous fat and hair roots.

Previous histological analyses of apparently normal skin from patients with NF1 revealed minute neurofibromas, presumably in the early stages of development, in the immediate vicinity of the hair follicular apparatus. In a recent study, we showed that human cutaneous neurofibromas contain multipotent NF1−/− precursor cells capable of differentiating in vitro into cell types found in neurofibromas, in-

![Figure 1](Image)

**Figure 1.** Proposed model for the development of cutaneous neurofibromas. As opposed to the traditional understanding of neurofibromas being formed by the dissociation of peripheral nerve components, the proposed model views neurofibromas as resulting from divergent cellular differentiation of multipotent precursor cells. The requirements for the development of neurofibromas include the presence of a clonal population of NF1−/− Schwann cells in a microenvironment harboring other cell types with an NF1−/− genotype. The potential sources of NF1−/− Schwann cells are small cutaneous nerve twigs, hair roots, and subcutaneous fat. The close proximity of hair and incipient neurofibromas may suggest that the multipotent NF1−/− cells residing in this area are the major source of neurofibroma-derived progenitors (NFPs), which can give rise to the different cell types found in the neurofibromas. Animal studies have suggested that the neurofibroma mast cells may be derived from bone marrow. These cellular components alone are, apparently, not sufficient to allow the cutaneous neurofibromas to develop into visible tumors because the latter do not appear before puberty, when marked changes in the hormonal status of an individual take place.
cluding Schwann cells, fibroblasts, and epithelial cells. Together, these results suggest that the multipotent cells present in the hair follicles may contribute to the development of cutaneous neurofibromas.

Conclusions

Figure 1 summarizes the interplay between multiple cell types with NF1+/− genotypes and NF1−/− Schwann cells. In addition to the NF1 nullizygous Schwann cells, neurofibromas contain a high number of other cells with an NF1+/− genotype, including Schwann cells, neurons, perineurial cells, fibroblasts, adipocytes, epithelial cells, and mast cells.

Although the mutations in the NF1 gene are sufficient to cause NF1 syndrome, the pathobiology of the multiple manifestations of the disease in different organ systems seems increasingly complex. The question emerges as to whether the different cellular phenotypes originate from disintegrated peripheral nerves or whether they represent divergent cellular differentiation pathways of multipotent precursor cells.

References

5. Ferber R: The NF1 neurofibromatosis. Prakt Neurol 2010, 10:82–93
16. Le L, Parada L: Tumor microenvironment and neurofibromatosis type 1: connecting the GAPs. Oncogene 2007, 26:4609–4616


