Tumorigenesis in neurofibromatosis: new insights and potential therapies

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The neurofibromatoses NF1 and NF2 are inherited cancer predisposition syndromes in which affected individuals are prone to development of mostly benign, but occasionally malignant, tumors. The NF1 and NF2 genes function as tumor suppressor genes (negative growth regulators), such that their loss of expression predisposes to tumor formation. Neurofibromin, the protein product of the NF1 gene, acts as a negative regulator of the ras proto-oncogene, to reduce cell growth. Merlin, the NF2 gene product, is involved in regulating cell proliferation and motility, and probably plays a role in integrating multiple cell-signaling pathways. By understanding the function involved in regulating cell proliferation and motility, and probably plays a role in integrating multiple cell-signaling pathways, we have a unique opportunity to develop targeted pharmacotherapeutic interventions for these disorders.

Neurofibromatosis 1 and 2 (NF1 and NF2) are autosomal dominant disorders involving the nervous system, in which affected individuals have a propensity for developing both benign and malignant tumors. Because affected individuals have an increased risk of tumor formation, these disorders are classified as inherited cancer syndromes. NF1 affects 1 in 3500 individuals worldwide, whereas NF2 affects 1 in 30 000–42 000 individuals. The prevalence of these syndromes is constant across all ethnic backgrounds and there is no gender predominance. NF1 and NF2 are clinically distinct disorders with different tumors developing in each disorder. Our ability to develop targeted clinical treatments for NF1 and NF2 is heavily dependent on an improved understanding of the molecular biological mechanisms underlying these disorders.

Neurofibromatosis 1
Clinical features
NF1 is also referred to as peripheral neurofibromatosis or von Recklinghausen’s disease and is characterized by the development of pigmentary abnormalities, such as café-au-lait macules, skinfold freckling and iris hamartomas (Lisch nodules). In addition to these features, individuals with NF1 can develop skeletal and vascular abnormalities, and have a greater incidence of certain learning disabilities. Tumors that develop in individuals with NF1 include neurofibromas, optic pathway gliomas, and pheochromocytomas. Although NF1 is inherited by an autosomal dominant mechanism, 50% of diagnosed cases appear to occur without a family history as a result of a new mutation. The diagnostic criteria for NF1 are listed in Box 1 (Ref. 2).

The most common tumor associated with NF1, the neurofibroma, occurs in nearly all patients diagnosed with NF1. Neurofibromas are benign tumors that arise in association with peripheral nerves and are composed of a mixture of Schwann cells, fibroblasts and other cells. They are rarely present in childhood, but develop during puberty and pregnancy, suggesting a hormonal influence on tumor growth. Although they do not transform into malignant tumors, they can cause significant discomfort and disfigurement. Neurofibromas can occur anywhere on the skin and can also arise internally. One subtype of neurofibromata, the plexiform neurofibroma, is thought to represent a congenital lesion. In contrast to the more common discrete neurofibromas, these tumors can grow to enormous proportions and can cause significant morbidity by stimulating underlying bone growth or by compressing surrounding tissues. Additionally, plexiform neurofibromas harbor a 5% lifetime risk of transformation into malignant peripheral nerve sheath tumors (MPNSTs). MPNSTs are highly malignant and metastatic cancers with a high mortality and a poor response to chemotherapy and radiation. Other less common tumors seen in NF1 include optic pathway gliomas and myelodysplastic disorders (see below).

NF1 gene
Since the identification of the NF1 gene by positional cloning in 1990 (Refs 3–5), we have learned a great deal about the action of the NF1 gene product and how its dysfunction might result in tumor formation. The NF1 gene is located on chromosome 17q11.2, comprises 350 kb of genomic DNA, with 60 exons and encodes an mRNA of 11 to 13 kb. The NF1 gene product, a protein called neuro-fibromin, is expressed in neurons, oligodendrocytes and Schwann cells. It is also found ubiquitously in white blood cells, the adrenal medulla and many other cell types. In the brain, it is expressed in neuronal dendrites and axons, and might be associated with the neuronal cytoskeleton. Sequence analysis of neurofibromin reveals a region of homology with p120-GAP, the GTPase-activating protein (GAP) for the ras family of oncogenes (Fig. 1a). Exons 20–27a of neurofibromin encode this ‘GAP related domain’ (GRD), which serves to accelerate the GTP-hydrolysis activity and thus downregulate p21-ras (ras). This is the only region of the neurofibromin protein whose biological function is known.
Ras is a 21-kDa protein located in the plasma membrane and is critical for the regulation of cellular growth and differentiation. Ras resides in an active form when bound to GTP (Fig. 1b). When GTP is hydrolyzed to GDP, ras is inactivated. In many cell types, the active form of ras can stimulate cell proliferation or differentiation. Additionally, activating mutations in ras are common in some sporadic tumors of non-NF1 patients, and have been implicated in malignant transformation.

The NF1 gene is expressed in many human tissues as several transcript isoforms that result from the variable inclusion of three alternatively spliced exons (9a, 23a and 48a). Neurofibromin containing exon 48a is found in muscle tissues, whereas exon 23a appears to be expressed in a wide variety of tissues. The neurofibromin isoform containing exon 9a is expressed only in forebrain neurons and appears to be developmentally regulated, with expression first detected early in human postnatal life. Further studies will be required to determine whether neurofibromin with exon 9a specifies a unique neurofibromin GAP-like activity and increased levels of activated ras (Refs. 12, 13). Increased ras activity in association with neurofibromin loss has now been described in NF1-associated leukemias, malignant peripheral nerve sheath tumors and low-grade gliomas. These results strongly suggest that neurofibromin functions as a growth regulator by inhibiting ras activity, independent of the expression of other ras regulators, like p120GAP.

Molecular pathogenesis of NF1-associated tumors
Understanding why particular tumor types arise in NF1 patients is an area of active research. For this reason, many groups have worked to develop mouse models of NF1. Mice with targeted abnormalities in both allelics of the Nf1 gene die during embryogenesis from a cardiac vessel defect, whereas Nf1 heterozygote mice die by 15 months from leukemias and pheochromocytomas. In an effort to develop more sophisticated models for NF1-associated tumors, mice have been generated that are chimeric for Nf1 loss, such that a small minority of the cells in their body have both copies of the Nf1 gene inactivated. These Nf1 chimeric mice are viable and some of these animals develop tumors that are histologically similar to the plexiform neurofibromas seen in NF1 patients. Further refinement of animal models using tissue-specific Nf1 gene inactivation is likely to provide critical insights into the roles of neurofibromin in specific cell populations relevant to tumor formation.

Benign cutaneous neurofibromas, comprised of fibroblasts and Schwann cells, are the most common tumor type in NF1. Recently, it was postulated that genetic errors is the unifying basis for development of NF1-associated features.

In support of the idea that neurofibromin functions as a rasGAP, several groups have demonstrated that cell lines derived from tumors of NF1 patients have undetectable levels of neurofibromin, decreased neurofibromin GAP-like activity and increased levels of activated ras (Refs. 12, 13). Increase in association with neurofibromin loss has now been described in NF1-associated leukemias, malignant peripheral nerve sheath tumors and low-grade gliomas. These results strongly suggest that neurofibromin functions as a growth regulator by inhibiting ras activity, independent of the expression of other ras regulators, like p120GAP.

Box 1. Diagnostic criteria for neurofibromatosis 1 (NF1)*

Two or more of the following features must be present for an individual to be diagnosed with NF1:

- Six or more café-au-lait spots with diameters greater than 0.5 mm before puberty or 1.5 cm after puberty
- Two or more neurofibromas or a single plexiform neurofibroma
- Freckling in the axillary and inguinal regions (Crowe’s sign)
- Optic pathway tumor
- Lisch nodules (hamartomas of the iris)
- A distinctive bony lesion: dysplasia of the sphenoid bone or dysplasia/thinning of long bone cortex
- A first-degree relative diagnosed with NF1


Fig. 1. Neurofibromin and ras regulation. (a) Neurofibromin contains a central region of sequence homology shared with a family of proteins that accelerate the conversion of ras from its active GTP-bound conformation to its inactive GDP-bound form. The inactivation of ras by neurofibromin GAP activity leads to regulated cell growth. (b) Loss of neurofibromin in tumors as a result of Nf1 gene inactivation leads to increased ras activity and increased cell growth.
S100+ cells might be primarily responsible for the development of neurofibromas\(^2\). These S100+ cells are believed to be Schwann cells, although they do not always express myelin P0, which is a specific marker of differentiated Schwann cells. S100+ cells from neurofibroma tissues of NF1 patients lack \(NF1\) mRNA and thus do not express the protein neurofibromin. Additionally, the Schwann cells in the neurofibromas are probably defective in ras regulation\(^2\). The fibroblasts from the same tumor tissues, however, carry at least one normal \(NF1\) allele, and thus make mRNA and neurofibromin. These findings argue that the defective cell type in the neurofibroma is the Schwann cell. The lack of expression of mature Schwann cell proteins (i.e. myelin P0) seen in S100+ cells raises the possibility that neurofibromin loss might occur in a precursor cell population or might cause the differentiated Schwann cell to revert to a more immature phenotype.

The difference in the mechanisms of tumorigenesis between benign cutaneous neurofibromas and plexiform neurofibromas is unclear, however research by Cappione et al. suggests that the amount of \(NF1\) mRNA processing could play a role in determining which tumor type develops\(^2\). In general, benign cutaneous neurofibromas seem to have the lowest amount of mRNA editing, plexiform neurofibromas have an intermediate amount and malignant peripheral nerve sheath tumors have the most mRNA editing of the \(NF1\) gene. These differences in mRNA processing could relate to the clinical variability of tumor expression in NF1 patients.

Malignant peripheral nerve sheath tumors (MPNSTs; formerly called neurofibrosarcomas) are likely to require more than one genetic alteration for tumor development. Previous work on human MPNSTs demonstrated that, in addition to inactivation of the \(NF1\) gene on chromosome 17, about half of the tumors also harbored mutations in another tumor suppressor gene (\(p53\))\(^2\). This genetic cooperativity suggests that multiple genetic events are required for the progression from benign neurofibroma to MPNST. In support of this hypothesis, two groups have shown that loss of both \(NF1\) and \(p53\) in mice genetically engineered with targeted disruptions of these tumor suppressor genes develop high-grade MPNSTs reminiscent of the human malignant tumors\(^2\). In these studies, mice with mutations in both \(NF1\) and \(p53\) developed soft tissue sarcomas with a 100% frequency between the ages of three and seven months. The requirement of two or more mutations for the development of an MPNST is consistent with the low incidence of these malignant tumors in clinical practice.

Optic gliomas represent the second most common tumor seen in NF1 patients\(^2\). Approximately 15% of children with NF1 will develop optic pathway tumors, which are WHO grade I pilocytic astrocytomas that tend to behave clinically in a benign or slowly-progressive fashion. These tumors most frequently involve the optic nerves and chiasm, but can also be observed in the hypothalamus or brainstem. In resting astrocytes, neurofibromin expression is low; however, in response to several physiologic stimuli, expression can be dramatically increased \(in\ \textit{vitro}\) and \(in\ \textit{vivo}\). Support for a role of neurofibromin as a growth regulator in astrocytes derives from several studies. First, increased astrogliosis has been observed in brains from patients with NF1 (Ref. 10). Second, mice heterozygous for a targeted mutation in the \(NF1\) gene demonstrate increased astrocyte proliferation \(in\ \textit{vitro}\) and \(in\ \textit{vivo}\), which is associated with increased ras pathway activation\(^2\). Third, increased ras activity was detected in one NF1 patient astrocytoma associated with loss of neurofibromin expression\(^1\). Based on these observations, it is likely that neurofibromin acts as a negative growth regulator for astrocytes.

As only a minority of NF1-associated astrocytomas exhibit clinical progression, it is possible that additional genetic alterations contribute to the pathogenesis of symptomatic clinically progressive optic pathway/hypothalamic tumors in NF1.

Malignant myeloid diseases, including juvenile chronic myeloid leukemia (JMML), preleukemic myelodysplastic syndrome (MDS) and myeloproliferative syndrome (MPS), are rarely malignant tumors seen in patients with NF1. Analysis of bone marrow DNA from children with NF1 and MPS confirmed that genetic disruption of both copies of the \(NF1\) gene occurred\(^2\). Further work on both the human and mouse NF1-associated leukemias has demonstrated that loss of neurofibromin was associated with increased ras activation. The critical role of ras in the pathogenesis of myeloproliferative disorders is underscored by the observation that children with these malignancies harbor either inactivating mutations of \(NF1\) (NF1 patients), or activating mutations in ras (sporadic cases)\(^3\).

**Neurofibromatosis 2**

**Clinical features**

NF2 is also referred to as central or bilateral acoustic neurofibromatosis\(^4\). Individuals with NF2 develop bilateral vestibular schwannomas affecting the VIIIth cranial nerve and meningiomas, as well as schwannomas involving other cranial or peripheral nerves. The vestibular schwannomas can often grow to affect the temporal bone or eighth cranial nerve function. Unlike in NF1, NF2 patients have few pigmentary abnormalities, such as café-au-lait spots, and the majority of their symptoms relate to tumor formation. Other associated features can include posterior lenticular opacities (juvenile cataracts) and retinal abnormalities. A number of proposed diagnostic criteria for NF2 have been suggested over the past 15 years to improve upon the early detection of individuals at risk for NF2 (Ref. 2) (Box 2).
The mean age of onset of NF2-associated clinical symptoms is in the early twenties. The presenting symptom is most often related to the development of vestibular schwannomas, with hearing loss and balance problems. There are individuals with NF2 who present early in life (‘Wishart’ variant) and often exhibit a more rapid, aggressive clinical course. These patients tend to have many tumors in addition to the bilateral vestibular schwannomas. Other individuals present later in life (‘Gardner’ variant) and their clinical course tends to be less severe, with fewer tumors.

**NF2 gene**

The *NF2* gene is located on chromosome 22q12.2. The *NF2* gene was identified in 1993 by two groups, and comprises 17 exons encoding a 595 amino acid protein (Fig. 2a). The protein product of *NF2* – termed merlin or schwannomin – has homology in the first 13 exons to the ERM (ezrin, radixin and moesin) family of proteins, which are thought to play a role in linking cytoskeletal components to cell membrane glycoproteins. However, unlike these other ERM proteins, merlin can suppress cell growth and thus acts as a tumor suppressor. Whereas ERM proteins bind strongly to the actin cytoskeleton through residues in the carboxyl terminus of the protein, merlin weakly associates with actin in vitro through a domain in its amino terminus.

Mutations in the protein product of the *NF2* gene may result from a single site mutation or from larger mutations (deletions or insertions), mostly in the first 13 exons that lead to a truncated and nonfunctional protein. Clinically, patients with mutations that result in premature protein termination tend to exhibit a more severe clinical course of NF2, with symptoms starting before age 20 and the development of two or more CNS tumors before age 30 (Ref. 36). Single amino acid changes (missense mutations), on the other hand, are often associated with more mild clinical disease.

**Molecular pathogenesis of NF2-associated tumors**

Several lines of evidence support the hypothesis that merlin is a negative growth regulator (tumor suppressor). Deficiency of merlin, secondary to disruption of the *NF2* gene, has been reported for all tumors associated with NF2, as well as many sporadic schwannomas and meningiomas. Regulated overexpression of merlin in schwannoma cells and viral transduction of the *NF2* gene into meningioma cells results in growth suppression in vitro and reduced tumor growth in vivo. Mice in which both copies of the *Nf2* gene are disrupted die early in embryogenesis as a result of a failure to induce mesoderm formation. This suggests that merlin might be necessary for regulating cellular growth and differentiation in early tissue formation during embryogenesis. Mice heterozygous for an *Nf2* mutation are viable, but succumb to malignant sarcomas.

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The association of merlin with the actin cytoskeleton could have functional relevance to the role of merlin as a tumor suppressor. In human and rat schwannoma and meningioma cells, merlin localizes with F-actin at the leading cell membrane in vitro, and is located in Schwann cells along paranodal incisures in the rat sciatic nerve in vivo. Loss of merlin expression in NF2-associated schwannoma cells is associated with dramatic alterations in the actin cytoskeleton. Regulated overexpression of wild-type, but not mutant, merlin results in dramatic alterations in actin cytoskeleton organization during cell spreading, abnormalities in cell attachment and reduced cell motility. Similarly, malignant tumors that arise in mice with a targeted mutation in the Nf2 gene have highly motile and metastatic cells.

Unlike neurofibromin, it is not clear how merlin regulates cell growth or motility. Recent work based on the identification of proteins that specifically interact with merlin suggests that merlin might function to integrate a number of intracellular signaling pathways (Fig. 2c). In this regard, merlin binds to CD44 – a hyaluronic acid receptor important in growth regulation – and promotes growth arrest. Additionally, merlin also associates with the hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate, HRS. HGF is one of the most powerful stimuli for Schwann cell proliferation and motility, suggesting that the association of merlin with HRS might inhibit HGF-mediated cell growth and movement (Fig. 2c). Further work is required to determine how merlin might link these two pathways in vivo.

As mentioned above, Nf2 knockout mice develop malignant tumors that are not seen in humans affected with NF2. Using a tissue-specific Nf2 gene disruption strategy, Giovannini and colleagues developed mice with loss of Nf2 specifically in Schwann cells. These mice develop cranial nerve schwannomas similar to the tumors seen in patients with NF2. The development of these more sophisticated animal models of NF2 might help to elucidate the function of merlin in cell growth and differentiation.

Future targeted therapies

Over the past few years, we have learned a tremendous amount about the function of the NF1 and NF2 gene products. Based on these insights, it is now possible to consider applying treatments that specifically target the cellular abnormalities in NF1 and NF2. In the case of NF1, we now appreciate the critical role of ras regulation in cell proliferation (Fig. 3a). Potential therapies include inactivation of ras by preventing it from undergoing secondary post-translational modifications, such as farnesylation. The addition of a farnesyl group to a specific region of the ras protein allows ras to translocate to the membrane and initiate ras pathways in vivo. Inhibition of the enzyme involved in this process using farnesyl transferase inhibitors has recently found its way into early clinical trials for NF1-associated plexiform neurofibromas. In addition, it might be important to consider targeting more specific downstream effectors of ras, such as vascular endothelial growth factor, mitogen activated protein kinase (MAPK), and HGF-R.

Outstanding questions

- Are there tissue-specific functions for the NF1 and NF2 gene products that need to be determined to design tissue-specific targeted therapies?
- Which molecules downstream of ras transduce its growth-promoting signal in response to the absence of NF1 protein (neurofibromin) in Schwann cells and astrocytes?
- How does the NF2 protein (merlin) function in Schwann and leptomeningeal cells?
- What are the other genetic influences that determine whether an affected individual with NF1 will manifest with a brain tumor versus leukemia?
kinase and other molecules activated by ras (e.g. Rac and Rho) to achieve better clinical results.

Because less is known about the specific pathways involved in transducing merlin’s growth suppressive signal, the design of future therapeutics will have to await improved insights into the function of merlin (Fig. 3b). Potential drug therapies could be directed at replacing the function of merlin relevant to CD44 and HRS signaling. A review with more detail about some of these therapies was recently written by Weiss et al. This exciting area of clinical research will continue to improve the quality of life for neurofibromatosis patients and could someday help to eliminate many of their most serious symptoms.