Current progress in genomics and targeted therapies for neurofibromatosis type 2

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[Review]

Current progress in genomics and targeted therapies for neurofibromatosis type 2

Ryo Hiruta[1)], Kiyoshi Saito[2)], Mudathir Bakhit[1)] and Masazumi Fujii[1)]

[1)]*Department of Neurosurgery, Fukushima Medical University, Fukushima, Fukushima, Japan,*
[2)]*Department of Neurosurgery, Fukushima Rosai Hospital, Iwaki, Fukushima, Japan*

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Abstract

Neurofibromatosis type 2 (NF2), a multiple neoplasia syndrome, is a manifestation of an impaired expression of the merlin protein, exerting inhibitory effects on cell proliferation signals due to abnormalities of the *NF2* gene located on chromosome 22. About half of patients inherit a germline mutation from a parent, and nearly 60% of *de novo* NF2 patients are estimated to have somatic mosaicism. The development of technical methods to detect *NF2* gene mutation, including targeted deep sequencing from multiple tissues, improved the diagnostic rate of mosaic NF2. With improved understanding of genetics and pathogenesis, the diagnostic criteria for NF2 were updated to assist in identifying and diagnosing NF2 at an earlier stage. The understanding of cell signaling pathways interacting with merlin has led to the development of molecular-targeted therapies. Currently, several translational studies are searching for possible therapeutic agents targeting VEGF or VEGF receptors. Bevacizumab, an anti-VEGF monoclonal antibody, is widely used in many clinical trials aiming for hearing improvement or tumor volume control. Currently, a randomized, double-masked trial to assess bevacizumab is underway. In this randomized control trial, 12 other Japanese institutions joined the principal investigators in the clinical trial originating at Fukushima Medical University. In this review, we will be discussing the latest research developments regarding NF2 pathophysiology, including molecular biology, diagnosis, and novel therapeutics.

Key words : neurofibromatosis type 2, merlin, somatic mosaicism, bevacizumab

Introduction

Neurofibromatosis type 2 (NF2) is a tumorigenesis syndrome characterized by bilateral vestibular schwannoma (VS). The phenotypic expression of NF2 varies in severity, onset, and tumor expression, including intracranial and/or spinal schwannomas, intracranial meningiomas, and ependymomas. Furthermore, the mutation type or location in the *NF2* gene can influence tumor phenotypic variations.

In this review, we will outline clinical characteristics, elucidate the genetic mutations, and discuss recent advances in genetic mutation analysis methods. We will also describe the molecular features of merlin, explore the relationship between genotype and phenotype, and provide an explanation of the diagnostic criteria. Finally, we will consider the potential for new therapeutic interventions and treatment modalities.

Epidemiology

The disorder is estimated to occur in one in 25,000-33,000 births[1,2)]. Although NF2 patients vary in age of onset and clinical phenotype, this disease causes early death due to the critical anatomical location of tumors and the temporal and spatial multiplicity of tumors. NF2-related tumors develop in susceptible organs, including the nervous sys-

Corresponding author : Ryo Hiruta E-mail address : ryo-h@fmu.ac.jp

tem, eyes, and skin, deriving from cells that have lost wild-type (normal) function due to mutation of both alleles of the *NF2* gene. The average onset age of symptoms is 18–24 years, and the average age at death is 36 years[2].

An analysis of the national NF2 registry of the Japanese Ministry of Health, Labour and Welfare, including 807 NF2 cases (44% male, 56% female) enrolled in the period between 2009 to 2013, showed that the ratio of NF2 patients with a family history of NF2 to those without was 1 to 2[3]. The same study found that the onset age varied widely from less than five years to 80 years, with a median age of 24 years. Among patients, 42% had onset at 25 years or older and 45% had onset at less than 25 years (13% were unknown). The prognosis of NF2 differs between the two age-of-onset groups, with the younger group being worse. According to a nationwide survey using personal clinical records in Japan in 1999[4], the patients with onset at an age younger than 25 years showed overall 5-, 10-, and 20-year survival rates following diagnosis of 80%, 60%, and 28%, respectively. Corresponding survival rates for patients with onset older than 25 years were 100%, 87%, and 62%[4]. The younger group is known as the Wishart type, while the older group is the Gardner type[5]. The problem with either type of NF2 is the progressive deterioration of activity or quality of life due to multiple tumors in the central nervous system and peripheral nerves. Although the tumor itself is benign, the disease represents a long-term threat to life expectancy.

Clinical features

The nervous system, eyes, and skin of NF2 patients are susceptible to lesions. Although bilateral VS is characteristic, schwannomas often occur in other cranial, spinal, and peripheral nerves. Meningiomas and non-vestibular schwannomas are other intracranial lesions associated with NF2. These patients could have ocular lesions involving cataracts, retinal epitheliomas, and retinal hyperplasia. Cutaneous lesions also present frequently[6]. It should be noted that "neurofibroma" might be present in NF2 patients but is quite rare. Historically, many have referred to this disease as neurofibromatosis, but this terminology is not representative of all NF2 clinical presentations. Thus, further consideration of the nomenclature for neurofibromatosis type 2 is necessary[7].

A Japanese survey based on personal clinical records[3] found that almost 90% of the cases were affected by vestibular schwannomas, the most frequent type of tumor in NF2, of which 81% were bilateral, and 6% were unilateral. Also, trigeminal schwannomas occurred bilaterally in 24% and unilaterally in 13% (unknown 17%). Intracranial meningiomas were found in 43% of the cases (unknown 11%). Hearing impairment was the most common symptom, with 61% of patients having severe hearing loss of >70 dB on at least one side of the ear. Other symptoms were spinal cord-related symptoms (48%), facial nerve palsy (34%), cerebellar ataxia, facial hypoesthesia, dysphagia or dysarthria, and others. In 58% of patients, progressive symptoms occurred, and only 3% experienced improvement.

Identifying NF2 gene

The first recorded case of bilateral VS was presented by Wishart in 1822[8]. These diseases were previously thought to be Mendelian disorders that mainly affect the nervous system, with two clinically distinct types : type 1 neurofibromatosis, also known as von Recklinghausen neurofibromatosis, and type 2, also known as bilateral acoustic neurofibromatosis. Family studies involving nearly 100 members affected by neurofibromatosis type 2 suggested that this disorder was characterized by bilateral VS caused by a single mutant gene that was distinct from the mutation seen in neurofibromatosis type 1[9].

However, frequent loss of alleles on chromosome 22 was reported not only in NF2 but also in sporadic VS and meningiomas[10], suggesting that tumor proliferation was initiated by the inactivation of a tumor suppressor gene on the NF2 locus[11]. The specific loss of alleles on chromosome 22 was detected in various tumor types, including VS, other cranial neurofibromas, and meningiomas from patients with bilateral VS (i.e., NF2). Therefore, the common chromosome deletion mechanism was considered in different tumor types[12].

It was later discovered that NF2 patients carry a mutated NF2 gene on chromosome 22q12[13,14], which encodes merlin, an analog of the moesin-ezrin-radixin family of cytoskeleton-associated proteins[15].

Gene mutation analysis

Germline *NF2* pathogenic variants are identified in up to 90% of patients with a family history of NF2. The penetrance of NF2 is 100%, and those

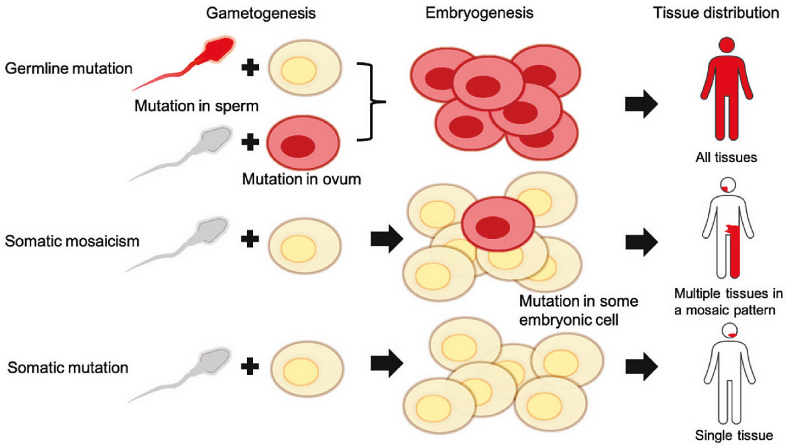

Fig. 1 Somatic mosaicism is a mosaic-like distribution of lesions resulting from genetic mutations in some embryonic cells during embryogenesis, which causes *de novo* NF2. Sporadic vestibular schwannoma follows somatic mutation.

with the *NF2* gene variant will develop the clinical disorder during their lifetime. On the other hand, more than 50% of NF2 patients have a *de novo NF2* gene variant without a family history, identifying *NF2* germline alterations are found in only 25%–60% of cases, and the remainder are thought to have somatic mosaicism[16–18]. Because somatic mutations occur at the embryonic cell stage, a mosaic of different phenotypes expresses in normal tissues with wild-type *NF2* and tumor tissues with mutant *NF2*[19] (Figure 1). A study including >1,000 de novo NF2 patients estimated that nearly 60% of de novo cases have somatic mosaicism.

With regard to identifying the mutation of *de novo NF2*, sanger sequencing can identify point mutations or small-size mutations, while multiplex ligation-dependent probe amplification (MLPA) is suited to detect copy number alterations due to large deletions. However, it is challenging to identify *NF2* variants with low variant allele frequency (VAF) in lymphocyte DNA. Thus, an analysis using these techniques may diagnose around 15% of cases as mosaic NF2[16]. Even with next-generation sequencing (NGS), the genetic diagnostic rate was only between 20.2% to 23.5%[16,18,19]. If a case has a *de novo* somatic mosaicism, whether a variant could be detected in any cell lineage DNA depends on when the mutations developed in embryogenesis[20]. Thus, targeted deep sequencing of DNA from multiple tissues (blood, buccal mucosa, hair follicle, and tumor) can improve the diagnostic rate of mosaic NF2 by up to 37.7%[21].

Merlin protein

Merlin is related to the ezrin-radixin-moesin (ERM) family of proteins that link plasma membranes and actin filaments. Merlin contains an N-terminal 4.1-ezrin-radixin-moesin (FERM) domain followed by a flexible coiled-coil domain and a carboxy-terminal hydrophilic tail (Figure 2). Merlin exists in two conformational forms, open and closed forms. Merlin switches from an active closed state to an inactive open state by phosphorylation at serine-518 on the carboxy-terminal domain[22].

Merlin's function in cell signaling

Merlin exerts inhibitory effects on multiple receptor tyrosine kinases (RTK) such as the ErbB receptor, platelet-derived growth factor receptor (PDGFR), insulin-like growth factor 1 receptor (IGF1R), and vascular endothelial growth factor receptor (VEGFR). This is confirmed by merlin-deficient schwannoma cell models strongly activating the downstream of these RTK, including oncogenic Ras/Raf/MEK/ERK and PI3K/AKT pathways[23,24]. Merlin also suppresses the activation of the mammalian target of rapamycin complex 1 (mTORC1) signaling in the mTOR pathway, which contributes to the control of cell survival and proliferation[25], and the yes-associated protein (YAP) in the Hippo pathway regulating the cell proliferation and apoptosis[26].

Cell signaling of activation and deactivation of merlin

One of the merlin activation mechanisms is contact inhibition, which regulates tissue growth when two cells contact each other. This important mechanism activates signals through membrane pro-

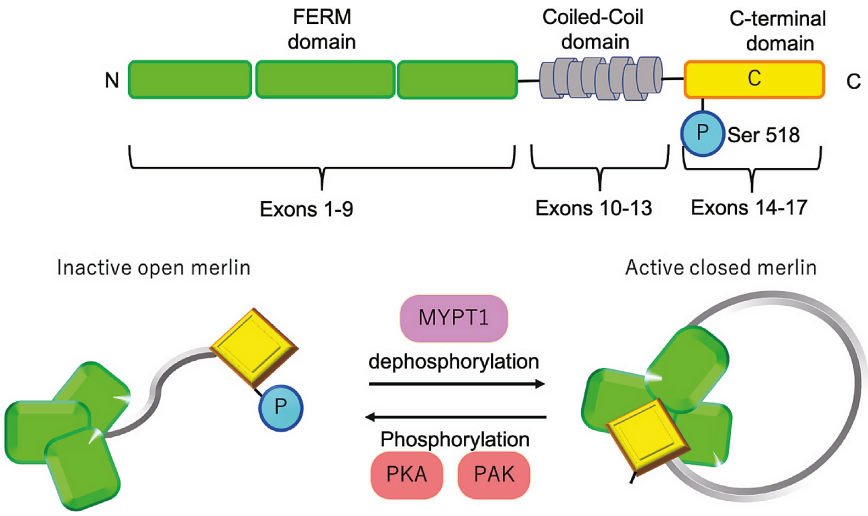

Fig. 2 Merlin consists of FERM, coiled-coil, and C-terminal domains. Dephosphorylation by MYPT1 closes the protein structure and activates merlin ; phosphorylation by PKA or PAK opens the protein structure and deactivates merlin.

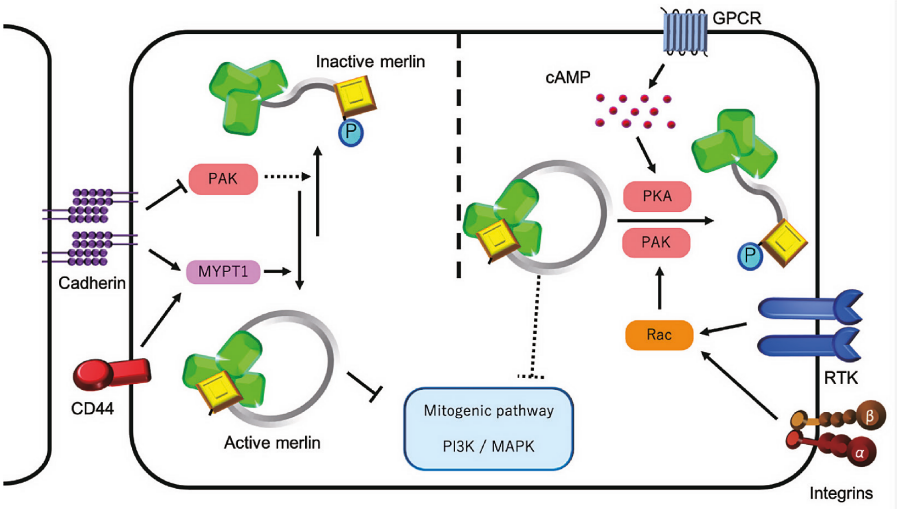

Fig. 3 (Left) Merlin is dephosphorylated (activated) by MYPT1, activated by cadherins and CD44, and suppresses cell proliferation signals. (Right) Tyrosine kinase receptors or integrins activate Rac and PAK, and G protein-coupled receptors signaling activate cyclic AMP and PKA, inducing phosphorylated (deactivated) merlin.

tein organization (i.e., CD44, epidermal growth factor receptor, and layilin), intercellular adhesion (i.e., cadherin), and cytoskeletal structures (i.e., spectrin and actin)[27]. The myosin phosphatase targeting subunit 1 (MYPT1) also dephosphorylates merlin protein into an active closed state[28] (Figure 3).

Conversely, the mechanisms of merlin inactivation include the following. Proliferation signals initiated by membrane-anchored integrins and RTKs activate the signaling protein Rac, which activates p21-activated kinase (PAK), which in turn phosphorylates and inactivates merlin. In addition, a high concentration of cyclic AMP mediated by stimulation of G protein-coupled receptors (GPCRs) activates protein kinase A (PKA), which phosphorylates merlin[29] (Figure 2).

Genotype-phenotype relationship

While phenotype and natural history of NF2 are often similar within the same family line, a different family line with other *NF2* mutations presents significant differences in phenotype, thought to be influenced by mutation type or location within the *NF2* gene[30]. Patients with *de novo* NF2 also present phenotypic differences, suggesting a correlation with the extent of *NF2* VAFs[21]. The type of NF2 mutation is responsible for the number of lesions of the intracranial meningiomas, spinal cord, and peripheral nerves. If nonsense and frameshift muta-

tions occur in *NF2*, protein (merlin) expression is impaired, and the clinical symptoms are severe.

Meanwhile, missense mutations and in-frame deletions are known to result in mild symptoms because merlin could have a partial effect. An analysis of 268 patients with *NF2* abnormalities shows the truncated protein expression is associated with younger age at diagnosis and with a higher prevalence of meningiomas, spinal cord tumors, schwannomas rather than vestibular schwannomas, and skin tumors[31]. The exon number where mutations occurred also affects the NF2 phenotype. An exon 14-15 mutation is associated with fewer clinical manifestations and meningiomas[32], whereas truncating mutations in exons 2-13 present a worse prognosis than exon 14-15 mutations[30].

The phenotypes are also different between germline mutation and somatic mosaicism. Mosaic NF2 patients show significantly lower VS growth rates, lower incidence rates of NF2-related lesions other than meningiomas, and higher age of onset. However, about half of mosaic NF2 patients have multiple large meningiomas, even with unilateral or mildly bilateral VS, suggesting that the phenotype is not always mild[21].

Clinical assessment for NF2 patients focused on hearing function. The more severe the NF2 mutation type, the more hearing is lost at a younger age[33]. However, other reports showed that such a relation between the mutation type and hearing loss is not necessarily related to the tumor volume or growth rate [34,35].

Differential diagnosis of NF2

The differential diagnosis of NF2 with significant clinical overlap includes schwannomatosis and familial syndrome of multiple meningiomas. Although schwannomatosis is similar to NF2 in presenting multiple schwannomas, it differs in the absence of bilateral VS. Schwannomatosis is associated with *SMARCB1* and *LZTR1* mutations located on chromosome 22. This disease has *NF2* somatic mutations in tumor tissue, suggesting that either *NF2* somatic mutations occur in each tumor or patients may have somatic mosaicism of *NF2*. It is reported that *SMARCB1* mutations do not cause VS, while *LZTR1* mutations do[36]. Thus, revised diagnostic criteria for NF2 are proposed to include these genomic alterations[17].

Diagnostic criteria for NF2

The Manchester criteria developed in 1992 are the most widely used for NF2 diagnosis. With improved understanding of the genetics and pathogenesis of NF2, criteria have been revised to allow identification and diagnosis of NF2 at an earlier stage as a significant proportion of patients without bilateral VS[17,36] (Table 1).

Current status of NF2 drug discovery

Angiogenesis as a target of treatment

As discussed in *NF2* gene function, merlin affects multiple pathways involved in cell growth, which can help to identify different therapeutic targets. Although VS is a benign tumor and grows relatively slowly, angiogenesis is required for the tumor to expand beyond a specific size[37,38]. Some angiogenesis-stimulating factors were identified, and among the most well-known is vascular endothelial growth factor (VEGF). A correlation has been found between the degree of VEGF expression and clinical parameters such as tumor growth, tumor volume, and microvessel density[38,39]. Currently, a number of translational research efforts for therapeutic targeting of VEGF or VEGF receptors have been conducted ; the main ones are discussed below. Table 2 summarizes reports regarding other molecular-targeted therapies.

Bevacizumab

Bevacizumab, an anti-VEGF monoclonal anti-

Table 1. Diagnostic criteria for NF2

1.	Bilateral vestibular schwannomas, or
2.	FDR family history of NF2 and unilateral VS, or
3.	FDR family history of NF2 or unilateral VS and two of meningioma, cataract, glioma, neurofibroma, schwannoma, cerebral calcification (if UVS+, ≥2 schwannomas only need negative LZTR1 test[a]), or
4.	Multiple meningiomas (2 or more) and two of unilateral VS, cataract, glioma, neurofibroma, schwannoma, cerebral calcification, or
5.	Constitutional pathogenic NF2 gene variant in blood or identical in two tumors[b]

NF2 : neurofibromatosis type 2, FDR : first-degree relative, VS : vestibular schwannoma, UVS : unilateral vestibular schwannoma, LZTR1 : leucine zipper-like transcription regulator 1

[a]Includes two of any tumor type, such as schwannoma

[b]Requires molecular analysis

Table 2. Recent clinical trials for NF2-related VS

Drug	Author	Trial design and result
Lapatinib	Karajannis, *et al*[50]	Phase II. 4/17 with >15% tumor size reduction 4/13 with >10 dB improved hearing
Imatinib	Stephanie, *et al*[51]	Case report. Prolonged VS progression for 4 months Due to adverse effects, changed to bevacizumab with prolonged progression for 8 months
Erlotinib	Plotkin, *et al*[52]	Phase II. 11 patients with progressive VS No radiographic or hearing responses Median time to tumor progression was 9.2 months
Rapamycin	Giovannini, *et al*[53]	A case presentation Arrested growth of progressive VS
Everolimus	Karajannis, *et al*[54]	Phase II. 9 patients with progressive VS No radiographic or hearing responses.
Everolimus	Goutagny, *et al*[55]	Phase II. No more than > 20% tumor size reduction 4/9 with progressive disease, 5/9 with stable disease Prolonged time to progression

body, is already approved for clinical use, including in Japan, for treating malignant gliomas and other carcinomas. Plotkin *et al.* investigated the effect of bevacizumab in NF2-related VS for the first time, showing a reduction in tumor volume in 9 of 10 patients and improved hearing in some cases[40]. Other reports showed the efficacy of bevacizumab in NF2-related VS[41-43]. Goutagny *et al.* reviewed the literature and reported that bevacizumab improved hearing and tumor shrinkage in more than 50% of progressive VS[44]. A study of bevacizumab administration in NF2-related VS in Japanese patients showed a maximum decrease in tumor volume to baseline occurring three months after receiving four doses of bevacizumab (5 mg/kg in two-week intervals)[45]. However, increasing the bevacizumab dose from 5 mg/kg every two weeks to 10 mg/kg was found to be ineffective[46]. The problem with bevacizumab therapy is the need for long-term administration for tumor control, which can result in side effects, including hypertension, proteinuria, and delayed wound healing.

Currently, a randomized, double-masked, placebo-controlled, multicenter trial to assess bevacizumab's efficacy and safety in NF2 patients is ongoing in Japan, with sixty patients already included with the evaluation of hearing improvement in the affected ear as the primary objective[47]. This study is the first of its kind regarding the administration of bevacizumab in NF2 in a randomized controlled trial. It is also the most extensive prospective study of this rare and intractable disease, and the results are eagerly awaited. For this randomized control trial, 12 other Japanese institutions have joined the principal investigators at Fukushima Medical University.

Peptide vaccine therapy for VEGF receptor

Another study investigating the development of peptide vaccines targeting the VEGF receptor (VEGFR) as a novel therapy is also underway in Japan. High VEGFR (VEGFR1 and VEGFR2) expression was detected on endothelial cells and tumor cells in NF2 schwannomas[48]. Thus, a subcutaneous injection of VEGFRs combined with a human leukocyte antigen (HLA) peptide vaccine could activate cytotoxic T lymphocytes (CTLs) specific for these and exert an antitumor effect. The CTLs attack tumor cells and blood vessels directly and are maintained as acquired immunity, expected to be a long-term effect. A Japanese clinical study showed that CTLs against both VEGFRs were induced in 6 out of 7 patients with eight doses of the peptide vaccine. Hearing improved in 40% of patients. Twenty-two out of 23 schwannoma lesions were reduced or had no change, but only one cystic schwannoma became enlarged. Furthermore, tumor specimens obtained from pre- and post-vaccination showed that the vaccination caused both loss of VEGFR expression and reduction of VEGF-A expression[49].

Since this vaccine consists of HLA-A-binding peptides, some patients with different HLA-A types cannot induce an immune response to VEGFR. It is expected that the number of patients eligible for this kind of therapy could increase with the development of peptide vaccines mixed with certain common HLA types to compensate for the variations available in the population.

Conclusion and future prospects

Although NF2 is rare, it is still a life-threatening disorder with severe symptoms. There is some overlap with phenotypic-similar diseases (e.g., schwannomatosis or familial syndrome of multiple meningiomas) ; thus, genetic diagnosis will be essential. NF2 patients are offered either tumor debulking surgery or stereotactic radiotherapy according to their clinical symptoms. However, understanding the pathogenesis and employing genetic analysis can help diagnose NF2 early, especially the *de novo* types. Further clinical trials are still needed to assist in developing new management plans to improve the NF2 patient's quality of life.

Conflict of Interest

The authors are members of a research team conducting a phase 2 clinical trial to assess the therapeutic effect of bevacizumab in neurofibromatosis type 2. The trial is financed by the Japan Agency for Medical Research and Development (AMED) and the CHUGAI PHARMACEUTICAL COMPANY.

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