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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 (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 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. This review aims to provide an overview of the potential modifier genes contributing to NF1 clinical variability.

Introduction

Neurofibromatosis type 1 (NF1) (OMIM#162200) is one of the most common autosomal-dominant disorders, affecting approximately 1 in 2000–3000 (Koczkowska et al., 2018) individuals in all ethnic groups worldwide. 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, NF1 gene (OMIM# 613113; chromosome 17q11.2) is considered to function as a tumor suppressor gene. NF1 gene is comprised of 61 exons distributed over 350 kb of genomic DNA (Trovo-Marqui & Tajara, 2006). Initially identified NF1 gene contained 57 exons (Marchuk et al., 1991). In the next following years four alternatively spliced exons, temporarily numerically assigned as 9a, 10a-2, 23a, and 48a were discovered. Recently, in order to get over inconsistencies and confusion which has led by this numbering system in clinical and scientific publications, this way of exon designations of NF1 gene has been updated and named according to their location, i.e. 11alt12 (formerly 9a), 12alt13 (formerly 10a-2), 30alt31 (formerly 23a), and 56alt57 (formerly 48a) (Anastasaki, Le, Kesterson, & Gutmann, 2017). 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 (LOH) 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 oncprotein 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, EVI12A, EVI12B, and OMGP, are embedded within intron 27 b 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 (Upadhya et al., 2003). Detecting mutation causing neurofibromatosis type 1 disease is hindered by lack of mutation hotspots and a wide mutation spectrum in NF1 gene (Figure 1 (Lower)) (Valero et al., 2011; Zhang et al., 2015; Zhu et al., 2016). However, Koczkowska et al. (2018) showed a hotspot for occurring missense mutation in NF1. However, these recurrent missense mutations affect ~0.8% of unrelated NF1 mutation-positive probands. NF1 patients have intragenic NF1 mutations with no clear genotype-phenotype correlations but there are some exceptions. It has been shown that patients with 3-bp in-frame deletion (c0.2970–2972 delAAT) in exon 17 show a mild clinical phenotype with no cutaneous neurofibromas or clinically obvious plexiform neurofibromas (PNFs) (Upadhya et al., 2007). NF1 patients with severe phenotype are usually associated mostly with microdeletion (Pasmant et al., 2010).
Figure 1. Upper: The location of proteins interacting with neurofibromin protein has been shown. The domains are shown in light blue. Proteins interacting with NF1 are shown in ovals. The color of the ovals is 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-b 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. Lower: Schematic illustration and an overview on general location and the frequency of different types of mutations in NF1 gene. The origin of this data has been taken from histogram of dispersion of NF1 gene mutations in The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk).
Furthermore, it has been shown that NF1 patients with c0.5425 C>T missense variant (p.Arg1809) are associated with multiple CALM and considerably low prevalence for NF1-associated benign and malignant tumors (Pinna et al., 2015; Rojnueangnit et al., 2015). On the other hand, it has been reported recently that missense mutations at the NF1 region 844–848 codons are associated with a more severe phenotype in NF1 patients (Koczkowska et al., 2018).

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 (Praxedes, 2008), China (Zhang et al., 2015), and Korea (Ko, Sohn, Jeong, Kim, & Messiaen, 2013) that challenge this assumption. Moreover, recurrent mutations reported in previous publications are not common in Turkish patients (Terzi, Oguzkan, Anlar, Aysun, & Ayter, 2007; Terzi, Sirin, et al., 2011).

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, Terzi et al. (Terzi et al., 2007) have identified the same exon 4b mutations, 496delTG and 499delTGT TT, 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. 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 (Upadhyaya et al., 2007). In addition, patients with NF1 microdeletions have increased numbers of early-developing dermal neurofibromas (Wu, Liu, Williams, & Ratner, 2017). 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. We have learned a lot about the biology of NF1 from animal models including Drosophila (King et al., 2016), Zebrafish (Wolman et al., 2014), Mouse (Parada, Kwon, & Zhu, 2005), and Swine (Meyerholz et al., 2017). These models are helping to advance our knowledge of disease progression and the identification of biomarkers for the development of suitable therapies. Research based on preclinical work has already been translated into therapeutic trials for various NF1 features including PNFs, gliomas, malignant peripheral nerve sheath tumors (MPNST), and neurocognitive disorders. Especially MPNST models provide benefits beyond evaluating responses to targeted monotherapies and identification of biomarkers’ sensitivity. Some modifier genes like p53 (Wilde, Sassone-Corsi, Grundstrom, Zenke, & Chambon, 1984), CXCR4 (Mo et al., 2013), and CXCL12 (Sun et al., 2010) were also studied in mouse models and Drosophila (Walker & Bernards, 2014).

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 counseling. There are several reports concerning the role of modifier genes in NF1 clinical variations (Bartelt-Kirbach, Wuepping, Dodrimont-Lattke, & Kaufmann, 2009; Pemov et al., 2014; Wiest, Eisenbarth, Schmegner, Krone, & Assum, 2003). However, the selection of which phenotype to study is a key challenge in modifier gene studies.

**Modifier genes in NF1 phenotypic variation**

In this review, 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 as shown in Figure 1. Besides modifier genes, environmental factors might also contribute to the variable disease phenotype. 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, Pasman et al. (2011) performed whole-genome high-resolution array-comparative genomic hybridization of NF1-associated 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 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, Pasman’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 (Pasman et al., 2011). 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. Some of these candidate modifier genes are listed in Table 1 (the table is a modified from Ratner and Miller (2015)). 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 (Diggs-Andrews et al., 2014).

It has also been shown that adjustment in the level of cAMP can change the OPG pathway stereotypically (Bajenaru et al., 2003; Warrington et al., 2007). NF1-associated OPG can be modified by polymorphisms in the adenylate cyclase 8 (ADCY8) gene in a sex-specific manner. Warrington, Sun, & Rubin (2015) reported that the growth of human 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.

This idea was also reinforced by the observation of higher incidence of OPG in female children than in male children. Riccardi and Lupski (2013) evaluated two different groups for detecting the duplication type of gene mutations in NF1 patients; 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

Table 1. List of some modifier genes and proteins that may be important in NF1 patients. This table is modified from Ratner and Miller (2015).

<table>
<thead>
<tr>
<th>Functional category</th>
<th>Partner</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular trafficking</td>
<td>LRPPRC</td>
<td>Complex in RNA granules, trafficking</td>
</tr>
<tr>
<td></td>
<td>Kinesin-1</td>
<td>Vesicle transport, APP?</td>
</tr>
<tr>
<td></td>
<td>APP</td>
<td>Melanosone transport?</td>
</tr>
<tr>
<td>Cytoskeletal interaction</td>
<td>LIMK1/LIMK2</td>
<td>Serine-threonine kinase; Rho/ROCK actin cytoskeletal remodeling, Phosphorylate cofillin and prevent the cleavage of F-actin</td>
</tr>
<tr>
<td></td>
<td>Tubulin</td>
<td>Microtubules; inhibits NF1 Ras-GAP activity</td>
</tr>
<tr>
<td>Neuronal differentiation</td>
<td>VCP</td>
<td>AAA (ATPase); dendritic spine formation</td>
</tr>
<tr>
<td></td>
<td>CRMP-2</td>
<td>Neurite outgrowth</td>
</tr>
<tr>
<td></td>
<td>DPF5L2</td>
<td>Enzyme; interact with collapsin response mediator protein</td>
</tr>
<tr>
<td></td>
<td>OMPG</td>
<td>Downregulate role of neurofibromin on Ras, learning disability?</td>
</tr>
<tr>
<td></td>
<td>LINGO-1</td>
<td>Leucine-rich repeat and Ig-like domain-containing Nogo receptor interacting protein-1, expressed in oligodendrocytes and neurons, upregulated in CNS disease and injury</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>Focal adhesion kinase (FAK)</td>
<td>cAMP polymorphisms, change the optic glioma pathway stereotypically</td>
</tr>
<tr>
<td>Ubiquitination</td>
<td>ETEA (FAF2)</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td></td>
<td>SAG/CUL1/FBXW7 (SCF)</td>
<td>SCF E3 ubiquitin ligase complex/ubiquitination</td>
</tr>
<tr>
<td></td>
<td>Cullin 3</td>
<td>E3 ubiquitin ligase complex/Ubiquitination</td>
</tr>
<tr>
<td>Membrane localization</td>
<td>Phospholipids</td>
<td>Membrane localization?</td>
</tr>
<tr>
<td></td>
<td>Caveolin-1</td>
<td>Localization and Ras/FAK/Akt signaling</td>
</tr>
<tr>
<td></td>
<td>Syndecan</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td></td>
<td>EVI2B</td>
<td>Target of C/EBPα, NF1/neuromelanoma syndromes?</td>
</tr>
<tr>
<td>Cell signaling</td>
<td>DDAH1</td>
<td>NO/NOS regulator</td>
</tr>
<tr>
<td></td>
<td>14-3-3</td>
<td>Negatively regulates NF1-GAP activity</td>
</tr>
<tr>
<td></td>
<td>Ras</td>
<td>Signaling</td>
</tr>
<tr>
<td></td>
<td>Spred1</td>
<td>Downregulation of Ras activity</td>
</tr>
<tr>
<td>Gender</td>
<td>Adenylate cyclase 8 (ADCY8)</td>
<td>cAMP polymorphisms, change the optic glioma pathway stereotypically</td>
</tr>
<tr>
<td></td>
<td>CXCL12</td>
<td>Enhance the growth of astrocytes in females</td>
</tr>
<tr>
<td>Microdeletions</td>
<td>RNF135</td>
<td>Responsible for overgrowth of NF1 patients</td>
</tr>
<tr>
<td>DNA repair</td>
<td>Mismatch; MSH2, MSH6, MSH3, MLH1, PMS2</td>
<td>Constitutional mismatch repair deficiencies (CMMRD), cause rare childhood malignancies</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Nonallelic homologous recombination (NAHR)</td>
<td>Cause microdeletions</td>
</tr>
<tr>
<td></td>
<td>Succinate dehydrogenase (SDH)</td>
<td>Cause gastrointestinal stromal tumor (GISTs)</td>
</tr>
<tr>
<td></td>
<td>TRAP1</td>
<td>Chaperon, downregulate mitochondrial respiration by reducing the activity of SDH</td>
</tr>
<tr>
<td>Bone mass</td>
<td>Vitamin D</td>
<td>Responsible for the regulation of calcium homeostasis</td>
</tr>
<tr>
<td></td>
<td>Vitamin D receptor (VDR)</td>
<td>G/A polymorphism in intron-8 result in a shorter VDR protein and decreased VDR receptor expression which decreases 1.25(OH)2D3 activity</td>
</tr>
<tr>
<td>MicroRNA’s</td>
<td>miR-29c</td>
<td>To distinguish malignant from benign tumors</td>
</tr>
<tr>
<td></td>
<td>miR-34a</td>
<td>Direct target of p53</td>
</tr>
<tr>
<td></td>
<td>miR-214</td>
<td>Induces cell survival and cisplatin resistance</td>
</tr>
<tr>
<td></td>
<td>miR-10b</td>
<td>Transforming benign NF1-associated neurofibromas to MPNSTs</td>
</tr>
<tr>
<td></td>
<td>miR-204</td>
<td>Contributes to the growth of MPNSTs</td>
</tr>
<tr>
<td></td>
<td>miR-21</td>
<td>Important in MPNST progression</td>
</tr>
<tr>
<td></td>
<td>miR-107</td>
<td>Regulates NF1 in gastric cancer</td>
</tr>
<tr>
<td>Telomerase</td>
<td>TERT mRNA and telomerase activity</td>
<td>High-grade malignancy in NF1-associated MPNST</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Apoptotic protease activating factor-1 (Apaf-1)</td>
<td>Tumor development</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma antigen 66 (HCA66)</td>
<td>NF1-microdeletion syndrome</td>
</tr>
<tr>
<td>Others</td>
<td>INK4A/ARF and P53</td>
<td>Regulates the cell cycle, underlie the development of MPNSTs in animal models</td>
</tr>
<tr>
<td></td>
<td>Epidermal growth factor receptor (EGFR)</td>
<td>Modify neurofibroma number</td>
</tr>
<tr>
<td></td>
<td>CXCR4/CXCL12 ligand</td>
<td>Cause metastasis and progression of cancer</td>
</tr>
</tbody>
</table>
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 (Theos & Korf, 2006). Indeed, in NF1, many phenotypic features such as the development of neurofibroma are age-dependent. Pemov et al. (2014) 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 Knudson’s ‘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

EV12A, EV12B, and OMGP are embedded within intron 27b of the NF1 gene, and Viskochil (2002) proposed that the transcriptional regulation of these embedded genes might play a role in the NF1 clinical phenotype. Both EV12A and EV12B 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 (Trovo-Marqui & Tajara, 2006). NF1 patients have a higher risk of developing juvenile chronic myelogenous leukemia (OMIM #607785) compared to the general population. There are a limited number of studies in the literature related to NF1-associated leukemia (Buchberg, Bediyan, Jenkins, & Copeland, 1990; Cawthon et al., 1991; Kuhn et al., 2012; Largaespada, Shaughnessy, Jenkins, & Copeland, 1995; Paulus, Koronowska, & Folster-Holst, 2017). It is possible that mutations involving both NF1 and EV12A (or EV12B) may cause NF1/leukemia syndromes (OMIM #607785) (Shannon et al., 1994). Indeed, EV12B was identified as a direct target gene of C/EBPa, a transcription factor critical for myeloid differentiation (Zjablovskaja et al., 2017). It is possible that these genes are related to 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 EV12A and EV12B in NF1 tumors and leukemias. Our preliminary results showed the possibility of viral integrations in EV12B in NF1-acute myeloid leukemia patients (Ayet, Anlar, Varan, & Cinin, 2017).

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 (Kavale & Forness, 2000; Levine, Materek, Abel, O’Donnell, & Cutting, 2006; Moore, 2009).

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 (Upadhyaya et al., 1998).

Neurofibromin 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 (Terzi, Oguzkan-Balci, Anlar, Erdogan-Bakar, & Ayter, 2011).

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 (Vourch et al., 2003). Despite all of these findings suggesting a role of OMGP in cognition, Terzi, Oguzkan-Balci, et al. (2011) did not observe any relationship between OMGP gene mutations and learning disability in NF1. Further studies are required with large populations of NF1 patients with learning disability.

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 (Kluwe et al., 2004). Patients with NF1-microdeletion syndrome (OMIM #613675) usually have more cutaneous, PNFs and a higher risk of developing MPNST compared to patients with NF1 (Ning et al., 2016). 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 (Pasman et al., 2009). Pasman
et al. (2010) showed that learning disabilities and facial dysmorphism were significantly associated with NF1-microdeletion syndrome. Spiegel et al. (2005) proposed that overgrowth was in fact a distinctive phenotype of patients with NF1-microdeletion syndrome. Alternatively, Douglas et al. (2007) suggested that RNF135 haploinsufficiency was responsible for the overgrowth of individuals with NF1 microdeletions. The results of Pasman et al. (2010) 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. (2016) 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 (Ning et al., 2016).

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 (Albert et al., 2008). 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 (Jiricny, 2006). 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 (Wimmer, Rosenbaum, & Messiaen, 2017).

Another DNA repair mechanism that is speculated to affect NF1 is nonallelic homologous recombination (NAHR) (Messiaen et al., 2011). 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 a NAHR mechanism (Messiaen et al., 2011). Type-2 NF1 deletions (1.2 Mb) have also been reported to occur predominantly because of intrachromosomal mitotic (post-zygotic) NAHR (Roehl et al., 2012).

Indeed, some hotspots of meiotic NAHR have been identified in both types of microdeletions (Raedt et al., 2006; Vogt et al., 2012).

The role of 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 (Penta, Johnson, Wachsman, & Copeland, 2001). 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 (Alonso et al., 1997; Fliss et al., 2000; Hibi et al., 2001; Liu et al., 2001; Polyak et al., 1998; Tan, Bai, & Wong, 2002). Analysis of mtDNA mutations and comparison of the presence of somatic mtDNA mutations in NF1 tumor and non-tumor cells (Kurtz et al., 2004) 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 (Alonso et al., 1997; Fliss et al., 2000; Hibi et al., 2001; Liu et al., 2001; Polyak et al., 1998; Tan et al., 2002; Wong, Lueth, Li, Lau, & Vogel, 2003).

According to Kurtz et al. (2004), 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) (OMIM#606864) (Pasini et al., 2008; Price, Zielenska, Chilton-MacNeill, Smith, & Pappo, 2005). GISTs arise in NF1 patients 150-times more often than they do in the general population (Bajor, 2009). 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 (Wang, Lasota, & Miettinen, 2011). This observation raised the possibility that SDHB could be a candidate modifier of the phenotypic variation in NF1. Sciacovelli et al. (2013) 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 (Gilkes, Semenza, & Wirtz, 2014). 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 (Frezza & Gottlieb, 2009) to allow the
cells to grow in a hypoxic condition (Hsu & Sabatini, 2008). 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 (Selak et al., 2005). HIF1 decreases the influx of pyruvate to the Krebs cycle and activates glycolysis (Semenza, 2010). Specifically, TRAP1, an evolutionarily conserved chaperone of the heat-shock protein 90 family (Altieri, Stein, Lian, & Languino, 2012), downregulates mitochondrial respiration by reducing the activity of SDH, which in turn stabilizes HIF1 by increasing succinate levels (Sciacovelli et al., 2013).

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 (Holmstrom & Finkel, 2014). To prohibit the harmful effect of ROS, a scavenging program that can protect tumor cells from cell death is elicited (Trachootham, Alexandre, & Huang, 2009). The inhibition of SDH, which is the main site of ROS generation, with TRAP1 can have an anti-apoptotic effect (Grimm, 2013). Notably, mutations in the SDH gene resulting in LOH are extremely rare. Nevertheless, TRAP1 has many post-translational modifications (Rasola, Neckers, & Picard, 2014; Yoshida et al., 2013), and any defect in these modifications can affect SDH enzymatic activity (Guzzo, Sciacovelli, Bernardi, & Rasola, 2014). 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, Masgras et al. (2017) 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 (Calvo, Whiting, & Barton, 2005; Holick, 2004). 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) (Heaney, Horst, Cullen, & Armas, 2009).

Vitamin D is responsible for the regulation of calcium homeostasis, which plays a critical role in bone metabolism (Holick, 2004). Approximately 10% of NF1 patients show focal bony abnormalities (Illes, Halmai, de Jonge, & Duboussset, 2001), severe osteomalacia (Brunetti-Pierri et al., 2008), and reduction in bone mass (Dulai et al., 2007), 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) (Lammert et al., 2005). Nakayama et al. (1999) observed some significant reduction in the pigmentation 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 (Lammert et al., 2006; Tucker et al., 2009). However, Schnabel et al. (2014) 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) (Miyamoto et al., 1997). Different series of polymorphisms have been reported in the VDR genes, which alter the biological processes of this receptor (Morrison, Yeoman, Kelly, & Eisman, 1992). Two of these polymorphisms were identified as a T/C transition located in a start codon (ATG) (Uitterlinden, Fang, Van Meurs, Pols, & Van Leeuwen, 2004) and a G/A polymorphism located on intron-8, which were detected using the FokI and BsmI restriction enzymes, respectively (Ingles et al., 1997). These two polymorphisms result in a shorter VDR protein with decreased expression. Bueno et al. 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 (Souza Mario Bueno et al., 2015). However, Souza Mario Bueno et al. (2015) did not detect correlations between low vitamin D levels and VDR gene polymorphisms and deregulation of osteoblast and osteoclast activity of NF1 patients.

MicroRNA’s (miRNA’s)

Approximately 20 years ago, miRNAs were discovered as new regulatory factors of the genome. This mechanism has greatly contributed to gain 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 (Bartel, 2004).

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 (Sedani, Cooper, & Upadhyaya, 2012).

There are several hundred 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 (Chen et al., 2012). Weng, Chen, Chen, Liu, and Bao (2013) 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 tumorogenesis to date, including miR-29c (used to distinguish malignant from benign tumors (Wang et al., 2011), miR-34a (a direct target of p53 (Subramanian et al., 2010), miR-214 (induces cell survival and cisplatin resistance (Yang et al., 2008), miR-10b (transforms benign NF1-associated neurofibromas to MPNSTs (Subramanian et al., 2010), miR-204 (contributes to the growth of MPNSTs (Gong et al., 2012), miR-21 (important in MPNST progression (Itani et al., 2012), and miR-107 (regulates NF1 in gastric cancer (Wang et al., 2016). Nevertheless, there is still much work to be done to gain a detailed understanding of the actual function and pathway of miRNAs in NF1 (Sedani et al., 2012).

Additional gene mutations in NF1-associated tumors

For the development of malignant transformation, especially in NF1-related MPNSTs, besides 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 (Cichowski et al., 1999). The loss of the P53 gene is responsible for the abnormalities in DNA damage-dependent cell cycle arrest and apoptosis. Mutations in p53 underlie the development of MPNSTs in animal models, but there are controversies remaining related to the role of P53 mutations in human MPNSTs (Gottfried, Viskochnik, & Couldwell, 2010; Legius et al., 1994).

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. (2017) 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. Karaozamanoglu et al. (2018), in her thesis studies, 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 up to 120-fold and the CXCL12 gene expression level was increased up to 512-fold in all tumors relative to normal human Schwann cells.

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 (Greider, 1998). The enzyme telomerase comprises two subunits: telomerase RNA and the telomerase reverse transcriptase (TERT) (Kilian et al., 1997). 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 (Kim et al., 1998). 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 (Jones, Grimstead, Sedani, Baird, & Upadhyaya, 2017; Mantripragada et al., 2008).

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 (Igney & Kramer, 2002). 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 (De Raedt et al., 2004). Patients with NF1-microdeletion syndrome have a distinct phenotype with a poor prognosis characterized by a low IQ, dysmorphic features, and numerous neurofibromas (Leppig et al., 1997). Piddubnyak et al. (2007) 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 (Dipple & McCabe, 2000). 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 with some exceptions as mentioned before (Koczkowska et al., 2018; Pasmany et al., 2010; Pinna et al., 2015; Rojnowegnit et al., 2015; Upadhyaya et al., 2007). 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 (Terzi et al., 2012). 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 biobanking 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 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.

Disclosure statement

The authors declare that they have no conflict of interest.

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