Neurofibromatosis Type 1 and tumorigenesis: molecular mechanisms and therapeutic implications

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Neurofibromatosis Type 1 (NF1) is a common autosomal dominant disease characterized by complex and multicellular neurofibroma tumors, and less frequently by malignant peripheral nerve sheath tumors (MPNSTs) and optic nerve gliomas. Significant advances have been made in elucidating the cellular, genetic, and molecular biology involved in tumor formation in NF1. Neurofibromatosis Type 1 is caused by germline mutations of the NF1 tumor suppressor gene, which generally result in decreased intracellular neurofibromin protein levels, leading to increased cascade Ras signaling to its downstream effectors. Multiple key pathways are involved with the development of tumors in NF1, including Ras/mitogen-activated protein kinase (MAPK) and Akt/mammalian target of rapamycin (mTOR). Interestingly, recent studies demonstrate that multiple other developmental syndromes (in addition to NF1) share phenotypic features resulting from germline mutations in genes responsible for components of the Ras/MAPK pathway. In general, a somatic loss of the second NF1 allele, also referred to as loss of heterozygosity, in the progenitor cell, either the Schwann cell or its precursor, combined with haploinsufficiency in multiple supporting cells is required for tumor formation. Importantly, a complex series of interactions with these other cell types in neurofibroma tumorigenesis is mediated by abnormal expression of growth factors and their receptors and modification of gene expression, a key example of which is the process of recruitment and involvement of the NF1+/− heterozygous mast cell. In general, for malignant transformation to occur, there must be accumulation of additional mutations of multiple genes including INK4A/ARF and P53, with resulting abnormalities of their respective signal cascades. Further, abnormalities of the NF1 gene and molecular cascade described above have been implicated in the tumorigenesis of NF1 and some sporadically occurring gliomas, and thus, these treatment options may have wider applicability. Finally, increased knowledge of molecular and cellular mechanisms involved with NF1 tumorigenesis has led to multiple preclinical and clinical studies of targeted therapy, including the mTOR inhibitor rapamycin, which is demonstrating promising preclinical results for treatment of MPNSTs and gliomas. (DOI: 10.3171/2009.11.FOCUS09221)

KEY WORDS • optic nerve glioma • loss of heterozygosity • malignant peripheral nerve sheath tumor • neurofibroma • plexiform neurofibroma

Neurofibromatosis Type 1 is a common autosomal dominant disease and is characterized by neural crest-derived tumors.18,101 The key feature of NF1, neurofibromas, are complex tumors arising from peripheral nerve sheaths.59,101 Neurofibromas consist primarily of Schwann cells, fibroblasts, and a large amount of extracellular matrix with collagen surrounding an axon, but they also contain many other cell types including perineurial cells, mast cells, pericytes, and endothelial cells.9,59,80,85,101 Malignant tumors can arise in either childhood or adulthood, with MPNSTs being most common.10 In addition to the lesions associated with the peripheral nervous system, patients with NF1 are also at risk for CNS tumors, specifically gliomas.2,42 In childhood, these gliomas are primarily located in the optic pathway and less frequently in the hypothalamus and brainstem; adults are more likely to develop higher-grade gliomas.2

Our group has previously published an overview of the genetics and pathogenesis of neurofibromas in NF1.38 In the present paper, we update the relevant information related to the genetic alterations that predispose individuals to this tumor and also discuss the mechanisms involved in the tumorigenesis of MPNSTs and optic nerve gliomas, with attention on implications for targeted therapies (not previously presented).

Genetics

As background information, we include here a brief summary of the genetics of this disease. In this auto-

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somal dominant–inherited condition, in which homozygosity is lethal to embryos, all affected individuals are heterozygous for an NF1 mutation. One NF1 allele carries a genetic alteration in all cells of a patient with NF1, and therefore, a loss of the second NF1 allele (loss of heterozygosity, LOH) results in complete functional loss of neurofibromin and risk of tumor formation. Many mutations in the NF1 gene result in truncation of the protein product, neurofibromin. Approximately half of cases represent new mutations, and a mutation at a given locus may provide a selective and proliferative advantage in a germ-cell precursor.

Positional cloning identified the NF1 gene at 17q11.2. It produces an mRNA that is expressed in almost all tissues but most highly in brain, spinal cord, and the peripheral nervous system. Neurofibromin, the protein product of the NF1 gene, is found in neurons, oligodendrocytes, and Schwann cells in adults, and is also expressed in other cell types, such as keratinocytes, adrenal medulla, and white blood cells. Neurofibromin is reduced or absent in neurofibroma cells, which are composed principally of Schwann cells without functional neurofibromin.

**Signal Transduction Pathways**

Although it only occupies a small area of the protein (360 amino acids), the Ras-GTPase activating protein (GAP)-related domain (GRD) is an important functional region of neurofibromin that stimulates the intrinsic GTPase of p21-Ras-GTP to hydrolyze GTP to GDP, inactivating the active Ras-GTP is the main function of neurofibromin.

The functional domain of neurofibromin, Ras, acts as...
part of a signal transduction pathway that is activated by growth factors and their receptors. Increased Ras-GTP leads to increased signaling through Raf kinase, which activates a kinase cascade involving MEK kinase and the Erk1 and Erk2 isoforms of MAPK resulting in cell proliferation. Increased Ras-GTP also protects cells from apoptosis by activating mTOR. Studies confirm that neurofibromin negatively regulates this mTOR pathway with loss of neurofibromin expression in established human neurofibroma cell lines associated with high levels of mTOR activity. The mTOR pathway is constitutively activated in both NF1-deficient primary cells and human tumor, is dependent on Ras and PI3K activation, and is mediated by the phosphorylation and inactivation of the TSC2-encoded protein tuberin by AKT. Overall, Ras is a key component of many growth factor signaling pathways, and in the absence of neurofibromin it is constitutively activated, resulting in increased cell proliferation and survival.

The Ras/MAPK pathway is critical to normal development by its regulation of cell proliferation, differentiation, motility, growth, apoptosis, and cell senescence. Interestingly, there are multiple developmental syndromes in addition to NF1 that form tumors by abnormalities in the Ras pathway, and they are referred to as the “RASopathies” or “neuro-cardio-facial-cutaneous syndromes.” These diseases include Noonan syndrome, LEOPARD syndrome, Costello syndrome, capillary malformation–arteriovenous malformation (CM-AVM) syndrome, cardio-facio-cutaneous (CFC) syndrome, and Legius syndrome, have considerable phenotypic overlap. Thus, targeted molecular treatment may be effective for multiple diseases. Figure reproduced with permission from Tidyman and Rauen.
increased signal transduction. Each syndrome exhibits unique phenotypic features; however, there are overlapping clinical features including characteristic facial features, cardiac defects, skin abnormalities, developmental delay, and a risk of malignancy. Interestingly, there are several NF1-related pathways that are significant in the pathogenesis of other developmental syndromes, including mTOR, which contributes to tuberous sclerosis. Denayer and colleagues noted that these syndromes with common molecular pathways demonstrated the important roles for evolutionarily conserved pathways not only in oncogenesis, but also in cognition, growth, and development.

Malignant Peripheral Nerve Sheath Tumors

Additional molecular cascades may be involved in tumorigenesis for malignant NF1 tumors. For example, Ral overactivation is a novel cell signaling abnormality in MPNSTs. Of note, overactivation of Ras and many of its downstream effectors occurred in only a fraction of MPNST cell lines, but Ral was overactivated in all MPNST cells and tumor samples. Silencing Ral or inhibiting it with a dominant-negative Ral caused a significant reduction in proliferation, invasiveness, and in vivo tumorigenicity of MPNST cells. Also, neurofibromin is shown to regulate Ral activation, and NF1-GRD treatment caused a significant decrease in proliferation, invasiveness, and cell cycle progression, and increased cell death. The mTOR pathway has also been shown to have an important role in NF1-associated malignancies including MPNST.

Among the genetic abnormalities previously reported to be involved in the transformation to malignancy in NF1, the loss or mutation of the P53 gene in NF1-related MPNSTs (but never in benign neurofibromas) is associated with a poor prognosis. The loss of the P53 gene results in abnormalities in DNA damage–induced cell cycle arrest and apoptosis. Approximately half of MPNSTs in patients with NF1 show homozygous deletions at the CDKN2A (also known as INK4A or INK4A/ARF) gene, which contributes to increasing cellular proliferation. Also, MPNSTs demonstrate a high frequency of microsatellite instability, and thus, additional loci become targets for mutations during the malignant transformation.

Gliomas

There is evidence that the NF1 gene is involved in the tumorigenesis of both NF1-related and sporadically occurring gliomas. For example, a genomic analysis of human glioblastoma multiforme identified that the NF1 gene was mutated in 15% of tumors. As in other NF1 tumors, biallelic NF1 gene inactivation is observed in NF1-associated gliomas and the heterozygous supporting cell or astrocyte is also important for tumorigenesis. Of note, McGillicuddy et al. found that NF1 function is inactivated in sporadic gliomas, and it occurs by both proteasomal degradation and genetic loss. For example, neurofibromin protein destabilization is triggered by the hyperactivation of protein kinase C (PKC) and thus, these tumors may be sensitive to PKC inhibitors. They found that complete genetic loss of NF1 occurs when p53 is inactivated and allows sensitivity to mTOR inhibitors. Yeh et al. found that there is variability in NF1 gene expression in distinct populations of glioma and increased glial cell proliferation in the optic nerve and brainstem but not in other areas after NF1 inactivation in vitro and in vivo, and thus differences in NF1 gene expression might contribute to the regional localization of brain tumors. Recent studies also demonstrated that the mTOR pathway is involved in tumorigenesis of gliomas in NF1 and its inhibitor rapamycin is effective in decreasing tumor proliferation in an animal model.

Neurofibromas

Neurofibromas are unique among tumors because they exhibit extensive cellular heterogeneity. Reciprocal signaling among the many cell types is known to occur in normal peripheral nerve sheath and in neurofibromas. and decreased neurofibromin in neurofibromas may result in altered responses. Despite this heterogeneity, the Schwann cell lineage has been recognized as the primary cell type for neurofibroma formation. This cell type is the major one amplified in neurofibromas and is the primary target of growth factors stimulating neurofibroma formation. In addition, the loss of both NF1 alleles occurs exclusively in the Schwann cell, whereas a wild-type gene is retained in the other neurofibroma cells, including fibroblasts. This Schwann cell, which lacks functional neurofibromin, has a substantial growth advantage and a loss of negative autocrine growth control.

The stage of differentiation of the Schwann cell as the progenitor cell for NF1 tumors is still the subject of some debate. In 2009, Le and coworkers proposed that the neural crest stem cell is the cell of origin for cutaneous neurofibromas after identifying a population of stem/progenitor cells residing in the dermis (called skin-derived precursors) that, through loss of NF1, form neurofibromas. Interestingly, in another study, Joseph et al. showed that stem cells did not persist postnatally in the location of future neurofibromas or MPNSTs and proposed that the cell of origin is a more differentiated glial cell. Finally, Zheng and coworkers noted that plexiform neurofibromas result from NF1 deficiency in progenitor cells of peripheral nerves. These cells did not cause hyperproliferation or tumorigenesis in the early postnatal period and peripheral nerve development appeared largely normal except for abnormal Remak bundles, the nonmyelinated axon-Schwann cell unit, identified in postnatal mutant nerves. Subsequent degeneration of abnormal Remak bundles occurred concurrently with expansion of nonmyelinating Schwann cells. These findings led the authors to suggest that abnormally differentiated Remak bundles might be the cell of origin for plexiform neurofibroma.

Haploinsufficiency of the NF1 gene in the microenvironment of neurofibromas contributes to its tumorigenicity. The single active NF1 allele in the heterozygous supporting cell does not generate enough functional protein.
Tumorigenesis in NF1 and therapeutic implications

to achieve an appropriate biological response, and this confers a growth advantage that contributes to tumorigenesis. The neurofibromin-deficient NF1-. Schwann cell and other heterozygous supporting cells are absolutely necessary for tumor formation and are critically involved in the pathogenesis of neurofibromas. Also, inflammatory cells, including mast cells, are important to tumor initiation, progression, and angiogenesis. Neurofibromin-deficient Schwann cells secrete increased kit ligand, which serves as a chemoattractant for mast cells expressing c-kit receptor. Also, heterozygous inactivation of NF1- promotes migration of mast cells on αβ1 integrins (mast cell surface proteins), in response to the kit ligand. Overall, the loss of NF1 in Schwann cells results in an increase in growth factor production that initiates a paracrine loop, and it is important for tumor initiation and progression.

Preclinical Models

The development of animal models for preclinical testing in NF1 is key to identifying novel targets for future therapies. Early xenograft models for studying neurofibroma formation produced tumors by injecting human neurofibroma tissue or Schwann cell preparations into the sciatic nerve of immunodeficient mice. Subsequently, NF1 transgene mouse models were developed based on a mutation identified in patients with NF1. It was hoped that these models would prove to be a suitable model for human NF1, but mice heterozygous for the NF1 mutation (NF1+/NF1+) do not develop the classic phenotype of NF1 disease including neurofibromas, and mice that are homozygous for the NF1 mutation die in utero. Later, chimeric mice (NF1/-;NF1/-) were developed by injection of NF1- homozygous mutant embryonic stem cells at an early developmental stage (blastocysts) to overcome the lethality of the germline homozygous genotype with some success. Although the mice developed multiple neurofibromas resembling human plexiform neurofibromas, the cell type in which the NF1 was deleted could not be controlled. Next, a conditional mouse model was developed that resulted in somatic inactivation of NF1 and ablation of neurofibromin function specifically in Schwann cells. This animal model, NF1flox/-;Krox20-cre, provided evidence for the necessity for haploinsufficient supporting cells in neurofibroma formation, and all mice developed plexiform neurofibromas with all of the typical supporting cells (NF1-/-).

A mouse model was developed for MPNSTs by generating mice with mutations in both NF1 and p53 genes. These mice (NF1-/-;p53-/-) developed soft-tissue sarcomas and MPNSTs in neural crest–derived tissue. This model demonstrates that a mutation in the p53 gene in addition to that in NF1 is required for malignant transformation of cells of neural crest origin.

There are also NF1-deficient animal models of NF1 that produce gliomas. In contrast to astrocyte-restricted NF1 conditional knockout mice, heterozygous mice lacking NF1 in astrocytes develop optic nerve gliomas. Thus, NF1 glioma formation requires additional cellular or genetic conditions. This mouse model demonstrates that NF1-/- cells contribute to the pathogenesis of gliomas in NF1 and provides a tool for the preclinical evaluation of potential therapeutic interventions.

Targeted Treatment

Targeted therapy will have a great impact for patients with numerous neurofibromas, very massive plexiform neurofibromas, or MPNSTs for which surgery and radiation have not been widely successful, and as an adjunct or primary treatment of gliomas. Currently, the principal mode of therapy for spinal neurofibromas or plexiform neurofibromas is surgery. Although isolated single–nerve root neurofibromas can be resected without significant morbidity, patients with multiple spinal neurofibromas often require multiple surgeries during their lifetime. Also, patients with large plexiform neurofibromas represent a surgical challenge; have a high recurrence rate if tumors are subtotally resected, intense adherence, and invasion into local tissue; and have a risk of malignant transformation.

Preclinical or clinical studies have targeted various aspects of mast cell functioning, the Ras signaling pathways, rapamycin, and growth factors and receptors, but future therapies may be targeted at almost any molecule involved in NF1 tumorigenesis. In the future, these medical therapies may be useful for treatment of residual tumors, or as a measure to reduce tumor size prior to surgery, or even to decrease the development of tumors. It may also be possible to apply new therapeutic agents to the local tumor environment to reduce the incidence of recurrences.

Preclinical or clinical studies have targeted various aspects of mast cell functioning, the Ras signaling pathways, rapamycin, and growth factors and receptors, but future therapies may be targeted at almost any molecule involved in tumorigenesis (Fig. 1). A number of strategies were discussed in our previous review. Proposed treatments that reduce the mast cell activity, migration, or numbers within neurofibromas and mast cell stabilizers have been considered as possible therapeutic strategies.

Options for treatment include pharmacological inhibition of PI3K, kit activity, or adhesion. The Ras signal transduction pathways have also been targeted for treatments. Anti-Ras therapies are an ideal target, because RAS is one of the most common oncogenes mutated in human malignancies, and Ras-GTP is elevated for both benign and malignant NF1 neurofibromas. Treatment options include the delivery of gene therapy vectors with functional NF1 GRD proteins or with dominant negative Ras, which blocks Ras activity.

Other options include the injection of neutralizing Ras antibodies, which inhibits cell proliferation, or viruses that target and infect cells with activated Ras signaling. For example, the human reovirus requires an activated Ras

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signaling pathway for infection of cultured cells and has been shown to cause tumor regression in mouse models. The downstream effectors of the Ras signaling pathway, including agents that inhibit MEK, PI3K, and P53, are promising targets for future neurofibroma treatment. Newer studies are focusing on the mTOR pathway and its inhibitor rapamycin. Importantly, tumor cell lines derived from patients with NF1, and a genetically engineered cell system that requires Nfi-deficiency for transformation, are highly sensitive to rapamycin. For example, Bhola et al. developed and characterized a human NF1-MPNST explant grown subcutaneously in mice to evaluate the effect of rapamycin. Rapamycin significantly inhibited human NF1-MPNST mTOR pathway activation and growth without systemic toxicities. It was effective at reducing NF1-MPNST proliferation and angiogenesis, but did not increase apoptosis. Rapamycin effectively decreased activation downstream of mTOR, but there was increased AKT activation. This study therefore demonstrated the therapeutic potential and limits of rapamycin in NF1-associated and possibly sporadic MPNST. Johansson et al. demonstrated that rapamycin suppresses the growth of aggressive NF1-associated malignancies in a murine model. Interestingly, they demonstrated that rapamycin does not function via mechanisms generally assumed to mediate tumor suppression, including inhibition of HIF-1α and indirect suppression of AKT, but does suppress the mTOR target Cyclin D1. Finally, in a genetically engineered mouse model of low-grade optic glioma resulting from inactivation of the NFi gene, pharmacological mTOR inhibition in vivo led to decreased tumor cell proliferation in a dose-dependent fashion associated with a decrease in tumor volume. It is important to realize that rapamycin is an effective treatment in other developmental syndromes with abnormalities of the mTOR pathway, including tuberous sclerosis, where it may be efficacious in the management of cognitive or developmental disorders and epilepsy and in preventing or limiting tumor development.

Overall, these medical therapies targeting specific genetic or molecular events involved in NF1 tumorigenesis may be useful for treatment of residual tumors, to reduce tumor size prior to surgery, to decrease the incidence of tumor formation, or to apply to the local tumor environment at the time of resection.

Conclusions

Remarkable progress has been made toward understanding the pathogenesis of neurofibromas since the cloning of the NFi gene in 1990. Neurofibromatosis Type 1 is caused by germline mutations of the NFi tumor suppressor gene, which generally results in decreased intracellular neurofibromin protein, leading to increased cascade Ras signaling to its downstream effectors, including Ras/ MAPK and Akt/mTOR. In general, a somatic loss of the second NFi allele in the progenitor cell, either the Schwann cell or its precursor, combined with haploinsufficiency in multiple supporting cells is required for tumor formation. Importantly, there is a complex series of interactions with these other cell types, such as NFi–heterozygous mast cells, in neurofibroma tumorigenesis. In general, for malignant transformation to occur there must be accumulation of additional mutations of multiple genes including INK4A/ARF and P53. Increased knowledge of molecular and cellular mechanisms involved with NF1 tumorigenesis has led to more accurate animal models of NF1 tumors to evaluate new agents for targeted molecular therapy, including the mTOR inhibitor rapamycin, which are demonstrating promising preclinical results for treatment of MPNSTs and gliomas.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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