EREDETI KÖZLEMÉNY

ORIGINAL ARTICLE

Neurological comorbidities and novel mutations in Turkish cases with neurofibromatosis type 1

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Background and purpose – Neurofibromatosis type 1 (NF1) is a rare, autosomal dominant multisystemic disease. The *NF1* gene is localized on chromosome 17q11.2. Patients with NF1 have different clinical presentations and comorbidities. The aim of the present study is to determine the novel mutations and neurological comorbidities of NF1.
Methods – Patients who were diagnosed with NF1 by clinical criteria of the National Institutes of Health were included in the study. After a detailed examination, the *NF1* gene was analysed with the help of next generation sequencing technology from peripheral blood samples via MiSeq (Illumina, USA). Bioinformatic analyzes were performed to evaluate the clinical significance of the detected variants via the international databanks in accordance with the ACMG (American College of Medical Genetics) guideline. In addition, cerebral-spinal MRI, cerebral angiography, and ENMG examinations were performed if deemed necessary.
Results – Twenty patients (12 female, 8 male) were included in the study. The mean age was 25.8±10 (10-56) years. Previously defined 13 different pathogenic mutations according to the ACMG criteria were identified in 18 patients. Also, two novel mutations were detected in 2 cases. Moreover, neurological comorbidities (moyamoya disease, multiple sclerosis, Charcot Marie Tooth Type 1A) were found in 3 patients with NF1.
Conclusion – In the present study two novel mutations and three different neurological comorbidities were identified in NF1.

Keywords: neurofibromatosis, novel mutations, CMT1A, moya-moya, multiple sclerosis

Neurológiai komorbiditások és új mutációk törökországi 1-es típusú neurofibromatosisos esetekben
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Háttér és cél – Az 1-es típusú neurofibromatosis (NF1) ritka, autoszomális domináns multiszisztémás betegség. Az *NF1* gén a 17q11.2 kromoszómán lokalizálódik. NF1 esetén a betegek különböző klinikai megjelenési formákkal és különböző társbetegségekkel jelentkeznek. A jelen tanulmány célja az NF1 új mutációinak és neurológiai társbetegségeinek meghatározása.
Módszerek – A vizsgálatba olyan betegeket vontunk be, akiknél az amerikai Nemzeti Egészségügyi Intézet (National Institutes of Health) klinikai kritériumai alapján NF1-et diagnosztizáltak. A részletes vizsgálatot követően az *NF1* gént perifériás vérmintákból újgenerációs szekvenálási technológia segítségével elemeztük MiSeq (Illumina, USA) használatával. A kimutatott variánsok klinikai jelentőségének értékelésére bioinformatikai elemzéseket végeztünk a nemzetközi adatbázisokon keresztül, az ACMG (American College of Medical Genetics) irányelvének megfelelően. Ezenkívül, szükség esetén cerebralis-spinalis MR-, agyi angiográfiás és ENMG-vizsgálatokat végeztünk.
Eredmények – Húsz beteg (12 nő, 8 férfi) került bevonásra. Az átlagéletkor 25,8 ± 10 (10–56) év volt. Tizennyolc betegnél az ACMG-kritériumok szerint korábban már meghatározott 13 különböző patogén mutációt azonosítottunk. Két új mutációt is kimutattunk két esetben. Ezenkívül, három NF1-es betegnél neurológiai társbetegségeket (Moyamoya-kór, sclerosis multiplex, Charcot–Marie–Tooth 1A típus) találtunk.
Következtetés – Jelen tanulmány két új mutációt és három különböző neurológiai társbetegséget azonosított NF1-ben.

Kulcsszavak: neurofibromatosis, új mutációk, CMT1A, Moyamoya, sclerosis multiplex

Neurofibromatosis (NF) is a genetic disease that affects the development of neural cells. There are three types of NF: type-1 neurofibromatosis (NF1), type-2 neurofibromatosis (NF2), and schwannomatosis[1]. The most common type is NF1 which is a neurocutaneous disease. The incidence of NF1 is approximately 1 in every 2,500 to 3,000 births[2]. Diagnostic features are café-au-lait spots, Lisch nodules, axillary freckling, neurofibromas, optic glioma, pseudoarthritis, central and peripheral nerve tumors[1]. *NF1* gene is located on chromosome 17q 11.2. NF1 is an autosomal dominant inherited disease with full penetrance and variable expressivity. Individuals with the disease have a 50% risk of transmission to their children. The mutations of *NF1* gene are determined by molecular analysis of more than 95% patients who meet the criteria of NIH. *NF1* gene produces a protein which is called neurofibromin and acts as a tumor suppressor gene. Neurofibromin levels are reduced by mutations of *NF1* gene. Decreased levels of neurofibromin lead to the development of tumor[1]. NF1 is diagnosed by clinical criteria of the National Institutes of Health (NIH)[3]. Until now different signs had been detected as well as genetic variations in NF1. The aim of the present study is to determine novel mutations and neurological comorbidities of NF1.

Method

The NF-patients were admitted to outpatient clinics (Medical Genetic/Neurology) from different departments for and with genetic evaluation. Patients diagnosed with NF1 by clinical criteria of National Institutes of Health[2] were included in the study. After detailed physical and brain examinations, if considered necessary, cerebral-spinal MRI, cerebral angiography, and electrophysiological studies were performed.

In addition, *NF1* whole gene sequence analysis was performed in all patients. Peripheral blood samples from patients were collected. Genomic DNA was extracted with QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Genomic DNAs were fragmented and then barcoded with molecular indexes for sample identification. All encoded exons and their neighboring regions were amplified via PCR primers (Scientific & Educational Software PRIMER © – Primer Designer v.2.0) for target enrichment. Library preparation steps were made using Illumina Nextera XT kit. Then, prepared library was next generation sequenced via Illumina MiSeq next generation sequencing platform (Illumina, San Diego, CA, USA). The sequences were aligned to the hg19 genome MiSeq Reporter software (Illumina Inc.) after quality control assessments of the raw data. SNVs (single nucleotide variations) and MNVs (multi nucleotide variations) excluding CNVs (copy number variations) were investigated via further analysis. The analyses were performed for the genetic variations using IGV 2.3 (Broad Institute) software using hg19 (GRCh37) reference sequence. Variant classifications were made accordingly with the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) Guideline[4]. *In silico* analyzes were made in cases with novel variations by using prediction tools such as *SIFT, PolyPhen 2, and MutationTaster*. In addition, *5 of 25* STR markers (AC005838, D17S2218, D17S2220, D17S2227, D17S2229) were investigated for the presence of *duplication in 17p11.2-12 PMP 22* in the case with Charcot Marie Tooth type 1A. STR marker sites on the duplicated region were amplified via polymerase chain reaction, electrophoresed through 8% polyacrilamide gel, and evaluated for duplication.

The local ethical committee approved the study (Ethical committee of Cukurova University, meeting no: 102), and all subjects (or legal guardians) signed to informed consent.

Results

Twenty patients (12 female, 8 male) were included in the study. None of the patients were related to each other. The mean age was 25.8 ± 10 (10-56) years. All of the patients had six or more café-au-lait macules, freckling in the axillary, and two or more Lisch nodules. All cases (n: 20) had disease-causing mutation, 18 of 20 cases had the previously identified mutations according to Human Gene Mutation (HGMD) (n: 10) and Clinical Variant (ClinVar) (n: 3), also two novel mutations were determined (case no: 1, 2) **(Table 1)**. Besides three different neurological comorbidities [Charcot-Marie-Tooth disease type 1A (CMT1A), Moya-Moya disease, and multiple sclerosis (MS)] were defined. Mutations of comorbidities were previously identified **(Tables 1., 2)**.

A female patient (case no: 1) who was 43 years old, was consulted by the dermatology clinic for neurological evaluation, she hadn't any neurological complaints. The result of the neurological examination was normal. Only dermatological signs of NF1 were seen on physical examination. p.1784_A1785del (c.5351_5356delATGCTT) (Heterozygous) mutation was detected in case no 1. This mutation was accepted as an uncertain change of clinical importance as a result of analyzes with in silico evaluations. The mutation was accepted as an etiology of NF1 due to frame shifting and early stop codon formation. The mutation was not previously identified and it was accepted as a novel mutation (NM_001042492) **(Tables 1., 2)**.

A male patient (case no: 2) who was 42 years old was accepted to the clinic with positional vertigo. The dix-hilpike test was positive on the examination. His complaint improved by the Epley maneuver. p.T1630* (c.4887_4890delGACT) (Heterozygous) mutation was detected in case no 2. The mutation was determined as

Table 1. *Mutation types of diagnosed with NF1*

CN	G-A	Mutation	Mutation ID
1	F-43	p.Y1784_A1785del (c.5351_5356delATGCTT)	NM_001042492
2	M-42	p.T1630*(c.4887_4890delGACT)	NM_001042493
3	F-36	p.R1176Sfs*18 (C.3525_3526delAA)	CD000971 (HGMD)
4	M-22	p.M991Dfs*29 (c.2970_2971delAA)	CD921025 (HGMD)
5	M-21	p.M991Dfs*29 (c.2970_2971delAA)	CD921025 (HGMD)
6	F-20	p.R1362* (c.4084C>T)	CD971046 (HGMD)
7	M-46	p.R1362* (c.4084C>T)	CD971046 (HGMD)
8	M-17	p.R1362* (c.4084C>T)	CD971046 (HGMD)
9	F-21	p.T586Vfs*18(c.1754_1757delTAAC)	RCV000467300.18 (CVDA)
10	F-32	p.L1511P (c.4532T>C)	VCV001209570.6 (CVDA)
11	M-35	p.E1020*(c.3058G>T)	VCV000839592.6 (CVDA)
12	F-35	p.K144E (c.4330A>G)	CM920506 (HGMD)
13	F-31	p.K144E (c.4330A>G)	CM920506 (HGMD)
14	F-28	p.K144E (c.4330A>G)	CM920506 (HGMD)
15	F-54	p.R1276*(C.3826C>T)	CI951961 (HGMD)
16	F-27	p.K2265Sfs*3 (c.6794_6794delA)	CD000994 (HGMD)
17	M-30	p.R440* (C.1318C>T)	CD950845 (HGMD)
18	M-33	IVS27-2A>G (c.3709-2A>G)	CS971825 (HGMD)
19	F-28	p.I679Dfs*21 (c.2033_2034insC)	CM950847 (HGMD)
20	F-29	p.H553R (c.1658A>G)	HM0703 (HGMD)

CN: case no, G-A: gender-age, F: female, M: fale, HGMD: human gene mutation database identity, CVDA: clinical variant database accesion, NM: novel mutation

the cause of NF1 according to MutationTaster data and early stop codon formation. The mutation was not previously identified and it was accepted as a novel mutation (NM_001042493) **(Tables 1., 2)**.

A female patient (case no: 3) who was 36 years old was accepted to the clinic with paresthesia on the left arm for one week. Bilateral Hoffman/Tromner reflexes were shown on examination. Multiple MS plaques were seen on cerebral and spinal MRI, and some of them were enhanced by gadolinium (Image: 1). Oligoclonal band was found as positive in cerebrospinal fluid. p.R1176Sfs*18 (C.3525_3526delAA) (Heterozygous) (HGMD ID: CD000971) mutation was determined by analysis of *NF1* gene. The mutation was previously identified **(Tables 1., 2)**.

A male patient (case no: 4) who was 22 years old was admitted to the clinic with leg weakness for 3 years. He was diagnosed with NF1 at the age of 9 years old. There were bilaterally glove and stocking hypoesthesia, absence of vibration, mild weakness (4/5) in knee flexion and extension, and bilaterally on lower extremities in dorsiflexion and plantar flexion (0/5 and 1/5). Deep tendon reflexes were absent in all extremities. Also atrophy (intrinsic and peroneal muscle groups), pes cavus, and hammer toes were shown. Demyelinating sensorimotor polyneuropathy was determined in an electrophysiological study **(Table 3)**. Duplication of the *PMP22* gene was determined by molecular analysis which was performed with STR17 method (D17S222017 marker) (Image: 2). This result support-

Table 2. *Neurological findings of diagnosed with NF1*

CN	Gender	Age	Mutation	Neurological Findings
1	female	43	novel	none
2	male	42	novel	positional vertigo
3	female	36	known	multipl sclerosis
4	male	22	known	Charcot-Marie-Tooth disease type 1A
6	female	20	known	Moya-moya disease

CN: case no

Table 3. *Electrophysiological study of case No 3*

Side	Motor/ Sensory	Nerve	Terminal Latency(ms) M	F	Interval (cm)	Velocity (m / sec)	Amplitude (mV)
right	motor	median (APB)	5.1	30.1	8.2	16.3	2.2
right	motor	ulnaris (ADM)	4.8	31.2	7.0	21.8	2.6
right	motor	tibialis (AHL)	–				
right	motor	peronal (EDB)	–				
right	sensory	median (2. finger)	–				
right	sensory	ulnaris (5. finger)	–				
right	sensory	suralis	–				
left	sensory	suralis	–				

Image 1: MS Plaques of Case No 3
Image 2: PCR amplification with STR marker
Band 1: AC005838 (4A), Band 2; D17S2218, Band 3: D17S2220
Band 4: D17S2227, Band 5, D17S2229
Image3: Case No 6; hamartomas (a, b) and puff of smoke sign (c, d)

M: motor, F: F response, APB: abductor pollicis brevis, ADM: abductor digiti mini, AHL: abductor hallucis longus, EDB: extensor digiti brevis

ed that the diagnosis of case no 4 was CMT1A. His parents were not relatives, and had no family history of CMT1A. Also p.M991Dfs*29 (c.2970_2971delAA) (Heterozygous) (HGMD ID: CD921025) was determined by analysis of *NF1* gene. That mutation was previously identified **(Tables 1., 2)**.

A female patient (case no: 6) who was 20 years old was accepted to the clinic with a headache. The results of the examination of sensation, motor, cerebellar, pyramidal, and extrapyramidal systems were in normal range. Cerebral hamartomas were shown on MRI. The puff of smoke sign of Moya-moya was shown on cerebral angiography (Image: 3). thep.R1362* (c.4084C>T) (Heterozygous) (HGMD ID: CD971046) was determined by analysis of *NF1* gene. The mutation was previously identified **(Tables 1., 2)**.

Discussion

The *NF1* is a large gene, encompassing 55 constitutive exons as well as five alternatively spliced exons. Until now more than 3600 different pathogenic *NF1* variants have been reported[5]. In the present study, 13 different known mutations were observed in 18 of 20 patients which were previously identified and associated with the disease pathogenesis. Also two novel mutations were determined in 2 of 20 cases (case no: 1, 2) (Table1). In addition, three different comorbidities (CMT1A, Moya-Moya, and Multiple Sclerosis) were observed in patients with NF1.

In case no 3, a mutation known as "p.R1362 (c.4084C>T) (Heterozygous) (HGMD ID: CD971046)" was determined. Case no 3 who had paresthesia in the left arm was diagnosed with MS+NF1. In the literature, fifteen cases have been reported with MS+NF1[6, 7]. Some authors have suggested that this comorbidity may be associated with mutations of *neurofibromin protein* or *oligodendrocyte-myelin glycoprotein (OMG)* genes[6].

In cases no 4, 5, a mutation known as "p.M991Dfs *29 (c.2970_2971delAA (Heterozygous) (HGMD ID: CD921025)" was detected **(Tables 1., 2)**. Case no 4 was diagnosed with CMT1A+NF1. CMT1A is an autosomal dominant inherited disease which is a hereditary demyelinating polyneuropathy. CMT1A has duplication of *PMP22* gene localized on chromosome *17p11.2-12*. In the literature, there are 4 cases with CMT1A+NF1[8–10]. Both *Onu* et al. and *Roos* et al. had described a patient, and *Lupski* et al. detected two patients with CMT1A+NF1. Lupski et al. suggested that by the molecular analysis of CMT1A and NFA they are autosomal dominant diseases that can occur in the same individual secondary to a chance phenomenon. The present study supported the opinion of Lupski et al., because case no 4 hadn't a family history of CMT1A, and had the same mutation as case no 5 who hadn't a family history **(Tables 1., 2)**.

In cases no 6, 7, 8, a mutation known as thep.R1362* (c.4084C>T) (Heterozygous) (HGMD ID: CD971046) was determined. Case no 6 was diagnosed with Moya-Moya+NF1. Both NF1 and Moya-moya patients can have cerebral artery stenosis, cerebral aneurysm, arteriovenous malformation, and fistula[11–14]. Both of them may be accompanied by cerebrovascular events due to artery stenosis. Despite comorbidity of NF1 and Moya-moya having been reported in childhood in literature[12, 14], our case was 20 years old. While Moya-moya disease is localized in chromosome*17q25*, NF1 is localized in chromosome *17q11.2*. Despite the nearness between *NF1 (17q11.2)* and *Moya-Moya (17q25)* genes, until now a definite relationship between these diseases couldn't be demonstrated.

Before the present study, few molecular studies were published about NF1 in Turkey[15–17]. *Ulusal* et al. presented 3 novel and 12 known pathogenic variants[15], *Ece SA* et

al. presented 17 novel and 41 identified variants[16]. *Sharifi S* et al. showed 11 novel and 11 identified variants[17]. In the present study, we detected two novel and 13 identified variants. Additionally, unlike the other 3 studies, we reported three comorbidities (Moyamoya Disease, Multiple Sclerosis, Charcot Marie Tooth Type 1A) in patients with NF1.

NF1 patients have shorter survival time than the general population. NF1 increases the risk of malignancy and vasculopathy. The fibroma of optic nerve and brain tumors are common in NF1[18]. Also stroke can be seen in NF1 patients more often than in the general population[11–14]. Molecular studies are essential for the diagnosis of NF1. If the type of mutation is known, possible complications and comorbidities can be detected early. This will reduce the treatment time and cost of NF1.

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