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POSSIBLE MODIFIER GENES IN THE VARIATION OF NEUROFIBROMATOSIS TYPE 1 CLINICAL PHENOTYPES

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ABSTRACT

Neurofibromatosis type 1 (NF1) is the most common neurogenetic disorder worldwide, caused by mutations in the neurofibromin 1 (*NF1*) gene. Although NF1 is a single-gene disorder with autosomal-dominant inheritance, its clinical expression is highly variable and unpredictable. NF1 patients have the highest known mutation rate of *NF1* among all human disorders, with no clear genotype–phenotype correlations. Therefore, variations in *NF1* mutations may not correlate with the variations in clinical phenotype. Indeed, for the same mutation, some NF1 patients may develop severe clinical symptoms whereas others will develop a mild phenotype. Variations in the mutant *NF1* allele itself cannot account for all of the disease variability, indicating a contribution of modifier genes, environmental factors, or their combination. Considering the gene structure and the interaction of neurofibromin protein with cellular components, there are many possible candidate modifier genes. The present review aims to provide an overview of the potential modifier genes contributing to NF1 clinical variability.

Keywords: Neurofibromatosis type 1; genotype–phenotype correlation; clinical variability; modifier genes

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common autosomal-dominant disorders, affecting approximately 1 in 3500 individuals in all ethnic groups worldwide [1]. It is clinically characterized by café-au-lait spots (CLS), Lisch nodules, axillary and inguinal freckling, multiple peripheral nerve tumors, bone lesions, and a predisposition to malignancy. Therefore, the causal gene neurofibromin 1 (*NF1*) is considered to function as a tumor suppressor gene. Neurofibromas, characteristic features of the disease, are found in cutaneous, subcutaneous, and plexiform types, and although they are benign, they could develop malignancy. NF1 is caused by dominant loss-of-function mutations in the *NF1* gene, which encodes neurofibromin, a negative regulator of Ras proteins. A 360-amino acid region of the gene product shows homology to the catalytic domain of the mammalian GTPase-activating protein (GAP). This region is referred to as the NF1–GAP-related domain (NF1-GRD) and is encoded by the central part of the *NF1* gene. The GAP proteins downregulate the activity of the Ras oncoprotein by stimulating its intrinsic GTPase activity. Therefore, neurofibromin is part of the Ras-mediated signal transduction mechanism. Several genetic disorders are caused by dysfunction in gene products associated with this pathway, and owing to their common phenotypes, they have been recently classified together and termed as "Rasopathies." Three genes, *EVI2A*, *EVI2B*, and *OMGP*, are embedded within intron 27b of the *NF1* gene. These genes are transcribed in the direction opposite to that of the *NF1* gene. However, little is known about the function of these genes. A hallmark of the *NF1* gene is its high mutation rate; almost half of all NF1 cases result from *de novo* mutations in *NF1* [2].

NF1 patients have intragenic *NF1* mutations with no clear genotype–phenotype correlations except for a 3-bp in-frame deletion (c.2970–2972 delAAT) in exon 17, which has been associated with a mild clinical phenotype [3], and microdeletions.

Although the frequency of NF1 is the same across all ethnic groups, and it is considered that the distribution of *NF1* mutations does not differ among populations, there are some reports from Brazil, China, and Korea that challenge this assumption. Moreover, recurrent mutations reported in previous publications are not common in Turkish patients [4,5].

Clinical signs are variable and the phenotypic complexity of NF1 is similar to that of multifactorial diseases, including epigenetic factors and heritable elements such as genetic modifiers. Some NF1

patients with the same mutation may develop severe clinical symptoms while others develop a mild phenotype. As an example, in our previous study, we identified the same exon 4b mutations, 496delTG and 499delTGTT, in two different NF1 families that showed completely different phenotypes. Both of these families have identical mutations that cause a premature stop codon leading to the same truncated proteins [5]. These phenotypic variations within and between families with the same genetic cause may be the effect of modifier genes or a second somatic mutation occurring in the tumor tissues. The scientific background for this clinical variability is not well understood. The allelic heterogeneity of the *NF1* mutation may be one of the reasons to explain the phenotypic variations associated with this disease. The absence of cutaneous neurofibromas with a 3-bp *NF1* internal deletion indicates that the *NF1* genotype itself can act as an NF1 modifier [3]. In addition, patients with *NF1* microdeletions have increased numbers of early-developing dermal neurofibromas [6]. However, it is also clear that variation in the mutant *NF1* allele itself does not account for all of the disease variability observed. This variation could be due to modifier genes, environmental factors, or their combination.

It is generally considered that genetic modifiers, distinct from the disease locus itself, play an important role in phenotypic variations of single-gene disorders. Identifying these genetic modifiers is very important in terms of both treatment and genetic counselling. There are several reports concerning the role of modifier genes in NF1 clinical variations. However, the selection of which phenotype to study is a key challenge in modifier gene studies.

MODIFIER GENES IN NF1 PHENOTYPIC VARIATION

In this paper, the term "modifier gene" is used to define any gene that may change several features of the NF1 phenotype, and the word "gene" is used for not only protein-coding sequences but also for microRNA (miRNA) and long non-coding RNA genes that may modulate the NF1 phenotype. Considering the gene structure and the interaction of neurofibromin protein with cellular components, there are many possible candidate modifier genes influencing NF1. Besides modifier genes, environmental factors might also contribute to the variable disease phenotype, as shown in Figure 1. There are two major strategies to detect the modifier genes in NF1: whole-genome scanning or focusing on specific candidate genes or pathways. In a recent study, Pasmant et al. [7] performed whole-genome

high-resolution array-comparative genomic hybridization of NF1-associated plexiform neurofibroma (PNF) to identify candidate modifier genes. Neurofibromas are complex benign tumors of the peripheral nerve sheath composed of heterogeneous cell types (Schwann cells, endoneurial fibroblasts, mast cells, and perineurial cells). Although they are composed of different cell types, neurofibromas arise from Schwann cells that undergo loss of heterozygosity (LOH) at *NF1* through somatic mutations. PNFs may undergo malignant transformation into neurofibrosarcomas, known as malignant peripheral nerve sheath tumors (MPNSTs). Therefore, it is very important to identify patients at high risk for developing PNF or MPNST in advance. For this reason, detection of modifier genes that may be important for the development of PNF may help to diagnose and treat these tumors. For this purpose, Pasmant's group used the tissue samples from PNF tumors for genome-wide array comparative genomic hybridization studies to identify the candidate modifier genes involved in PNF development [7]. However, this high-throughput technology is not available for all laboratories, and therefore there are several reports dealing with possible candidate modifiers. Accumulation of information about these modifier genes would have great diagnostic and prognostic value. The present review discusses these candidate modifier genes and their effect on NF1 clinical phenotypes and potential for future therapies.

VARIATIONS ACCORDING TO SEX

Some specific gene mutations and symptoms are more prevalent in females. For example, female patients with NF1-associated optic glioma (OPG) require treatment for visual decline more often than their male counterparts. Mouse models also show similar sex differences in some NF1 symptoms. Furthermore, only male NF1 mice showed learning/memory deficits, increased Ras activity, and reduced dopamine levels [8].

It has also been shown that adjustment in the level of cAMP can change the OPG pathway stereotypically [9,10]. NF1-associated OPG can be modified by polymorphisms in the adenylate cyclase 8 (*ADCY8*) gene in a sex-specific manner. Warrington and coworkers reported that the growth of male and female *NF1*-/- astrocytes varied in response to inhibition of ADCY by dideoxyadenosine; moreover, CXCL12 enhanced the growth of astrocytes in females but not in males [11].

This idea was also reinforced by the observation of higher incidence of OPG in female children than in male children. Riccardi and Lupski [12] evaluated two different groups for detecting the duplication type of gene mutations; a total of 21 patients were included in this study, and 18 of them were females. This suggests that there may be some modifying factors protecting males from this type of mutation. Collectively, these data suggest that sex is a major factor contributing to the extent of the neural dysfunction in NF1, which should be considered as a modifier when developing a new therapeutic strategy and planning a clinical study.

VARIATIONS ACCORDING TO AGE

Age and the hormonal environment are additional critical factors contributing to the clinical variation in NF1. Many disease features are more prevalent in older patients. Some clinical features such as dermal neurofibromas begin to develop around puberty, and the number and size of neurofibromas increase during pregnancy. Indeed, in NF1, many phenotypic features such as the development of neurofibroma are age-dependent. Pemov et al. [13] measured a variety of phenotypic features with particular focus on the number of CLS, because these spots are easy to quantify and their number tends to reach a maximum after early childhood. CLS are ‘‘tumor-like’’ in that they follow the Knudsen two-hit hypothesis: melanocytes in these lesions acquire a second somatic mutation in *NF1*. Thus, genes that modify the number of CLS may also possibly modify the risk of tumor development. Variations in the number of dermal neurofibromas among NF1 patients, even within a family carrying the same *NF1* mutation, support the idea of the existence of some modifiers of dermal neurofibroma. Second hits almost always require an additional time course, and therefore age is likely another important modifier in the variation of CLS counts beside *NF1* mutations.

GENES EMBEDDED IN THE *NF1* GENE

EVI2A, *EVI2B,* and *OMGP* are embedded within intron 27b of the *NF1* gene, and Viskochil et al. proposed that the transcriptional regulation of these embedded genes might play a role in the NF1 clinical phenotype. Both *EVI2A* and *EVI2B* encode putative transmembrane proteins. The mouse homologs (*Evi-2a* and *Evi-2b*; ecotropic viral integration sites) are associated with viral insertions

involved in leukemia in mice, although their relationships to NF1 symptoms are unknown [14]. NF1 patients have a higher risk of developing juvenile chronic myelogenous leukemia compared to the general population. There are a limited number of studies in the literature related to NF1-associated leukemia. It is possible that mutations involving both *NF1* and *EVI2A* (or *EVI2B*) may cause NF1/leukemia syndromes [15]. Indeed, *Evi2b* was identified as a direct target gene of C/EBPa, a transcription factor critical for myeloid differentiation. It is possible that these genes are related to the leukemia observed in NF1 patients, although there are no data confirming this association in the literature. Expression of this gene may be altered by viral integration, which could predispose cells to myeloid diseases. Therefore, we investigated the expression of *EVI2A* and *EVI2B* in NF1 tumors and leukemias. Our preliminary results showed the possibility of viral integrations in *EVI2B* in NF1-acute myeloid leukemia patients [16].

Another embedded gene in the *NF1* structure is *OMGP*, which encodes the oligodendrocyte-myelin glycoprotein (OMGP), expressed only in the oligodendrocytes of the central nervous system. To date, no mutations have been detected in the *OMGP* gene except for some cases of large deletions of the gene. Specific learning disabilities are the most common neurological complication in children with NF1. The frequency of learning disability is approximately 7–10% in the general population, and is 50% among NF1 patients. Therefore, the *OMGP* gene may be a possible candidate modifier of NF1-associated learning disability for several reasons: (1) it is located within the *NF1* gene, (2) is related to axon myelinization, and (3) contains binding sites for the transcription factors involved in neuronal development and synaptic plasticity, which are also important for the learning process. Neurofibromin and OMGP are both expressed in the same type of cells, and their interaction may help to explain the central nervous system findings in NF1 patients. NF1 and OMGP proteins are regulated by common mechanisms, and both may synergistically inhibit Ras activity. Therefore, OMGP might contribute to the downregulation of Ras by neurofibromin, which is impaired in NF1. Another observation of our group is that the unaffected siblings of NF1 patients obtained lower scores in certain cognitive tests compared to healthy controls, supporting the presence of another modifying gene or genes.

There have also been some reports of patients with NF1 and multiple sclerosis. *OMGP62* polymorphisms have also been associated with autism and non-syndromic mental retardation. Despite

all of these findings suggesting a role of OMGP in cognition, Terzi et al. [17] did not observe any relationship between *OMGP* gene mutations and learning disability in NF1.

MICRODELETIONS

Approximately 5–10% of NF1 cases are due to microdeletions, in which the entire *NF1* gene is deleted together with a number of other genes [18]. Patients with NF1-microdeletion syndrome usually have more cutaneous, plexiform neurofibromas and a higher risk of developing MPNST compared to patients with other forms of NF1. Three typical and seven atypical forms of these microdeletions have been detected: type-1 (1.4-Mb long, resulting in 14 deleted protein-coding genes and *NF1*), type-2 (1.2-Mb long, resulting in 13 deleted protein coding genes and *NF1*), and type 3 (1.0-Mb long, with 8 deleted protein coding genes and *NF1*) microdeletions. Pasmant et al. [19] showed that learning disabilities and facial dysmorphism were significantly associated with NF1-microdeletion syndrome. Spiegel et al. [20] proposed that overgrowth was in fact a distinctive phenotype of patients with NF1-microdeletion syndrome. Alternatively, Douglas et al. [21] suggested that *RNF135* haploinsufficiency was responsible for the overgrowth in individuals with *NF1* microdeletions. The results of Pasmant et al. [19] confirmed this suggestion, because four patients with NF1-microdeletion syndrome presented childhood overgrowth, although the *RNF135* gene was not included in the microdeletion interval (no whole gene deletions of *RNF135*). Ning et al. [22] conducted the largest study to date on the growth of NF1 patients with microdeletions. They measured the weight, length, and head circumference of 56 patients with *NF1* microdeletions and 226 NF1 patients with other kinds of mutations. They detected that children with microdeletions were taller and heavier than the non-deletion NF1 patients; however, they noted that no differences were observed in early infancy. The head circumference measurements and age at puberty were similar in both groups of NF1 patients, those with and without microdeletions [22]. This study represents yet another example of how the *NF1* genotype itself can act as an NF1 modifier.

DNA REPAIR SYSTEM

DNA repair is a series of mechanisms that protect the cells' DNA from developing detriments due to environmental factors and normal metabolic processes inside the cell, and try to correct such damage in

DNA molecules [23]. One of these mechanisms is mismatch repair (MMR), which is present in all cells to correct errors that are not otherwise corrected by the proofreading mechanism [24]. Any functional abnormality of this system results in replication errors that remain uncorrected, leading to a mutator phenotype and sporadic mutation formation. According to the existing information, the association between constitutional mismatch repair deficiencies (CMMRD) and NF1 seems to be incorrect, because some CMMRD patients with rare childhood malignancies have been erroneously included in presumed NF1 cohorts. Therefore, to re-evaluate the likely association between NF1 and childhood malignancies such as central nervous system tumors and rhabdomyosarcomas, all prospective and retrospective studies should be repeated to exclude cases of CMMRD. It should also be pointed out that the incidence of postzygotic *NF1* mutations in some CMMRD patients can be responsible for the observed NF1 features [25].

Another DNA repair mechanism that is speculated to affect NF1 is nonallelic homologous recombination (NAHR). As mentioned above, different types of microdeletions have been identified in NF1 depending on the size of the deleted region. It has been shown that the majority of type-1 *NF1* microdeletions (1.4 Mb) occur in the germline through an NAHR mechanism [26]. Type-2 *NF1* deletions (1.2 Mb) have also been reported to occur predominantly because of intrachromosomal mitotic (post-zygotic) NAHR [27]. Indeed, some hotspots of meiotic NAHR have been identified in both types of microdeletions [28,29]. Therefore, genetic differences in the DNA damage repair capacity might act as modifiers of NF1 disease severity.

MITOCHONDRIA

Mitochondrial DNA (mtDNA): mtDNA does not contain any protective histone molecules in its structure and thus has a limited capacity to repair damage, making it particularly sensitive to damage from reactive oxygen species (ROS) and accumulation of mutations [30]. Recent reports have shown that these accumulated somatic mtDNA mutations have an important role in the development of tumors in the brain, ovary, esophagus, breast, and colon [31-36]. Analysis of mtDNA mutations and comparison of the presence of somatic mtDNA mutations in tumor and non-tumor cells [37] showed that all mtDNA mutations occurred in the hypervariable D-loop region of the mitochondrial genome, which is unique

because the mutations detected in tumors related to other organs are mostly located in coding regions [31-36,38]. According to Kurtz et al. [37], cutaneous neurofibromas could be formed from cells already carrying somatic mtDNA mutations.

Succinate dehydrogenase (SDH) and TRAP1: Succinate dehydrogenase subunit B (SDHB) is one of the ubiquitously expressed proteins in the mitochondria. The lack of SDHB expression was observed in cases of Carney triad and Carney Stratakis syndrome-associated gastrointestinal stromal tumors (GISTs) [39,40]. GISTs arise in NF1 patients 150-times more often than they do in the general population [41]. Similar to the majority of adult GISTs, NF1-associated GISTs express SDHB; however, unlike the majority of GISTs, they do not respond well to imatinib treatment [42]. This observation raised the possibility that SDHB could be a candidate modifier of the phenotypic variation in NF1. Sciacovelli and co-workers [43] proposed that inhibition of SDH with the mitochondrial chaperone TRAP1 could promote neoplastic growth. TRAP1 expression is restricted to the mitochondria, and has a protective role in oxidative stress. TRAP1 is highly expressed in many tumors, and therefore may also be a crucial factor in NF1 tumors. Cancer cells require a high amount of oxygen so that they can expand their own blood supply rapidly [44]. Yet, because their growth rate is so high, cancer cells have acquired a mechanism, known as the Warburg effect, which decreases the rate of mitochondrial respiration [45] to allow the cells to grow in a hypoxic condition [46]. This effect is initiated by the induction of hypoxia-inducible transcription factor-1 (HIF1), which is activated by hypoxia together with the accumulation of succinate and fumarate, the Krebs cycle metabolites [47]. HIF1 decreases the influx of pyruvate to the Krebs cycle and activates glycolysis [48]. Specifically, TRAP1, an evolutionarily conserved chaperone of the heat-shock protein 90 family [49], downregulates mitochondrial respiration by reducing the activity of SDH, which in turn stabilizes HIF1 by increasing succinate levels [43].

It has been proposed that the inhibition of SDH by TRAP1 has both anti-oxidant and anti-apoptotic effects on tumor cells. The conditions of the tumor microenvironment, such as abnormal activation of signal transduction pathways, increase the accumulation of ROS in cancer cells [50]. To prohibit the harmful effect of ROS, a scavenging program that can protect tumor cells from cell death is elicited [51]. The inhibition of SDH, which is a main site of ROS generation, with TRAP1 can have an anti-apoptotic effect [52]. Notably, mutations in the *SDH* gene resulting in LOH are extremely rare.

Nevertheless, TRAP1 has many post-translational modifications [53,54], and any defect in these modifications can affect SDH enzymatic activity [55]. Some of these interactions are summarized in Figure 2.

Moreover, a recent report showed that activation of the Ras/extracellular signal-related kinase (ERK) signaling pathway in neurofibromin-deficient cells in the pro-tumorigenic stage boosted the glycolysis reaction and downregulated mitochondrial oxidative phosphorylation by inhibition of SDH through TRAP1. Specifically, Rasola and coworkers [27] demonstrated that a fraction of the active ERK located in the mitochondrial matrix of cells with a neurofibromin deficit phosphorylated TRAP1, which in turn inhibited SDH and promoted tumor growth. This suggests that any detriment in the RAS/ERK signaling pathway that could affect its activity in cells lacking neurofibromin can lead to inhibition of mitochondrial respiration.

VITAMIN D AND BONE MASS

Vitamin D is a fat-soluble secosteroid whose function is to increase the absorption of calcium, iron, magnesium, phosphate, and zinc from the intestine. The most important forms of vitamin D are vitamin D2 (cholecalciferol) and vitamin D3 (ergocalciferol). Both forms can be ingested from foods and supplements, but cholecalciferol can also be synthesized naturally in the skin from cholesterol under sun exposure [56,57]. The precursor of vitamin D in the skin is 7-dehydrocholesterol, which is synthesized upon exposure to ultraviolet irradiation. Next, vitamin D is hydroxylated to 25-hydroxyvitamin D3 (25(OH)D3) in the liver, and further hydroxylation takes place in the kidney to form the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) [58].

Vitamin D is responsible for the regulation of calcium homeostasis, which plays a critical role in bone metabolism [57]. Approximately 10% of NF1 patients show focal bony abnormalities [59], severe osteomalacia [60], and reduction in bone mass [61], suggesting a link to vitamin D deficiency.

Indeed, more than 60% of patients with NF1 displayed a severely low vitamin D level (20 ng/ml) [62]. Nakayama et al. [63] observed some significant reduction in the pigmentations of the CLS in response to vitamin D treatment besides bone abnormalities. Therefore, vitamin D can be another factor affecting the phenotype of NF1 patients. Correspondingly, the level of 25(OH)D3 was measured in NF1 patients

and compared to that in healthy people. There are some conflicting data in the literature concerning the seasonal effect of vitamin D levels [64,65]. However, Schnebel et al. [66] observed a low level of 25(OH)D3 in adults with NF1 both in summer and winter. They also emphasized that dermal neurofibromas did not block the dermal synthesis of 7-dehydrocholesterol.

Vitamin D receptor (VDR) is another factor that is considered to affect the vitamin D levels and bone mass in NF1 patients. VDR, also known as calcitriol receptor, is encoded by genes located on chromosome 12 (12q12-14) [67]. Different series of polymorphisms have been reported in the VDR genes, which alter the biological processes of this receptor [68]. Two of these polymorphisms were identified as a C/T transition located in a start codon (ATG) [69] and a G/A polymorphism located on intron-8, which were detected using the *Fok*I and *Bsm*I restriction enzymes, respectively [70]. These two polymorphisms result in a shorter VDR protein with decreased expression. Bueno et al. [71] hypothesized that the reduced VDR expression might decrease 1.25(OH)2D3 activity, even when vitamin D levels are normal, which would in turn decrease the bone turnover rate in NF1 patients due to the lack of calcium absorption in the duodenum. However, Bueno and co-workers did not detect correlations between low vitamin D levels and VDR gene polymorphisms and deregulation of osteoblast and osteoclast activity of NF1 patients. Nevertheless, their sample population was not large enough to conclusively rule out the possibility that VDR is a modifier in NF1.

MIRNAs

Approximately 20 years ago, miRNAs were discovered as new regulatory factors of the genome. This mechanism has greatly contributed to gaining a better understanding of the pathogenesis of many cancers at the molecular level. MiRNAs function in RNA silencing and negatively regulate gene expression at the post-transcriptional level [72].

It is now well-established that miRNAs play a critical role in cancer development, given their involvement in cell differentiation, developmental control, neural development, cell proliferation, and apoptosis. The miRNAs also have a crucial function in tumor progression such as in cancer invasion and metastasis [73].

There are several hundreds of types of miRNAs, which have several hundred target genes, making them useful biomarkers in cancer diagnosis, prognoses, as well as candidate therapeutic targets. In addition, certain types of stable miRNAs have also been found in human serum, whose altered expression can contribute to human diseases such as lung cancer [74]. Weng et al. [75] reported the significant upregulation of serum miRNA-24 levels only in NF1-MPNST patients, and suggested that this miRNA could be a biomarker for NF1-MPNST detection

Although the role of miRNA in the NF1 phenotypic variation can be considered as a new subject of study, several miRNAs have already been detected in NF1 tumorigenesis to date, including miR-29c (used to distinguish malignant from benign tumors [76]), miR-34a (a direct target of p53 [77]), miR-214 (induces cell survival and cisplatin resistance [78]), miR-10b (transforms benign NF1-associated neurofibromas to MPNSTs [77]), miR-204 (contributes to the growth of MPNSTs [79]), miR-21 (important in MPNST progression [80]), and miR-107 (regulates NF1 in gastric cancer [81]). Nevertheless, there is still much work to be done to gain a detailed understanding of the actual function and pathway of miRNAs in NF1 [73].

ADDITIONAL GENE MUTATIONS IN NF1-ASSOCIATED TUMORS

For the development of malignant transformation, especially in NF1-related MPNSTs, beside the *NF1* mutations, additional mutations in several genes, including *INK4A/ARF* and *P53*, are required. A mouse model was developed for MPNSTs by generating mice with mutations in both the *Nf1* and *p53* genes [82]. The loss of the *p53* gene is responsible for the abnormalities in DNA damage-dependent cell cycle arrest and apoptosis. Mutations in *TP53* underlie the development of MPNSTs in animal models, but there are controversies remaining related to the role of *p53* mutations in human MPNSTs [83,84].

Changes in growth factor expression create secondary genetic events contributing to malignant transformation. Growth factors help to suppress cell death in Schwann cell precursors. Abnormal growth factor receptor expression also plays a role in tumor development, progression, and malignant transformation. Wu et al. [6] found that epidermal growth factor receptor (EGFR) levels modified the neurofibroma number. Neurofibroma development requires biallelic *NF1* mutations in Schwann cells and/or Schwann cell precursors. In mouse models, when *NF1* was mutated in Schwann cells and

Schwann cell precursors, all animals formed neurofibromas, and the number of tumors increased in proportion to the expression level of EGFR. These data demonstrate that neurofibroma formation increases when EGFR is overexpressed and *NF1* is mutated. Amplification of the receptor and disturbed Ras signaling both contribute to benign tumor formation.

Studies of different tumor types have demonstrated that the expression levels of chemokine receptors such as CXCR4 are increased in tumor tissues, which are linked to the metastasis and progression of cancer. We analyzed the gene expression of *CXCR4* and its ligand *CXCL12* in human neurofibromas. The results of this experiment showed that the *CXCR4* gene expression level was increased by 3 to 120-fold and the *CXCL12* gene expression level was increased by 97 to 512-fold in all tumors relative to normal human Schwann cells [85].

Telomerase can also be considered as a potential modifier of NF1. Owing to the shortening of telomeres beyond a certain level, cells are arrested and enter cellular senescence [86]. The enzyme telomerase comprises two subunits: telomerase RNA and the telomerase reverse transcriptase (TERT) [87]. However, it has been reported that the *TERT* mRNA expression and telomerase activity can be detected in most cancer cells, and this property can be used as a biomarker for cancer screening [88]. A recent study demonstrated a correlation between the high-fold expression of TERT, telomerase activity, and high-grade malignancy in NF1-associated MPNST. These data can provide a new approach for the modifying effect of telomerase and the treatment of NF1-associated malignant tumors [89].

Apoptosis can also be a modifier of NF1. Apoptosis can be initiated in the intrinsic and extrinsic pathways. Both pathways induce cell death by activating caspases. One of the important proteins in the intrinsic pathway is apoptotic protease activating factor-1 (Apaf-1), and any loss of expression of this protein can result in tumor development [90]. Hepatocellular carcinoma antigen 66 (HCA66) is one of the proteins that interacts with Apaf-1 and can regulate apoptosis. The *HCA66* gene, located on chromosome 17q11.2, is one of the genes that is deleted in NF1-microdeletion syndrome [91]. Patients with NF1-microdeletion syndrome have a distinct phenotype with a poor prognosis characterized by a low IQ, dysmorphic features, and numerous neurofibromas [92]. Piddubnyak et al. [93] examined the effect of the modulated expression of HCA66 on the apoptosis of cell lines derived from NF1-microdeleted patients. In this study, they showed that HCA66 seems to regulate apoptosis at the level of

the Apaf-1-induced activation of caspase-9 in the apoptosome following cytochrome c/dATP stimulation. Likewise, they presumed that the binding of HCA66 can also induce a conformational change that would increase the recruitment of caspase-9. Therefore, the reduced expression of HCA66 could make cell lines derived from NF1-microdeleted patients less susceptible to apoptosis. Accordingly, not only the *HCA66* gene but also all of the proteins involved in the apoptotic pathway should be considered as possible modifiers in NF1 tumors.

CONCLUSION

The borders between “single”-gene disorders and multiple-gene disorders are becoming less clear. In other words, there is no obvious clear distinction between simple Mendelian and complex traits [94]. In reality, even if there is generally one gene that is primarily responsible for the pathogenesis, one or more independently inherited modifier genes will ultimately influence the phenotype. The term "modifier gene" is thus used to define any gene that may change the disease phenotype. When we try to understand the clinical complexity of certain single-gene disorders such as NF1, it is not possible to explain all of the phenotypic variations of the genes by the allelic heterogeneity alone. Indeed, NF1 patients have a large number of *NF1* mutations with no clear genotype–phenotype correlations, except a 3-bp in-frame deletion (c.2970–2972 delAAT) in exon 17, which has been associated with a mild clinical phenotype, and microdeletions [3]. Variations in *NF1* mutations may not correlate with the variations observed in the clinical phenotype. Therefore, detection of mutations is not a big help in a clinical setting in terms of genetic counseling, since it is not possible to accurately predict the severity of disease simply by identification of the specific causal mutation. Clinical signs are variable and the phenotypic complexity of NF1 is similar to that of multifactorial diseases, including epigenetic factors and heritable elements. Some NF1 patients with the same mutation may develop severe clinical signs while others develop a mild phenotype. Moreover, some patients carrying two different pathological mutations will still have a mild phenotype [95]. The most important issue to consider in the search for modifier genes is to select a specific clinical phenotype and a relevant study population. Besides the selection of modifier genes, there are some other problems associated with the collection and bio-

banking of samples, since any variations in the collection and conservation techniques may change the results. Therefore, conflicting data exist in the literature. Considering the gene structure and the interaction of neurofibromin protein with cellular components, there are many possible candidate modifier genes. In addition, environmental factors might also contribute to the diverse phenotype observed clinically. Most of the studies dealing with modifier genes have thus far concentrated on tumors, which indeed show sub-phenotypic variations in terms of type, size, location, and the age of development. Several candidate modifier genes (Table 1) have already been studied and supporting data exist in the literature. Studies that can accurately mimic the effects of naturally occurring genetic modifiers might lead to the development of new therapeutics. Gaining a deeper understanding of the molecular basis of variable phenotypes may improve the prediction, treatment, and prevention of several NF1-related complications. These new findings will be crucial in providing more accurate genetic counseling.

Figure 1: The location of proteins interacting with neurofibromin protein has been shown (upper). The domains are shown in light blue. Proteins interacting with NF1 (ovals) are shown in ovals. The colour of the ovals are associated with functions ascribed to them. CSRD: cysteine–serine-rich domain; TBD: tubulin-binding domain; GRD: GTPase-activating protein-related domain; PH: pleckstrin homology; CTD: carboxy-terminal domain; SBD: syndecan-binding domain; DDAH1: dimethylarginine dimethylaminohydrolase 1; P: Phosphorylation, protein kinase A substrates; APP: amyloid-β precursor protein; DPYSL2: dihydropyrimidinase-related protein 2; FAF2: FAS-associated factor 2; FAK: focal adhesion kinase; LIMK2: LIM domain kinase 2; LRPPRC: leucine-rich pentatricopeptide motif-containing protein; SCF: Skp, Cullin, F-box-containing complex; VCP: valosin-containing protein.
The General location and the frequency of different types of mutations in Neurofibromin gene (NF1) has been summarized (Lower).

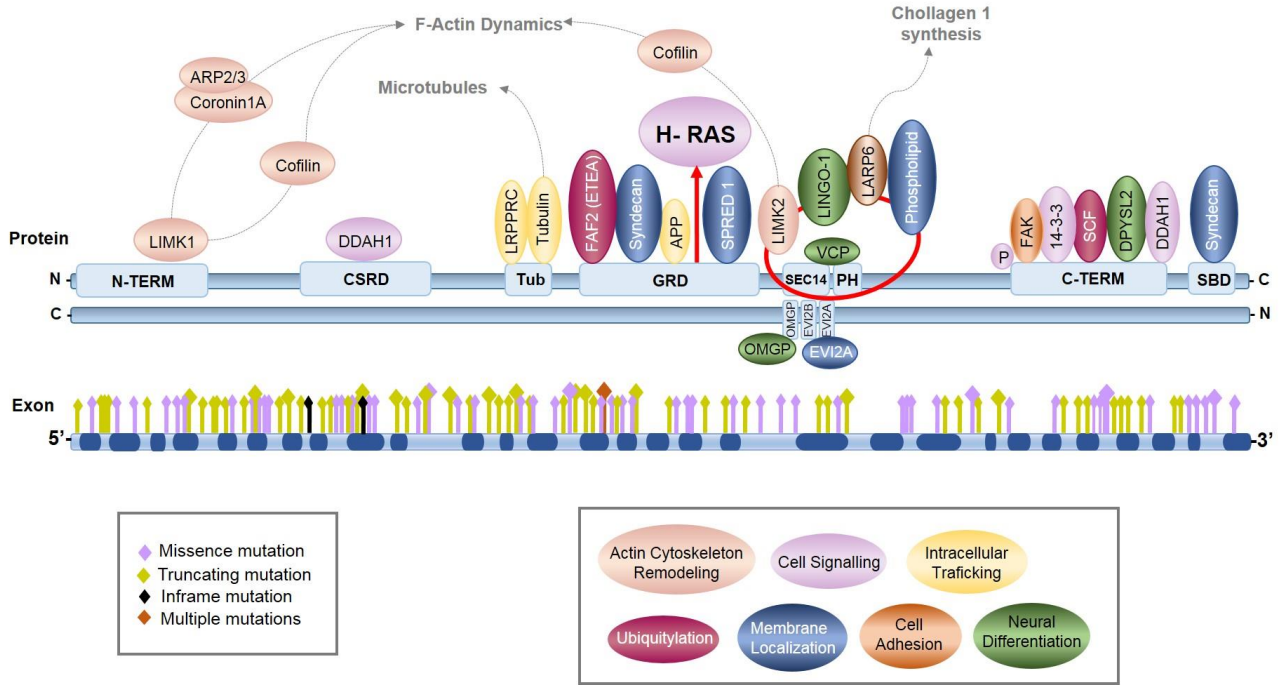
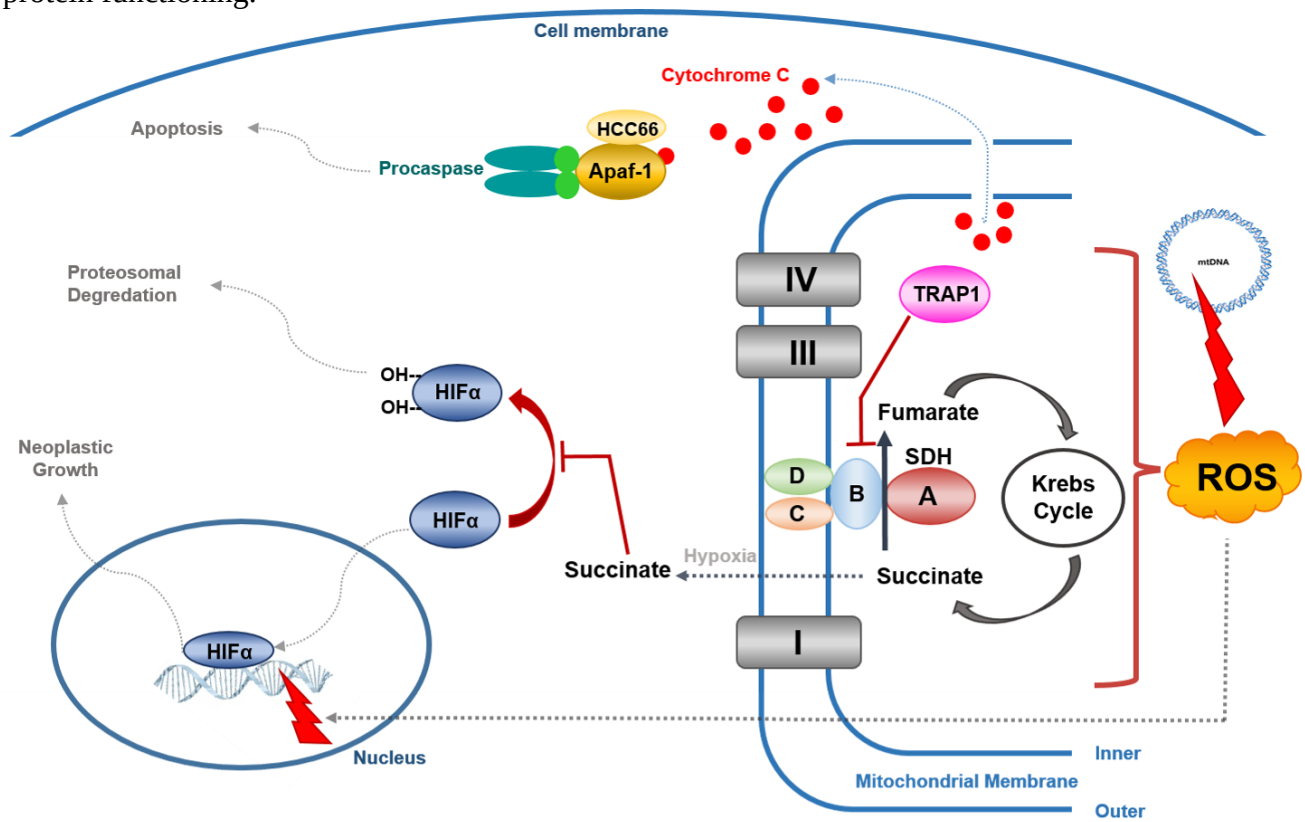


Figure 2: A summary of Mitochondrial mediated pathways which affect the neurofibromin protein functioning.



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