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Molecular and Clinical Overview of Type 1 Neurofibromatosis: Single Center Study and Mini Review on NF1-Associated Vasculopathy and Juvenile Myelomonocytic Leukemia

Tip 1 Nörofibromatozise Moleküler ve Klinik Genel Bakış: NF1 ile İlişkili Vaskülopati ve Jüvenil Miyelomonositik Lösemi Üzerine Tek Merkez Çalışması ve Kısa Gözden Geçirme

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ABSTRACT

Objective: Neurofibromatosis type 1 (NF1) is a genetic disorder presenting primary with variable patterns of skin pigmentation, neurofibromas and iris Lisch nodules. In addition, likely pathogenic/pathogenic mutations of the *NF1* gene predispose to multiple tumors. Juvenile myelomonocytic leukemia (JMML) is also associated with NF1. Molecular diagnosis is important in patients with an atypical presentation, as well as in children who have not yet developed sufficient characteristic features or for providing prenatal diagnosis. The purpose of this study was to define *NF1* gene mutations in the northeastern part of Türkiye and to contribute to the mutational spectrum of NF1. In addition, rare findings, such as cerebral vasculopathy and JMML, were discussed over the phenotypic findings.

Methods: In this study, *NF1* gene sequence analysis was performed using next-generation sequencing in 32 unrelated Turkish patients with a prediagnosis of NF1.

Results: Disease-causing variants were found in 68.75% (n=22/32) of the patients, whereas two of them were novel. Our study was also important in the aspect of vasculopathy regarding the frequency which was 9.1% of in a relatively small patient group. Another aspect was the distinct distribution of malignant tumors. In contrast to central nervous system malignancies, which are the most common malignancies apart from malignant peripheral nerve sheath tumors in the literature, JMML was the most common in our study.

Conclusion: The aim of this study is to draw attention to rare symptoms, such as vasculopathy and JMML, in NF1 in a small cohort. Although

ÖZ

Amaç: Nörofibromatozis tip 1 (NF1), sıklıkla değişken deri pigmentasyonları, nörofibromlar ve iris Lisch nodülleri bulguları gösteren genetik bir hastalıktır. Ayrıca *NF1* genindeki olası patojenik/patojenik mutasyonlar tümör gelişimine zemin hazırlarlar. Juvenil miyelomonositik lösemi (JMML) de NF1 ile ilişkilidir. Atipik prezentasyonlu hastalarda ve yeterli karakteristik bulguları ortaya çıkmamış çocuklarda veya prenatal tanının sağlanmasında moleküler tanı önemlidir. Bu çalışmanın amacı Türkiye'nin kuzeydoğusundaki *NF1* gen mutasyonlarını tanımlamak ve NF1'in mutasyon spektrumuna katkıda bulunmaktır. Ayrıca NF1'in serebral vaskülopati ve JMML gibi nadir görülen bulguları da fenotipik bulgular üzerinden tartışılmıştır.

Yöntemler: Bu çalışmada NF1 ön tanısı ile, aralarında akrabalık olmayan 32 Türk hastada yeni nesil dizileme tekniği ile *NF1* geni dizi analizi yapıldı.

Bulgular: Hastaların %68,75'inde (n=22/32) hastalığa neden olan varyant saptanırken, bunlardan ikisi novel idi. Çalışmamız aynı zamanda nispeten küçük bir hasta grubunda %9,1 oranında görülen vaskülopati açısından da önemliydi. Diğer bir önemli sonuç ise malign tümörlerin dağılımı idi. Literatürde malign periferik sinir kılıfı tümörleri dışında en sık görülen maligniteler santral sinir sistemi maligniteleri iken bizim çalışmamızda en sık JMML görüldü.

Sonuç: Bu çalışmanın amacı küçük bir kohortta NF1'de vaskülopati ve JMML gibi nadir görülen bulgulara dikkat çekmektir. JMML nadir görülen bir çocukluk çağı kanseri olmasına rağmen RASopatilere

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ABSTRACT

JMML is a rare childhood cancer, it is accompanied by RASopathies. It is important to investigate this association because JMLL treatment approaches change in the presence of germline mutations.

Keywords: Neurofibromatosis type 1, NF1, cerebrovascular stenosis, leukemia, JMML

ÖZ

eşlik eder. JMLL ile RASopati ilişkisinin araştırılması oldukça önemlidir çünkü germ hattı mutasyonlarının varlığında JMML tedavi yaklaşımları değişmektedir.

Anahtar Sözcükler: Nörofibromatozis tip 1, NF1, serebrovasküler stenoz, lösemi, JMML

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most frequent human genetic disorders. The incidence of NF1 is 1 per 2000 and 3000 births worldwide (1,2). Heterozygous pathogenic variants of the *NF1* gene cause this disease (3,4). The most common symptoms are variable skin pigmentation, multiple benign neurofibromas and iris Lisch nodules. Plexiform neurofibromas, malignant peripheral nerve sheath tumors, optic nerve and other central nervous system gliomas, vasculopathy, scoliosis, tibial dysplasia, and learning disabilities are less common but serious manifestations of NF1 (3).

The diagnosis of NF1 can be made in an individual who does not have a parent diagnosed with NF1 if two or more of the following are present according to revised diagnostic criteria for NF1: Six or more café-au-lait macules (CALMs) (>5 mm in greatest diameter in prepubertal individuals and >15 mm in greatest diameter in postpubertal individuals), freckling in the axillary or inguinal region [at least one of the two pigmentary findings (CALMs or freckling) should be bilateral], two or more neurofibromas of any type or one plexiform neurofibroma, optic pathway glioma, two or more iris Lisch nodules identified by slit lamp examination or two or more choroidal abnormalities defined as bright, patchy nodules imaged by optical coherence tomography/near-infrared reflectance imaging, a distinctive osseous lesion such as sphenoid dysplasia, anterolateral bowing of the tibia, or pseudarthrosis of a long bone, a heterozygous pathogenic NF1 variant with a variant allele fraction of 50% in apparently normal tissue such as white blood cells. In addition, a child of a parent who meets the diagnostic criteria specified above merits a diagnosis of NF1 in the presence of one or more of the criteria are present (5). The majority of patients have only mild manifestations of NF1, but symptoms increase with age. Because many features of NF1 increase in frequency with age, most patients without a family history meet the criteria by the age of eight (3,5). Findings that are common and can be determined objectively by many physicians are included in the diagnostic criteria. However, there are many findings other than those specified in the diagnostic criteria. Of these findings, juvenile myelomonocytic leukemia (JMML) and vasculopathy are critical findings for patient management. Therefore, it is important to examine patients for symptoms in all systems that may accompany NF1, apart from the diagnostic criteria (1).

NF1 is characterized by extreme clinical variability both among patients and within families (3,6). Modifier genes and/or environmental factors are possible reasons for this variability (7).

The average life expectancy is reduced in patients with NF1 compared to general population, and vasculopathy and malignant peripheral nerve sheath tumors are the most important causes of early death (3).

The diagnosis of NF1 is established by "revised diagnostic criteria for NF1". Molecular genetic testing is necessary in patients with an atypical presentation or in children who have not developed the most characteristic features to provide prenatal diagnosis (3).

The *NF1* gene comprises 60 exons and spans 350 kb of genomic DNA (3). More than 2500 mutations have been reported in the *NF1* gene in the human gene mutation database. Although diverse mutations are observed, most cause loss-of-function of the gene product and protein truncation (8). Among these, 5% were deletions encompassing the entire *NF1* locus (9). Almost half of all affected individuals have NF1 because of *de novo* mutations. Many genetic changes are unique to a particular family (3).

NF1 encodes neurofibromin, which is expressed in many tissues, but highly in neurons, non-myelinating Schwann cells, oligodendroglia cells, and dorsal root ganglia (10). Neurofibromin is an Ras GTPase-activating protein (GAP), which down regulates ras protein activity. Therefore, GAP acts as a tumor suppressor by controlling cellular proliferation. Several mutations diminish GAP activity, stimulate cellular proliferation, indicating the importance of RAS regulation in NF1 (4,6). Due to its effects on Ras-MAPK signaling via GAP, NF1 has been considered in RASopathy syndromes (4). In this study, patient data for which the diagnosis of NF1 was confirmed via mutation analysis were discussed. The aim of this study was to identify *NF1* gene mutations in northeastern Türkiye and to contribute to the mutational spectrum of NF1. In addition, rare findings, such as cerebral vasculopathy and JMML, were discussed over the phenotypic findings.

MATERIALS AND METHODS

Patients

A total of 32 patients with a pre-diagnosis of NF1 who were consulted to the medical genetics departments of the University of Health Sciences Türkiye, Trabzon Kanuni Training and Research Hospital and Karadeniz Technical University Faculty of Medicine, from 2017 to 2019, were taken to the study. All patients were born in northeast Türkiye. In addition, they were descended from families in the same region. This study was approved by the Institutional Ethics Committee by University of Health Sciences Türkiye, Trabzon Kanuni Training and Research Hospital (approval number: 2019/22, date: 25.04.2019). Informed written consent was obtained from the patient's parents. Each patient underwent a detailed evaluation by a medical geneticist. Prenatal and birth histories as well as family history were recorded, and pedigree analysis besides detailed physical examination, was performed. Dysmorphic features were evaluated in detail and noted. Cranial magnetic resonance imaging (MRI) was offered to all patients, but only pathological findings

were mentioned in the neurologic finding section of Table 2. On the other hand, MR angiography was planned in cases with any clinical suspicion of cranial vascular pathology.

Peripheral blood samples were collected from the patients and their parents, if possible. Patient DNA were extracted by QIAcube® automated DNA isolation system (Qiagen) according to the manufacturer's instructions.

All coding exons, including flanking intron regions, of the *NF1* gene were amplified by polymerase chain reaction. After library enrichment (MiSeq Reagent Kit v2, MS-102-2003) and quality control, the samples were sequenced using the MiSeq platform (Illumina, San Diego, California, United States). Raw reads were quality-trimmed with Trimmomatic and mapped to the reference human genome (hg19) with using Burrows-Wheeler Alignment Tool. Duplicates were removed using SAMTools and realignment across indels and base quality recalibration were performed with GATK.

Sanger sequencing was used to confirm low-quality variants, insertions/deletions, and splice site alterations on Applied Biosystems 3130 Genetic Analyzer. In addition, Sanger sequencing was used for segregation analysis.

Statistical Analysis

Descriptive study. Statistical analysis were not performed.

Data analysis and variant classification: The sequence was analyzed from both forward and reverse *stands* and compared with the *NF1* reference sequence NM_001042492.3 (https://www.ncbi.nlm.nih.gov/nuccore/NM_001042492). Reads were analyzed using the Integrative Genomics Viewer program, and read alignment was done according to the hg19/GRCh37 human reference genome. A minimum of 100x coverage was accepted read depth. Variants with a frequency higher than 0.5% were filtered out.

The identified variants were queried by browsing the HGMD (8), ClinVar (11) databases. Common variants were excluded by minor allele frequency (MAF >1) score by using 1000 Genomes Project (http://www.1000genomes.org/), gnomAD (https://gnomad.broadinstitute.org/) and NCBI dbSNP (database of Single Nucleotide Poylmorphisms https://www.ncbi.nlm.nih.gov/snp/) database. In silico prediction algorithms [PolyPhen2 (12), SIFT (13), MutationTaster software (14), and conservation scores (PlyloP 100)] (https://ccg.epfl.ch/mga/hg19/phylop/phylop.html) were used to interpret novel variants. The American College of Medical Genetics and Genomics and the Association for Molecular Pathology 2015 guidelines were used for the final variant classification (15).

RESULTS

NF1 gene sequence analysis was performed in 32 unrelated patients (18 males-14 female age: 1-47 years) in this study. Of them, a disease-causing variant was found in 22 and (68.75%) (Table 1). Twenty-two discrete mutations were detected. One mutation was observed twice (c.1019_1020delCT, p. Ser340CysfsTer12), whereas one patient harbored two pathogenic variants (c.1185+1G>T, IVS10+1G>T; c.8059_8060delAG, p. Ser2687CysfsTer5, which both were *de novo* but, cis/trans location could not be determined).

No mutational hotspot region was detected. Two mutations were novel (2/22), 13 were single nucleotide variants (13/22), eight were deletions (8/22) and one was insertion (1/22). Most variants were single nucleotide change mutations that cause disease by creating frameshift or premature stop codons. Concisely, the mechanism underlying this disease was the altered coding that led to truncated neurofibromin in the majority of mutations.

Family study could be performed for 20 of the patients, and eight mutations were found to be inherited (five paternal vs. 3 maternal), whereas 12 were *de novo*. No family history was noted for cases with *de novo* mutations. Mosaicism was not detected in the DNA extracted from the peripheral blood specimens of the patients and parents.

Fifteen out of 22 patients with pathogenic variant and 3 out of 10 patients without pathogenic variant fulfilled the diagnostic criteria. The diagnostic utility of NGS was 83% (15/18) for patients who fulfilled the NIH criteria in this study. The ages of seven patients who had molecular diagnosis without meeting the NIH criteria were 1-6 and none of them had a family history and harbored *de novo* mutations (cases: 5, 8, 11, 13, 15, 17 and 22).

The medical history and clinical findings of patients with molecular diagnosis is as follows (Table 2). Eight out of 22 patients had a parent who meets the diagnostic criteria. All patients had skin manifestations. The most common skin finding was CALMs. Although all patients had CALMs, in seven patients they were not sufficient to be a diagnostic criterion (case 5, 8, 11, 13, 15, 17, 22). The second was freckling (13/22) and the third was neurofibroma (9/22). Lisch nodules were detected in nine patients. Macrocephaly, which was detected in 13 patients, was also common in our cohort. The presence of focal areas of signal intensity (FASI) were the most common brain MRI findings (10/22). The other findings were scoliosis (2/22), short stature (2/22), learning disability (3/22), developmental delay (1/22), dural ectasia (1/22), and hydrocephalus (1/22). Cranial vascular pathology was detected in two patients (case 9 and 11) (Figures 1, 2), who had also intellectual disability and epilepsy. Malignancies were found in four patients. One patient had high-grade glioma, one had optic glioma, and two had JMML. Because of the wide distribution of mutations on the *NF1* gene, genotype-phenotype correlation could not be established.

DISCUSSION

NF1 mutation analysis is difficult due to the large size of the gene, presence of pseudogenes, and lack of mutation hotspots (16). In addition, approximately in 5% of patients have NF1 resulting from whole gene deletions (17). Current laboratory methodologies should be combined to detect mutations (18). RNA analysis is more sensitive than DNA methods with a detection rate of 95.8% (19). On the other hand, NGS was involved in diagnosis, providing a less costly method than Sanger sequencing and faster turnaround. Deletion/duplication analysis of the *NF1* and *SPRED1* gene sequence analysis should be performed in undiagnosed cases (16,18). Besides, alternative diagnoses, including, but not limited to, Legius syndrome, Noonan syndrome with multiple lentigines, and constitutional mismatch repair deficiency syndrome should be considered (5).

Table 1. List of detected disease-causing variants

Case no	Nucleotide change	Amino acid change	Location	Coding impact	Variant type	Reference	Inheritance	Pathogenicity (ACMG criteria)
1	c.479G>T	p. Arg160Met	Exon 4	Missense variant	SNV	HGMD	*De novo*	Pathogenic
2	c.1019_1020delCT	p. Ser340CysfsTer12	Exon 9	Frameshift variant	Deletion	ClinVar, HGMD	*De novo*	Pathogenic
3	c.1019_1020delCT	p. Ser340CysfsTer12	Exon 9	Frameshift variant	Deletion	ClinVar, HGMD	Maternal	Pathogenic
4	c.1393_1394delAG	p. Leu466TyrfsTer3	Exon 13	Frameshift variant	Deletion	ClinVar	*De novo*	Pathogenic
5	c.1722-3C>A		Intron 15	Splicing variant	SNV	ClinVar	*De novo*	Likely pathogenic
6	c.1885G>A	p. Gly629Arg	Exon 17	Missense variant	SNV	ClinVar, HGMD	NA	Pathogenic
7	c.2325G>C	p. Glu775Asp	Exon 19	Splicing variant	SNV	ClinVar, HGMD	*De novo*	Pathogenic
8	c.2466dupA	p. Gly823ArgfsTer8	Exon 21	Frameshift variant	Insertion	Novel	*De novo*	Pathogenic
9	c.3445A>G	p. Met1149Val	Exon 26	Missense variant	SNV	ClinVar, HGMD	*De novo*	Pathogenic
10	c.3457_3460delCTCA	p. Leu1153MetfsTer4	Exon 26	Frameshift variant	Deletion	ClinVar	Paternal	Pathogenic
11	c.3525_3526delAA	p. Arg1176SerfsTer18	Exon 27	Frameshift variant	Deletion	ClinVar, HGMD	*De novo*	Pathogenic
12	c.3826C>T	p. Arg1276Ter	Exon 28	Non-sense variant	SNV	ClinVar, HGMD	NA	Pathogenic
13	c.3897delA	p. Lys1299AsnfsTer10	Exon 29	Frameshift variant	Deletion	ClinVar, HGMD	*De novo*	Pathogenic
14	c.3916C>T	p. Arg1306Ter	Exon 29	Non-sense variant	SNV	ClinVar, HGMD	Paternal	Pathogenic
15	c.4084C>T	p. Arg1362Ter	Exon 30	Frameshift variant	SNV	ClinVar, HGMD	*De novo*	Pathogenic
16	c.4769T>G	p. Leu1590Ter	Exon 36	Non-sense variant	SNV	ClinVar, HGMD	Paternal	Pathogenic
17	c.4931A>G	p. Asp1644Gly	Exon 37	Missense variant	SNV	ClinVar, HGMD	*De novo*	Pathogenic
18	c.5347_5350delTATT	p. Tyr1783MetfsTer10	Exon 38	Frameshift variant	Deletion	ClinVar	Maternal	Pathogenic
19	c.5489G>T	p. Arg1830Leu	Exon 38	Missense variant	SNV	ClinVar, HGMD	Paternal	Pathogenic
20	c.5902C>T	p. Arg1968Ter	Exon 40	Non-sense variant	SNV	ClinVar, HGMD	Maternal	Pathogenic
21	c.6854_6855delAC	p. Tyr2285Ter	Exon 46	Non-sense variant	deletion	Novel	Paternal	Pathogenic
22	c.1185+1G>T // c.8059_8060delAG	IVS10+1G>T // p. Ser2687CysfsTer5	Intron 10 // exon 55	Splicing variant // frameshift variant	SNV // deletion	ClinVar, HGMD // ClinVar, HGMD	*De novo*	Pathogenic // pathogenic

ACMG: American College of Medical Genetics, HGMD: Human gene mutation database, SNV: Single nucleotide variant.

Table 2. Medical history and clinical findings of patients with molecular diagnosis. Table was designed according to revised diagnostic criteria for NF1 Legius et al. (5)

Case no	Age/ sex	A parent who meets the diagnostic criteria	Café-au-lait macules*	Freckling**	Neurofibromas***	Optic pathway glioma	Lisch nodules or choroidal abnormalities¶	Distinctive osseous lesion₮	Other	Neurologic	Neoplasia other than optic pathway glioma
1	8/M	-	+	+	-	+	-	-	Macrocephaly	Learning disability, presence of FASI detected by brain MRI	-
2	9/F	-	+	+	+	-	-	-	Macrocephaly	Presence of FASI detected by brain MRI	-
3	38/F	+	+	+	-	-	Lisch nodules	-	Macrocephaly	-	-
4	40/F	-	+	+	-	-	Lisch nodules	-	-	Learning disability	-
5	6/F	-	-#	-	-	-	-	-	Macrocephaly	Presence of FASI detected by brain MRI	-
6	47/F	-	+	-	+	-	Lisch nodules	-	Osteopenia	-	-
7	14/M	-	+	+	+	-	Lisch nodules	-	Macrocephaly, short stature	Presence of FASI detected by brain MRI	-
8	2/F	-	-#	+	-	-	-	-	Macrocephaly	Presence of FASI detected by brain MRI, hydrocephalus	High grade glioma
9	11/M	-	+	+	+	-	Lisch nodules	-	-	Intellectual disability, severe stenosis, and poor filling from the distal m1 segment of the right medial cerebral artery, epilepsy	-
10	30/F	+	+	+	+	-	Lisch nodules	-	Short stature	-	-
11	3/M	-	-#	-	-	-	-	-	Macrocephaly	Intellectual disability, prominent sulcus of right frontoparietal region, cortical atrophy and thinning in the right middle cerebral artery branches were observed in T2-weighted MRI, epilepsy	-

Table 2. Continued

Case no	Age/ sex	A parent who meets the diagnostic criteria	Café-au-lait macules*	Freckling**	Neurofibromas***	Optic pathway glioma	Lisch nodules or choroidal abnormalities¶	Distinctive osseous lesion₮	Other	Neurologic	Neoplasia other than optic pathway glioma
12	36/M	-	+	+	+	-	Lisch nodules	-	-	-	-
13	2/M	-	-#	-	-	-	-	-	Macrocephaly, juvenile xanthogranuloma	-	-
14	21/F	+	+	+	+	-	Lisch nodules	-	-	Learning disability	-
15	1/M	-	-#	-	-	-	-	-	Macrocephaly	-	Juvenile myelomonocytic leukemia
16	2/F	+	+	-	-	-	-	-	Juvenile xanthogranuloma	Presence of FASI detected by brain MRI	-
17	6/M	-	-#	-	-	-	-	-	Macrocephaly	-	-
18	3/M	+	+	-	-	-	-	-	Scoliosis	Presence of FASI detected by brain MRI, dural ectasia	-
19	2/M	+	+	+	-	-	-	-	Macrocephaly	Developmental delay, presence of FASI detected by brain MRI	-
20	33/M	+	+	+	+	-	Lisch nodules	-	Macrocephaly	-	-
21	14/M	+	+	+	+	-	-	-	Macrocephaly, scoliosis	Presence of FASI detected by brain MRI	-
22	3/M	-	-#	-	-	-	-	-	-	Presence of FASI detected by brain MRI	Juvenile myelomonocytic leukemia

*Six or more café-au-lait macules over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals. At least one of the two pigmentary findings (café-au-lait macules or freckling) should be bilateral. -# Although patients had café-au-lait macules, none was sufficient to be a diagnostic criterion. **Freckling in the axillary or inguinal region. At least one of the two pigmentary findings (café-au-lait macules or freckling) should be bilateral. ***Two or more neurofibromas of any type or one plexiform neurofibroma. ¶Two or more iris Lisch nodules identified by slit lamp examination or two or more choroidal abnormalities. ₮Such as sphenoid dysplasia, b anterolateral bowing of the tibia, or pseudarthrosis of a long bone.
FASI: Focal areas of signal intensity, MRI: Magnetic resonance imaging, M: Male, F: Female.

A Turkish population study from Ulusal et al. (20) and Bildirici et al. (21) suggested that NGS and multiplex ligation-dependent probe amplification (MLPA) methods are practical and helpful tools for genetic diagnosis of NF1. Sharifi et al. (22) used a multi-step process of NGS, MLPA, and array-comparative genomic hybridization in the analysis of NF1 patients.

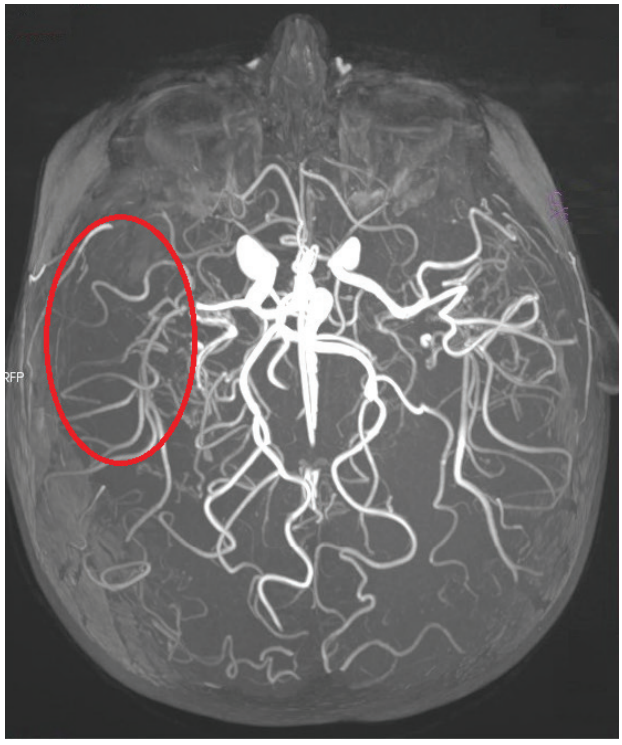

Figure 1. Cranial magnetic resonance imaging of case 9. Severe stenosis from the distal of the right middle cerebral artery M1 segment in 3D image and poor filling compared to its symmetry.

Although three patients fulfilled the revised diagnostic criteria for NF1, no pathogenic variant was detected in our study: 4-year-old male with eight CALMs >5 mm, axillary freckling, two Lisch nodules, and optic glioma; 15-year-old male with multiple CALMs >15 mm, axillary freckling, three neurofibromas, three Lisch nodules, family history of NF1; 27-year-old female with multiple CALMs >15 mm, axillary freckling, multiple neurofibromas, two Lisch nodules, scoliosis. Extensive genetic tests should be performed to detect possible deletions, deep intronic or non-coding region mutations.

All patients who harbor a pathogenic mutation and do not fulfill the revised diagnostic criteria are between 1-6 years of age (cases: 5, 8, 11, 13, 15, 17, 22). NF1 mutation analysis was performed in these patients' variable set of clinical features (CALMs, freckling, juvenile xanthogranuloma, macrocephaly, JMML, presence of FASI detected by MRI). Further clinical and genetic evaluation is recommended if the patient's diagnostic criteria do not meet NF1, despite NF1 pathogenic variants. Two cases met the diagnostic criteria after serial observations and follow-up (case 8 and 11). Further genetic analysis is planned for the remaining patients to verify whether the variant is germline, somatic, or mosaic. Cases 15 and 22 did not continue follow-up at our center. Since both patients did not have a parent who met the diagnostic criteria with NF1, further genetic analysis from a second tissue such as buccal must be performed.

In this study, disease-causing variants in the *NF1* gene were identified at a rate of about 68.75% with NGS, which is consistent with the sequencing method analysis in different studies (23,24). Our study emphasizes the benefits of NGS, which is very suitable in terms of producing rapid results economically and we suggest that it can be used as a first-step test.

According to family studies, 60% (12/20) of patients had the disorder caused by *de novo* mutations, which is also compatible with literature (3).

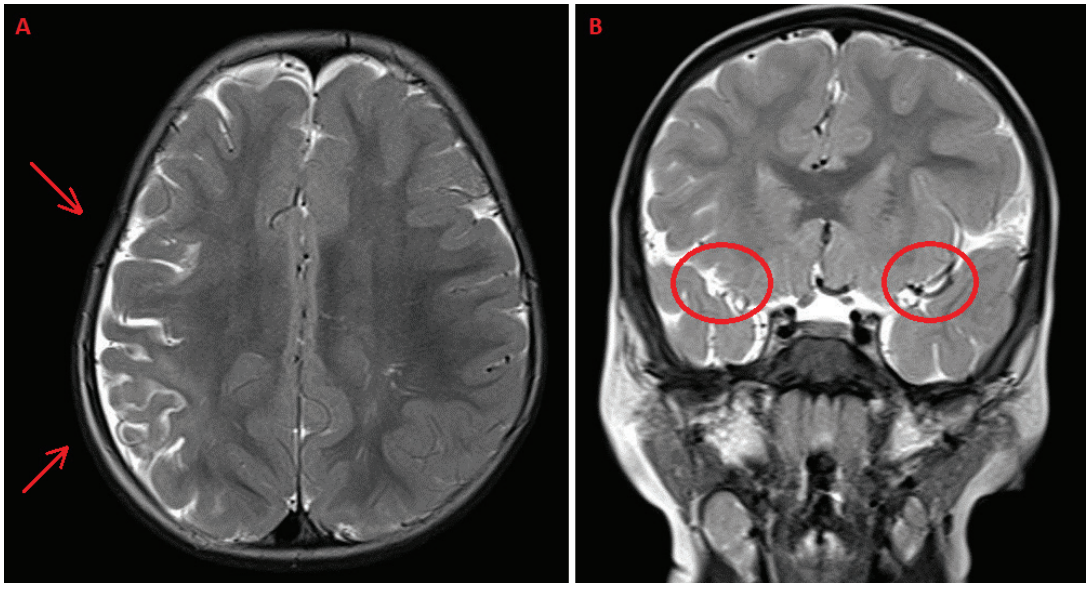

Figure 2. Cranial imaging of case 11. (A) Cortical atrophy and prominent sulcus of right frontoparietal region in axial T2-weighted magnetic resonance imaging (MRI). (B) Thinning in the right middle cerebral artery branches were observed in coronal T2-weighted MRI.

Table 3. List of in silico analysis of putative novel variants

Case no	Nucleotide change	Amino acid change	Splice distance	Coding impact	Mutation taster	PhyloP100
8	c.2466dupA	p. Gly823ArgfsTer8	58	Frameshift variant	Disease causing (prob:1)	5.708
21	c.6854_6855delAC	p. Tyr2285Ter	36	Non-sense variant	Disease causing (prob:1)	8.618

Two variants were considered as novel pathogenic according to population data, predictive impact, and segregation analysis (Supplementary Figure 1). The distribution according to their coding impacts was frameshift and non-sense. These variants were evaluated by comprehensive in silico analysis (Table 3). Evolutionary sequence homology and splice distance were shown for all novel variants. These variants were detected in cases 8 and 21. Case 8 was a two-year-old female. She was consulted by our department after being diagnosed with high-grade glioma. She had three CALMs >5 mm in greatest diameter, bilateral axillary freckling, macrocephaly, presence of FASI detected by brain MRI, and hydrocephalus. Neither of the parents was diagnosed with NF1. CALMs were increased in number and size during serial observations and follow up and she met revised diagnostic criteria for NF1. Case 21 was fourteen-year-old male who had ten CALMs >15 mm in greatest diameter, bilateral freckling in the axillary and inguinal regions, and multiple neurofibromas in his trunk. He also had macrocephaly, scoliosis, presence of FASI detected on brain MRI. His father was also diagnosed with NF1.

Genotype-phenotype correlation is not easy to discuss because of the high clinical variability in NF1. No clear genotype-phenotype correlation has been recognized in NF1 so far, apart from four alterations: Whole NF1 deletion associated with severe phenotype with facial dysmorphism, cognitive deficits, early onset of multiple cutaneous neurofibromas, and cardiovascular anomalies. In addition, patients have a higher lifetime risk of developing malignant peripheral nerve sheath tumors. A 3-bp in-frame deletion of c.2970-2972delAAT is associated with typical pigmentary features of NF1 but with few cutaneous or surface plexiform neurofibromas (2). Missense variants affecting *NF1* codon Arg1809 in exon 29 (exon 38 of NM_001042492.3) are associated with multiple café au lait spots in the absence of cutaneous neurofibromas or clinically apparent plexiform neurofibromas (25). Missense variants affecting codons 844-848 are associated with a more severe phenotype and higher risk of developing malignancies (2). These mutations were not detected in our cohort, and no genotype-phenotype correlation could be made within detected mutations.

Patients with NF1 are known to have a predisposition for various benign and malignant tumors. Neurofibromas are the most prevalent benign tumors. Nervous system malignancies are the most common malignant tumors, occurring more frequently than in the general population in patients with NF1. In addition, gastrointestinal stromal tumors, pheochromocytomas, and JMML are known to associate with NF1(26,27). Although the heterozygous state of NF1 is known to cause abnormal cell growth, tumor predisposition in NF1-associated malignancies is largely explained by the loss of heterozygosis (10).

Malignancies were found in four patients in our study. One patient had high-grade glioma, one had optic glioma, and two had JMML. Our study was interesting regarding the distribution of malignant tumors. Although central nervous malignancies are the most common malignancies apart from malignant peripheral nerve sheath tumors in the literature, leukemia was also common in our study with a rate of 9.1% (2/22). Leukemia is an interesting subtitle in NF1 because of its irrelevance to the nervous system. *NF1* gene has tumor suppressor role in early myelopoiesis (28). Somatic second hits of NF1 were also shown in patients with NF1 and myeloid disorders (29,30). In addition, NF1 knockout mouse embryos showed aberrant growth in myeloid hematopoietic cells, which were hypersensitive to granulocyte-macrophage colony-stimulating factor (31). Elucidating the underlying cause of JMML is important to determine treatment strategies. The management of patients with germline mutations differs from patients with only somatic mutations (32). Although the association of JMML in NF1 is not common, it is important to keep JMML in mind in NF1 as it determines treatment strategies. JMML and juvenile xanthogranulomas frequently co-exist in NF1. Observing juvenile xanthogranulomas in children with NF1 may raise awareness to actively search for other alarming signs of JMML (33). Chronic myelomonocytic leukemia was detected in 9% of patients with NF1 in the Great Britain cohort (34). Myeloid malignancies (1 acute lymphocytic leukemia, 1 JMML, 1 acute myeloid leukemia) were detected at a rate of 15.7% in an NF1 cohort from southern Türkiye (35). Interestingly no JMML was not observed in a large Finnish cohort of pediatric malignancies in NF1 (26). JMML incidence is <1 according to large series (36,37). Our hospital is not a regional center for pediatric hematological malignancies; however, the high frequency might be a bias due to the small sample size.

Cranial vascular pathology was detected in two patients (9.1% 2/22) in our study. Vascular abnormality-incidence that is associated with NF1 is increasing. NF1 vasculopathy can cause pseudoaneurysm, aneurysm, arteriovenous malformations, stenosis, and occlusion. Vasculopathy of the major arteries can have serious consequences. Cerebrovascular lesions can also be seen in NF1 patients; arteriovenous malformations, pseudoaneurysm, aneurysm, vascular stenosis, occlusion, and Moyamoya disease have been reported (3). Neurofibromin is expressed in blood vessel endothelial and smooth muscle cells, and vascular abnormalities in NF1 might be caused by the modification of neurofibromin function in these cells (38).

The frequency of vasculopathy is not known, due to both insufficient extensive studies and the presence of asymptomatic patients. Rea et al. (39) found the prevalence of cerebrovascular lesion to be 6% over 266 patients with NF1 who underwent cranial MRI. Rosser et al. (40) reported 2.5% of 316 and Cairns and North (41) reported 5% of 144 NF1 patients with a cerebrovascular lesion, which was detected on cranial MRI. D'Arco et al. (42) studied the usefulness of cranial MRA in patients with NF1. They found intracranial stenosis in 7.4% of 125 patients with NF1 who underwent both MRI and MRA. The higher prevalence in D'Arco et al. (42) might be due to the use of cranial MRA in addition to MRI.

The prevalence of vascular pathology was 9.1% (2/22) in our study. The patients were three- and 11-year-old males with intellectual disability and epilepsy (case 9, 11). Other causes of vasculopathy were excluded from these patients. Although case 9 fulfilled the

diagnostic criteria, case 11 was diagnosed after follow-up. The higher prevalence of vascular pathology (9.1%) compared with the current literature (2.5-7.4%) might be a bias due to the small sample size. Seizure and ID were interpreted to be secondary to vasculopathy. Both proteins are known to accompany NF1 (3,43). Intellectual disability is seen 6-7% of patients with NF1 (43). The etiology of ID in our study might be due to cerebral atrophy secondary to stenosis. In addition, seizures might be a result of cerebral ischemia.

Sobata et al. (44) grouped NF1 cerebrovascular lesions into three as stenotic, aneurysmal, and both stenotic and aneurysmal. Stenotic lesions are known to occur in younger patients (41,42). The patients had stenosis in our study, which is consistent with the literature.

Although lesions in other arteries have been reported (39-42,44), only middle cerebral artery was affected in both patients in our study. This result was probably due to the small sample size in our study and not using cranial MRA in all cases.

Some cerebrovascular lesions progress and eventually require surgical and medical treatment. Early medical/surgical intervention was suggested to prevent complications (39). Cerebrovascular lesions can be detected in asymptomatic patients, but some do not progress. Asymptomatic patients represent a clinical dilemma (41). Further comprehensive studies are necessary to gain more experience in the cerebrovascular pathology of NF1.

Study Limitations

It is important to acknowledge the limitations of the current study. Sample size is small in the current study. Further studies that include larger cohort is necessary.

CONCLUSION

We report three years of data on the molecular and clinical findings of NF1 in patients from northeast Türkiye. This study will contribute to the characteristics of patients with NF1. Among the detected mutations, two were novel. Further definition of the mutations will contribute to a better understanding of disease pathogenesis and manifestations. Rare features were detected as well as classic features of NF1. This study is also important for showing importance of NF1 vasculopathy. Although it can be detected in asymptomatic patients, NF1 vasculopathy can cause serious complications. Medical and/or surgical procedures are needed in some cases. Keeping this in mind could enable timely therapy. Our study shows that genetic analysis improved follow-up and counseling.

Ethics

Ethics Committee Approval: This study was approved by the Institutional Ethics Committee by University of Health Sciences Türkiye, Trabzon Kanuni Training and Research Hospital (approval number: 2019/22, date: 25.04.2019).

Informed Consent: Informed written consent was obtained from the patient's parents.

Authorship Contributions: Concept: Ş.A., Design: Ş.A., A.H.Ç., Supervision: Ş.A., A.H.Ç., Resources: Ş.A., A.H.Ç., Material: Ş.A., A.H.Ç., Data Collection or Processing: Ş.A., A.H.Ç., Analysis or Interpretation: Ş.A., A.H.Ç., Literature Search: Ş.A., A.H.Ç., Writing: Ş.A., Critical Review: Ş.A., A.H.Ç.

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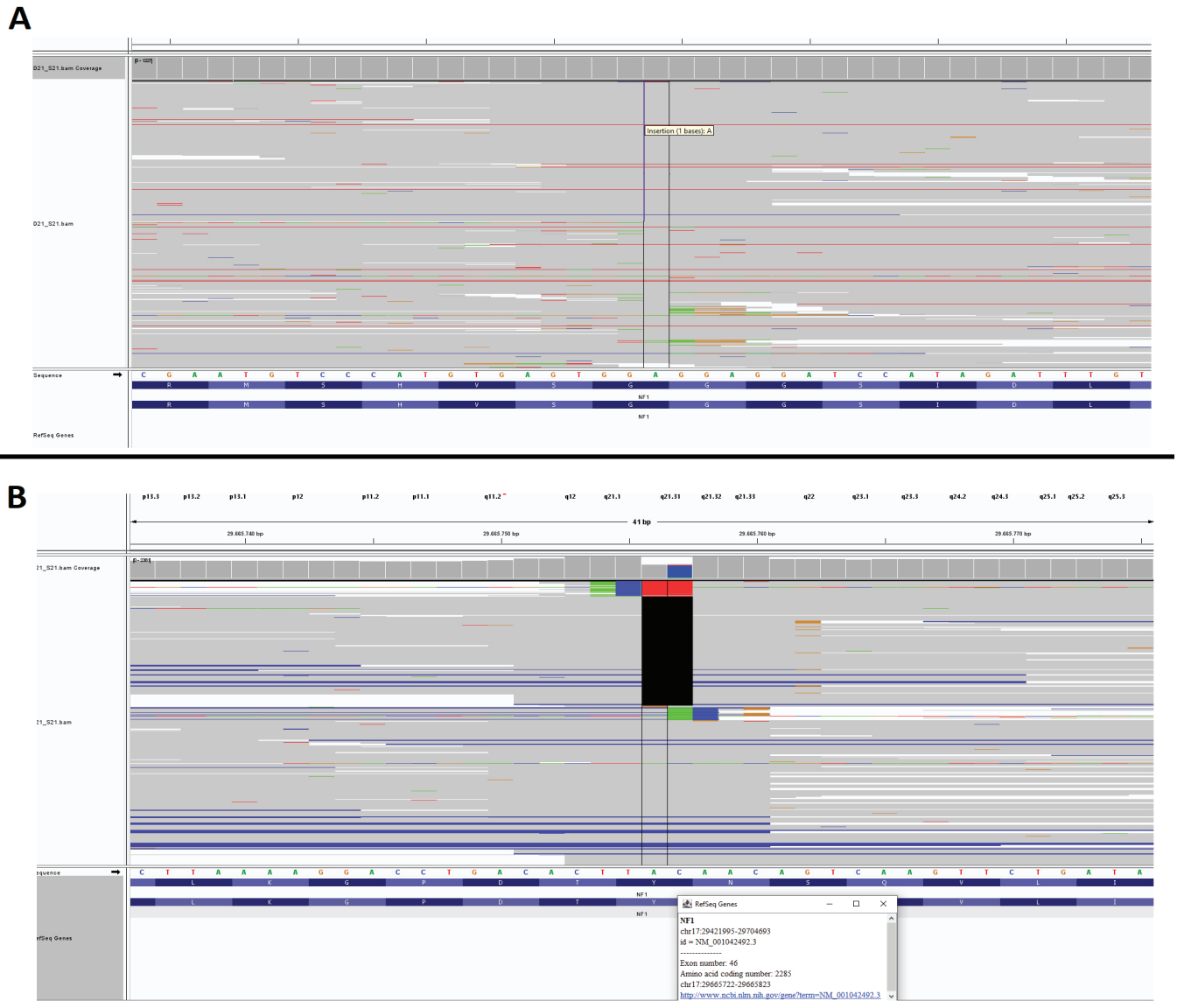

Supplementary Figure 1. Integrative genomics viewer images of novel mutations. (A) c.2466dupA, (B) c.6854_6855delAC.