

ORIGINAL ARTICLE

A NOVEL DISEASE-CAUSING *NF1* VARIANT IN A CROATIAN FAMILY WITH NEUROFIBROMATOSIS TYPE 1

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Abstract: Neurofibromatosis type 1 (NF1) is the most common autosomal dominant neurocutaneous syndrome with the estimated prevalence ranging from 1 in 3000 to 1 in 4000 individuals and wide phenotypical variability. NF1 is caused by autosomal dominant heterozygous mutations in the neurofibromin gene which is located on the chromosome 17 (17q11.2). Phenotypically, NF1 patients have a very heterogeneous clinical phenotype. In this study, a novel frameshift NF1 variant was identified in a Croatian family with NF1 (mother and two daughters). The novel variant c.4482_4483delTA leads to sequence change that creates a premature translational stop signal (p.His1494Glnfs*7) in the NF1 gene. Our study showed that even when the same germline NF1 variant has been identified, there is still huge phenotypic variability in patients even within the same family, and it makes prognosis of the disease more complex. The development of next-generation sequencing technologies which allow rapid and accurate identification of disease-causing mutations becomes crucial for molecular characterization of NF1 patients as well as for patient follow-up, in the context of genetic counseling and clinical management of patients.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is the most common autosomal dominant neurocutaneous syndrome with the estimated prevalence ranging from 1 in 3000 to 1 in 4000 individuals and wide phenotypical variability.^{1, 2} Clinical diagnosis of NF1 is suspected with the appearance of the following major features: occurrence of café-au-lait macules, Lisch nodules of the iris, cutaneous and plexiform neurofibromas, axillary freckling and skeletal abnormalities.³ Phenotypically, NF1 patients have a very heterogeneous clinical phenotype. Besides skin lesions as the most noticeable manifestation, NF1 may affect many organs and cause psychiatric and psychological disorders.^{4, 5} NF1 is caused by autosomal dominant heterozygous mutations in the neurofibromin gene which is located on the chromosome 17 (17q11.2). The NF1 gene is a tumor suppressor with the function of stimulating the GTPase activity of the RAS protein serving as a negative regulator of the cellular Ras/MAPK (mitogen-activated protein kinases) signaling pathway.^{6, 7} More than 3000 different pathogenic allelic variants have been identified in the NF1 gene so far (The Human Gene Mutation Database), with half of the variants arising *de novo*, which is an expected observation since NF1 has one of the highest mutation rates reported in humans.⁸ Molecular diagnosis in NF1 should be of great value for confirming the diagnosis. However, the large size of the gene (257 Kb), its high mutation rate, the existence of 15 pseudogenes and no mutation hot-spots present a big challenge, and, therefore, molecular testing of the NF1 gene is usually time-consuming and expensive.⁹⁻¹¹ The development of next-generation sequencing (NGS) technologies which allows for rapid identification of disease-causing mutations and high-risk alleles has recently been introduced into NF1 diagnosis.¹²⁻¹⁵

MATERIAL AND METHODS

Study subjects, disease criteria, and clinical evaluation

A family (mother and two daughters) with unusual clinical presentations of NF1 were recruited at the Clinical Hospital Center Zagreb for diagnostic analysis of the NF1 gene (Figure 1). The NF1 diagnosis was established based on the diagnostic criteria of the National Institutes of Health consensus statement.¹⁶ Peripheral blood specimens were collected from the patients. Clinical data including available medical histories, imaging, and histopathological examinations were obtained. The study was carried out in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participating individuals.

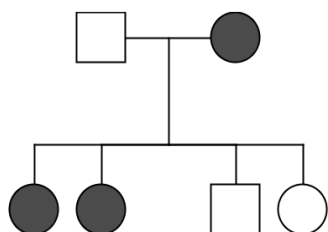


Figure 1. Pedigree of the family.

Legend: Squares indicate males, circles indicate females, respectively; open symbols indicate unaffected individuals, filled symbols indicate affected individuals.

Targeted gene enrichment and high-throughput sequencing

The gene panel was designed using the Design Studio Tool (Illumina, San Diego, CA, USA). The coding regions of 142 genes were selected for targeted gene enrichment. The coordinates of genomic regions were based on NCBI build 37 (UCSC hg19). Total DNA was extracted from peripheral blood samples using the Zymo Universal DNA kit (ZR; Zymo Research) according to the manufacturer's instructions. The DNA concentration of each sample was determined using a Qubit 3 Fluorimeter and the dsDNA HS kit (Invitrogen, Thermo Scientific, Wilmington, DE, USA). Custom targeted gene enrichment and DNA library preparation were performed using the Nextera Rapid Capture Custom Enrichment kit (Illumina) according to the manufacturer's instructions. The targeted regions were sequenced using the Illumina MiSeq platform, generating approximately 14 million of 150-bp paired-end reads for each sample (Q30 \geq 90%).

Variant calling, filtering, and classification

The FASTQ files generated by the MiSeq were streamed to Illumina BaseSpace where the data was assembled

with the BWA Genome Alignment Software and the variants called according to the GATK Variant Caller. This produced a Variant Call Format (.VCF) file, which was further imported into Illumina Variant Interpreter. Variants were considered disease-causing under strict criteria in accordance with the published Sherlock guidelines for the interpretation of sequence variants. After sequencing data submission, the pipeline executed the following steps: the quality checks and filter of the reads; the alignment on the reference genome hg19; variant preprocessing; coverage statistics and metrics; variant calling; variant annotation. Genetic variants predicted to alter the protein, such as non-synonymous variants, nonsense variants, canonical splicing site variants (affecting the donor or acceptor splice sites), in-frame and frameshift insertion/deletions were selected. To assess the potential functional impacts of variants, two bioinformatics algorithms were used: Sorting Intolerant From Tolerant (SIFT) and Mutation Taster. Using the online multiple protein sequence alignment tool COBALT we analyzed the conserved domain among *Homo sapiens* (human), *Rattus norvegicus* (Norway rat) and *Mus musculus* (mouse) to see whether the NF1 mutation was located in the conserved region of the human NF1 protein.

Variant databases and prediction programs

The list of variant databases and prediction programs used in the study is presented in Table 1.

Table 1. List of variant databases and prediction programs

Variant databases:
1000 Genomes Project http://browser.1000genomes.org/index.html
NCBI dbSNP http://www.ncbi.nlm.nih.gov/SNP/
Exome Variant Server http://evs.gs.washington.edu/EVS/
Genome Aggregation Database (gnomAD) http://gnomad.broadinstitute.org
ClinVar http://www.ncbi.nlm.nih.gov/clinvar/
Human Genome Mutation Database (HGMD) http://www.biobase-international.com
Prediction programs:
SIFT https://sift.bii.a-star.edu.sg/www/SIFT_indels2.html
Mutation Taster http://www.mutationtaster.org/
COBALT https://www.ncbi.nlm.nih.gov/tools/cobalt/

RESULTS

Clinical Features

Our first patient is a woman with positive diagnostic criteria for NF1. Her two daughters that we also describe later in the text have positive diagnostic criteria for NF1.

Family history is positive for NF1 (her mother and grandfather had numerous café-au-lait spots and cutaneous neurofibromas). At the age of 45, she was admitted to hospital because of a large tumorous mass on the right side of the head and neck. Besides a large, hard and elastic subcutaneous tumor mass on the right side of the head and neck, physical examination revealed numerous café au lait spots, cutaneous and subcutaneous neurofibromas and axillary freckling. Large (around 5 cm in diameter), well-circumscribed spherical tumorous mass, located between the internal carotid artery and the internal jugular vein, reaching the jugular foramen in the cranial direction was found intraoperatively. It was hardly detachable from the vagal nerve. Pathohistological examination revealed an encapsulated tumorous mass, 5.5x4x3.5 cm in diameter, histologically consisting of multiplied stromal cells, elongated and spindle-shaped, moderately polymorphic, with a few areas of high cellularity. Some cells that resembled ganglionic cells were also present. The tumor stroma was abundant and collagenous. Some areas showed the presence of cartilage tissue and necrosis. Immunohistochemical staining showed focal S-100 positivity. Diagnosis of malignant peripheral sheath tumor was established. Radiation therapy was administered postoperatively. Positron emission tomography scan performed 4 months postoperatively showed no signs of tumor dissemination. A brain MRI performed 1 year postoperatively revealed focal areas of signal intensity (FASI) in the anteromedial part of right thalamic nuclei, splenium of corpus callosum and bilaterally in both dentate nuclei of the cerebellum - all lesions typically found in NF1 patients. Currently, the patient is followed up regularly and she is without signs of tumor relapse.

The second patient is the older daughter with positive diagnostic criteria for NF1, delayed psychomotor development and suspected convulsions. She was born from an unremarkable pregnancy and labor. The pediatrician noticed some hypotonia and delayed psychomotor development in the infancy period and later. Language development was markedly delayed. In infancy she had several episodes of generalized convulsions that resembled affective crises and the EEG was repeatedly normal. A brain MRI was performed at the age of 12; it revealed FASI bilaterally in both dentate nuclei of cerebellum. At the age of 13 she was diagnosed with thoracic kyphoscoliosis. The MRI of thoracic spine revealed dural ectasia at the level of anterior coalition of the body of the 6th and 7th thoracic vertebra with hypoplastic intervertebral discus and arcuate kyphosis. Ophthalmologic examination revealed multiple Lisch nodules of the iris. Physical examination performed at the age of 13 showed short stature (3rd centile), sinistroconvex kyphoscoliosis of thoracic spine, café au lait spots on the front and back of the chest and several subcutaneous neurofibromas. Signs of mild cognitive impairment were also present.

The third patient is the younger daughter with positive diagnostic criteria for NF1 and very rare presentation of



Figure 2. Magnetic angiography in the younger daughter: magnetic angiography showed left frontal, temporal and parietal pial angiomatosis and a hypoplastic spheroidal and opercular segment of the left middle cerebral artery (MCA), with many collaterals of lenticular and thalamostriate arteries.



Figure 3. Digital subtraction angiography: DSA showed bilateral stenosis of the internal carotid artery. Catheterization of the right common carotid artery and right inner carotid artery in the younger daughter showed terminal part stenosis of the right inner carotid artery (ICA) immediately before bifurcation (arrow); Filiform filling of the residual part of the right inner carotid artery lumen with a weaker filling of the distal segments of the circulation, i.e. right middle cerebral artery and right (MCA) and anterior cerebral artery (ACA).

NF1, moyamoya syndrome (MMS). She was born from an uneventful pregnancy and labor. Her mother noticed café au lait spots already in the infancy period. Her psychomotor development was moderately delayed, especially in the domain of cognitive and language development. At the age of 3, she experienced the first episode of epileptic seizures and she was given valproate as an antiepileptic drug. At the age of 4, she was diagnosed with occlusive cerebral angiopathy; MMS of the left internal carotid artery, middle cerebral artery, right internal carotid artery, right anterior and middle cerebral artery (Figure 2, Figure 3). Soon after the diagnosis, she experienced a transitory ischemic attack with left hemisyndrome, and she was neurosurgically treated in the foreign center (direct and indirect revascularization of affected blood vessels). After the operation her clinical state is stable, without new deficits in neurological examination and new epileptic attacks. Her school performance is very poor and there are signs of low moderate cognitive impairment. Physical examination at the age of 10 revealed short stature (3rd centile), numerous café au lait spots, several subcutaneous neurofibromas and signs of moderate cognitive impairment.

Genetic analysis

Targeted sequencing of the three probands generated a mean of 14 million total effective reads, with an average of 99.88% mapping to the reference genome. The average sequencing depth on the target sequence region per individual was tenfold. 230 SNPs and 2 InDels were detected in the proband 1, 234 SNPs and 2 InDels were detected in the proband 2, 231 SNPs and 3 InDels were detected in the proband 3. Commonly known variants, documented in the 1000 Genomes Project, dbSNP, NHLBI ESP6500 along with synonymous variants, were removed in order to find the candidate disease-causing variants. *In silico* programs were employed to predict the possible effects of nonsynonymous variants on protein functions. A heterozygous frameshift variant c.4482_4483delTA (p.His1494GlnfsTer7) in the *NF1* gene was selected as a potential disease-causing variant for the probands of pedigree 1,2 and 3. Predicted pathogenicity analyses performed by different *in silico* programs (MutationTaster and SIFT) indicated that this variant was deleterious. After aligning the three protein

sequences for the conserved region, this shared NF1 frameshift mutation was discovered to be located in a conserved region (Figure 4).

DISCUSSION

In this study, a novel frameshift NF1 variant was identified in a Croatian family with NF1 (a mother and two daughters). The novel variant c. 4482_4483delTA leads to sequence change that creates a premature translational stop signal (p.His1494Glnfs*7) in the NF1 gene. This variant is not present in population databases (ExAC no frequency). Loss-of-function variants in NF1 are known to be pathogenic.^{17, 18} Multiple sequence alignment programs showed that the residue p.His1494 is phylogenetically conserved, and *in silico* programs indicated that they are damaging.

The variant is located in the GTPase-activating protein-related domain (GRD) of the protein, the best characterized functional domain of neurofibromin. Several studies have shown that the GRD domain has Ras-GAP activity both *in vitro* and *in vivo*.¹⁹ Current data suggest that about 30–65% of patients with NF1 have specific learning deficits.²⁰ Although a direct correlation between specific mutations in NF1 and phenotypes has not been established, missense mutation that disrupts the Ras-GAP function of NF1 was found in patients with multiple symptoms including learning disability and cognitive impairment.²¹ In general, in most NF1 patients with learning difficulties global cognitive impairment is not a very common feature.²² Although carrying the same variant, our three patients show a range from normal cognitive function in the mother and mild cognitive impairment in the older daughter to moderate cognitive impairment in the younger daughter.

Only a few NF1 genotype-phenotype correlations have been identified due to its wide phenotypic diversity and the extreme variability of the mutation spectrum. The clearest genotype-phenotype correlation show gross constitutional deletions of the NF1 gene associated with a severe form of the disease and increased susceptibility to malignant peripheral nerve sheath tumors (MPNST).^{23, 24} It was recently shown that patients with missense mutations in codons 844-848 have a high prevalence of a severe phenotype, including plexiform

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P21359.2 1439 LKLMSKILQSIANHVLFTKEEHRPFDNFVKSNFDAARRFFLDIASDCPTSDAVNHSLSFISDGNVLAHRLLLWNNQEKI 1518
P97526.1 1420 LKLMSKVLQSIANHVLFTKEEHRPFDNFVKSNFLARRFFLDIASDCPTSDAVNHSLSFISDGNVLAHRLLLWNNQEKI 1499
Q04690.1 1441 LKLMSKVLQSIANHVLFTKEEHRPFDNFVKSNFLARRFFLDIASDCPTSDAVNHSLSFISDGNVLAHRLLLWNNQEKI 1520
PROBANDS 1439 LKLMSKILQSIANHVLFTKEEHRPFDNFVKSNFDAARRFFLDIASDCPTSDAVNSFLHKSTOP

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Figure 4. Location of the frameshift variant detected in mother and two daughters.

The mutated site was referred against NF1 or NF1 homolog gene from *Homo sapiens* (P21359.2), *Rattus norvegicus* (P97526.1) and *Mus musculus* (Q04690.1). The position of the frameshift mutation marked by an arrow; black letters show altered amino acids caused by the frameshift variant.

and symptomatic spinal neurofibromas, symptomatic optic pathway gliomas, other malignant neoplasms, as well as bone abnormalities.²⁵ Patients with deletion of Met 992 or missense mutation of Arg 1809 lack plexiform or cutaneous neurofibromas making these two germline mutations associated with milder disease outcomes in NF1.²⁶⁻²⁸ Our patients showed intrafamilial phenotype variability: mother with rare malignant peripheral sheath tumor, older daughter with usual clinical presentation and younger daughter with very rare MMS and early severe presentation. This suggests that NF1 clinical phenotypes may be influenced not only by NF1 variants, but also by other factors, including second hit mutations, mosaicism, genetic modifiers, epigenetic and environmental factors.^{27, 29, 30} Studies of twins with NF1 have revealed that each major symptom associated with NF1 is likely to be affected by distinct genetic modifiers.³¹

In our first patient (the mother), diagnosis of MPNST was established. MPNST are biologically aggressive soft tissue sarcomas that are challenging to treat effectively. About 10% of NF1 patients develop MPNST, and it is known that this type of tumors have high metastatic potential and poor prognosis.^{32, 33} Large tumor size at presentation (typically >5 cm) has been the most consistently determined adverse prognostic factor.³⁴ Early diagnosis and surgery offer the most efficiency in treatment so far, chemotherapy and radiotherapy show no clear benefits for patient survival. Currently our patient is followed up regularly and she is without signs of tumor relapse. Discovery of the biomarkers for early detection of MPNSTs would be of great importance. As seen in our case presentation, it is hard to predict whether two daughters have a higher risk of developing MPNST. Besides biomarkers for early detection, patients with MPNSTs would benefit from accurate molecular prognostics markers. Study from Zou and colleagues showed that the expression level of p53 was significantly associated with a worse outcome.³⁵ Another study detected the AKT and TOR pathway, activated in a broad range of malignancies including sarcoma, with a negative prognosis proposing the inhibition of mTOR as a potential treatment target for both NF1-related and sporadic MPNSTs.³⁶ MET activation has been suggested as a molecular marker of inferior prognosis which is in line with data showing that MET targeting inhibits invasive phenotype of MPNST cells both *in vitro* and *in vivo*.³⁷

MMS is a rare cerebrovascular disorder developing by stenosis and occlusion of small anastomotic vessels in the distal branches of bilateral internal carotid arteries. MMS occurs in 2.3-6% of children with NF1. Symptoms include neurological findings such as epileptic seizures, headache, paresthesia, dysphasia, nystagmus, aphasia, and borderline mental level.^{35, 36} There are certain evidence indicating that MMS is related to genetic factors in familial cases, although the involvement of the NF1 gene in the occurrence of MMS remains controversial.³⁷ NF1 and MMS could be associated by close proximity of the responsible genes

on chromosome 17.³⁸ Our patient was diagnosed with MMS at the age 4, which is slightly earlier than observed in previous patients (mean age between 5.2 and 11.4 years). Apart from NF1, MMS is rarely observed in connection with other RASopathies. There is only one report on the genetic background of patients with NF1 showing no correlation between the NF1 genotype and MMS phenotype.³⁹

In conclusion, we identified a novel frameshift variant c. 4482_4483delTA in the NF1 gene in a mother and two daughters. Our study showed that even when the same germline NF1 variant has been identified, there is still huge phenotypic variability in patients, and it makes prognosis on the disease more complex. The development of next-generation sequencing technologies which allow rapid and accurate identification of disease-causing mutations becomes crucial for molecular characterization of NF1 patients as well as for patient follow-up in the context of genetic counseling and clinical management of patients.

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