Clinical Characteristics and Mutation Spectrum of Neurofibromatosis Type 1 in 27 Turkish Families

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Background: Neurofibromatosis type 1 (NF1) is a neurocutaneous disorder that results in a predisposition to the growth of multiple tumors in the central nervous system, the peripheral nervous system, and the skin. The clinical manifestations of neurofibromatosis are associated with loss of neurofibromin expression which causes the upregulation of the RAS pathway. Although neurofibromatosis type 1 can be diagnosed based on the National Institutes of Health criteria, sometimes the diagnosis is difficult, in cases where the characteristic features do not develop. Moreover, other RAS-related disorders may present with significantly overlapping clinical features.

Aims: To determine the clinical and molecular genetic characteristics of Turkish patients with neurofibromatosis type 1.

Study design: Cross-sectional study.

Methods: For the genetic analysis of 27 Turkish families clinically diagnosed with NF1 between 1990 and 2019, we used a multi-step process consisting of next-generation sequencing, multiplex ligation-dependent probe amplification, and array-comparative genomic hybridization.

Results: In this study, we identified 11 novel and 11 previously reported single-nucleotide variants in 22 families. Whole gene deletions were detected by multiplex ligation-dependent probe amplification analysis in 3 families. Of those, array comparative genomic hybridization analysis defined a 17q11.2 deletion in 4 patients from 2 families and 1.2-Mb involving 1 unrelated patient. All patients with a deletion had facial dysmorphism, suggesting a peculiar phenotype in this group. We could not find any pathogenic variant in the 2 families that met the National Institutes of Health criteria.

Conclusion: The novel pathogenic variants identified in this study broaden the spectrum of pathogenic variants in NF1 and provide better clinical characterization of NF1. RNA-seq experiments are recommended in patients who meet the National Institutes of Health diagnostic criteria for NF but have not identified any variants in next-generation sequencing, multiplex ligation-dependent probe amplification, or array-comparative genomic hybridization analysis.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a neurocutaneous disorder that results in a predisposition to the growth of multiple tumors in the CNS, including the brain, spinal cord, the peripheral nervous system, and the skin.1,2 It is related to heterozygous germline pathogenic variants in the NF1 gene which causes activation of the RAS/MAPK pathway (mitogen-activated protein kinases), predisposing patients to developing multiple tumor types and other organ system anomalies.3 The pathogenic variant can affect cognition and behavior, causing anxiety and attention deficit disorders as well as learning disabilities. It can lead to café-au-lait spots, which are brown birthmarks on the skin. Axillary and inguinal freckling is a very characteristic pattern and involves the armpits and groins. Lisch nodules are small benign hamartomas of the iris. Scoliosis, sphenoid wing dysplasia, and tibial bowing are the most common skeletal manifestations of this disorder.4 Other manifestations, such as vascular abnormalities, vitamin D deficiency, and growth delay are less common.5 The prevalence of the disease is estimated to be about 1 in 3000.6 It is one of the most common genetic disorders and there is no predilection for any specific geographic location or ethnic or cultural group. Although NF1 is usually diagnosed based on diagnostic criteria, clinical diagnosis may be challenging in individuals with atypical findings or in children at an early age, in whom the characteristic features have not developed fully yet.6 In addition, members of RAS-related disorders may present with overlapping clinical findings, which can make
diagnosis difficult, especially in the prenatal period. Therefore, due to the complexity of clinical diagnosis, understanding the mechanism of molecular pathogenesis in NF1 would be useful to characterize this disorder. The pathogenic variants identified in NF1 mostly lead to decreased protein levels or a truncated protein. Other pathogenic variants include single or multiple exon deletions/duplications and larger microdeletions involving NF1 and neighboring genes, which affect 4% of the patients. Therefore, we suggest that a multi-stage variant detection procedure that involves target sequencing of NF1 with next-generation sequencing (NGS), multiplex ligation-dependent probe amplification (MLPA) analysis, and array comparative genomic hybridization (a-CGH) is essential for the identification of NF1 variants and the classification of patients. Such a strategy will provide not only follow-up opportunities and early detection of benign and malignant tumors but also appropriate genetic counseling, including prenatal or pre-implantation genetic diagnosis.

MATERIAL AND METHODS

Patients

This study included 27 unrelated Turkish families that had at least 1 patient clinically diagnosed with or suspected to have NF1 based on the NIH criteria, between June 1990 and 2019 at the Istanbul University, Istanbul Faculty of Medicine (NIH, 1988). The study was approved by the Istanbul Medical Faculty Ethics Committee (June 30 2017, 825 issues) and informed written consent was obtained from each patient. After the patients’ approval, a physical examination was performed. For each family, a pedigree involving at least 3 generations was drawn and all the available medical records (radiographic images, photographs, laboratory test results, etc.) from each family were obtained.

Methods

Sample Preparations

A targeted-panel gene test was designed for the coding exons of the NF1 gene (NM_001042492.3) in 70 amplicons with 100 coverage. DNA isolations were performed by using the commercially available DNA Isolation Kit for Mammalian Blood (Roche11667327001/Magnapure Large Volume kit).

Next-Generation Sequencing

The DNA Library was prepared using the Ion Ampliseq Library Kit 2.0 (Thermo Fisher Scientific) according to the manufacturer’s instructions. After primer sequences were partially digested, adapters and barcodes (Ion Express™ Barcode) (Thermo Fisher Scientific) were ligated to the amplicon using the Ion Xpress Barcode Adapters Kit (Life Technologies) procedure. Following equalization of barcoded libraries using the Ion Library Equalizer Kit (Thermo Fisher Scientific), the emulsion-based PCR process was applied with the PGM Template Hi-Q™ View kit on the Ion OneTouch 2 Instrument (Thermo Fisher Scientific). The enrichment of template-positive Ion Sphere™ Particles was performed with Ion PGM™ template preparation kits using the Ion OneTouch™ ES Instrument (Thermo Fisher Scientific). Sequencing of enriched particles was performed by Ion PGM Hi-Q View Sequencing Kit, according to the manufacturer’s instructions (Thermo Fisher Scientific).

Data Analysis

For reference genome alignment, base calling, and filtering of weak signal reads, we used the IT platform-specific pipeline software Torrent Suite (v 4.2) with the plug-in ‘variant caller’ program (Life Technologies). Raw data were processed and aligned to the hg19 human reference genome (GRCh37) using Torrent Suite Software (v5) (Thermo Fisher Scientific) for a reliable variant calling. For annotation of the variants, Ion Reporter (v 4.0) software was used. Integrated Genomics Viewer (IGV) (http://software.broadinstitute.org/software/igv/) was used to see the coverage and quality of the variants. A cut-off of 100X coverage was applied to all analyses. In silico analysis programs (MutationTaster, PolyPhen and SIFT) as well as Combined Annotation-Dependent Depletion (CADD) (https://cadd.gs.washington.edu) were used for the prediction of the disease-causing status of novel variants. Variant frequency observed from the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/about). The phenotypic impact was searched from ClinVar and a literature search. The guidelines of the American College of Medical Genetics and Genomics (ACMG) were used for variant classification.

Sanger Sequencing

The confirmation and segregation analysis of the candidate pathogenic variants found with NGS were carried out with PCR followed by Sanger sequencing. Polymerase chain reaction (PCR) (Thermo Scientific) was performed only for exons with pathogenic variants for the NF1 gene. PCR products were sequenced utilizing the BigDye Terminator 3.1 Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Sequence analyses were performed using ABI3500 Sequencing System (Applied Biosystems).

Multiplex Ligation-dependent Probe Amplification

NF1-MLPA reaction kits (SALSA P081/P082) (MRC-Holland, Amsterdam, The Netherlands) targeting whole exons in NF1 were used according to the manufacturer’s protocol. Fragment analyses were conducted utilizing the Genetic Analyzer 3730 capillary electrophoresis system (Applied Biosystems, CA, USA). Data analysis was performed using Coffalyser.Net software.

Array Comparative Genomic Hybridization

The a-CGH analysis was performed by using Agilent SurePrint G3 CGH+SNP Microarray Kit (4x180K) (Agilent Technologies, Inc., Santa Clara, CA, USA). Data analysis was performed using Agilent Genomics Workbench (v 5.0.2.5). Genomic linear positions were given relative to hg19. The deleted genomic regions of the patients were evaluated using some specific databases such as Online Mendelian Inheritance in Man (https://www.omim.org), The Database of Genomic Variants (DGV, http://dgv.tcag.ca/dgv/app/home), The Database of genomic Variations and Phenotype in Humans using Ensembl Resources (DECIPHER, https://decipher.sanger.ac.uk), and DISEASES (https://diseases.jensenlab.org/Search).
RESULTS

Clinical Findings
Overall, 44 individuals from 27 families who had a clinical diagnosis or were suspected of NF1 based on NIH criteria were included in our study group (Table 1). Café-au-lait spots were the most common skin finding, which was present in 38 patients. Lisch nodules were diagnosed in 8 patients. Thirty-one patients had axillary and inguinal freckling. Optic pathway gliomas (astrocytomas of the optic pathway) were present in 3 patients. Neurofibromas of a variety of different types, either cutaneous neurofibromas, plexiform neurofibromas or malignant peripheral nerve sheath tumors were detected in 39, 9, and 1 patient(s) respectively. Distinctive bone abnormalities such as scoliosis and local bony overgrowths were identified in 6 and 1 patient(s), respectively.

A 20-year-old female (A1-III-3) had a history of multiple exostoses for which she had been operated on previously (Figure 1A), although there were 5 NF1 patients in this family, this finding was detected only in this patient. She was tested for exostosin-1 gene (EXT1) variants, based on a clinical diagnosis of multiple exostoses. We found a frameshift pathogenic variant [8q24.11, NM_000127, C.130_131insGA (p.N44Rfs*93)] in EXT1 by Sanger analysis (Figure 1B), which had not been reported before. The parents tested negative for this variant.

In our cohort, a female patient (A2-III-1) was diagnosed with breast cancer at the age of 35 years. The risk of breast cancer in NF1 is especially increased in patients younger than 50 years of age compared to the general population. Although breast cancer is a rare entity, it is the most common cause of death in NF1 patients. Of those, 3 [(A11-III-4), (A11-IV-3), (A11-V-1)] belonged to the same family (Figure 3), whereas in the 2 others (A3 and A19), the deletion had occurred de novo (Table 2). The a-CGH analysis defined a 17q11.2 deletion of about 1.4 Mb in 4 of them; and a 1.2 Mb deletion in 1 patient (family A3). To evaluate the clinical significance of the deleted region, we searched OMIM, DGV, DISEASE, and DECIPHER databases. The genes CRLF3, ATAD5, TEFM, ADAP2, RNF135, OMG, EVI2B, EVI2A, RAB11FIP4 SUZ12, LRRC37B, and NF1 were found to be located in these regions.

With the molecular approach used in our study, we did not detect any pathogenic variants in 2 patients who met the NIH criteria for a diagnosis of NF1 (A12-III-1 and A26-III-1).

DISCUSSION
NF1 is one of the most common genetic disorders with a broad spectrum of phenotypic manifestations that may progress throughout the life span of the patients. Although NF1 can be diagnosed readily based on criteria defined by NIH, diagnosis can sometimes be difficult, especially when a phenotype does not meet the diagnostic criteria of NIH. Therefore, the genetic analysis should be included in the diagnostic algorithm of this disorder. Loss of function of NF1 is responsible for the etiopathogenesis of clinical manifestations associated with this disorder. Most of the pathogenic variants detected in NF1 are single-base substitutions, insertions, or deletions. Other variants arise from multiple exon deletions or amplifications as well as microdeletions including NF1 and neighboring genes. Due to the numerous types of variants, it may not be possible to confirm the diagnosis with a single method. Therefore, a multi-step process is required for a comprehensive analysis of the gene. In this scope, we utilized a multi-step process consisting of NGS, MLPA, and a-CGH. In the first step, NGS yielded 22 pathogenic variants which were detected in 81% (22/27) of our patients. Additionally, in the (A1-III-3) patients from this study, a novel pathogenic variant in the EXT1 gene was found by NGS analysis. The EXT1 gene is associated with hereditary multiple exostoses (HME) disease. HME is a rare autosomal dominant disorder. The incidence is estimated to be about 1 in 5000. The disease is characterized by multiple exostoses, skeletal deformities, and scoliosis. It is predominantly caused by a pathogenic variant in EXT1 and exostosin-2 (EXT2) genes, that encode heparan sulfate (HS) glycosyltransferases. Therefore, this patient (A11-III-3) had both NF1 and HME. This shows the significance of considering additional diagnoses.

In the second step, MLPA analyses detected a deletion in 5 patients, 3 of them being members of the same family (3/27; 11%). In the third step, the a-CGH study showed a large deletion involving NF1 and multiple genes in all these patients.

For 2 patients who completely met the clinical diagnostic criteria, no NF1 variants could be identified with this multi-step approach. Previous studies have reported NF1 pathogenic variants located in deep intronic or regulatory regions, which are not covered by NGS. Other possible explanations for the absence of pathogenic
TABLE 1. Demographics, Clinical Findings and NF1 Mutational Spectrum of the Proband from NF1 Families by the NGS Study

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<td>Prediction-disease-causing</td>
</tr>
<tr>
<td>Family 15</td>
<td>A15-III-4</td>
<td>M/38</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>47</td>
<td>17:2060172</td>
<td>a.22695234 A</td>
<td>c.4925delT</td>
<td>p.Tyr1642 fs</td>
<td>Frame-shift</td>
<td>Deletion</td>
<td>Novel</td>
<td>NA</td>
<td>PS1</td>
<td>23.7</td>
<td>Np</td>
<td>Np</td>
<td>-</td>
<td>Prediction-disease-causing</td>
</tr>
</tbody>
</table>
The phenotypes of patients with NF1 microdeletion may be influenced by the loss of function of the genes TEFM, ADAP2, RNF135, OMC1, LRRC37B, TETRA, SUZ12, LRC72, and NFI in the deleted region. Based on literature data, the dysmorphic facial features of patients in our study may be due to the haploinsufficiency of the RNF135 gene. Moreover, the expression of only one copy of OMM61 (15q38) gene plays a significant role in intellectual disability traits in these patients. Similarly, COQ10, a gene coding for cytochrome c oxidase subunit 6, may contribute to intellectual disability in patients with NF1 microdeletion. The risk of NF1 microdeletion may be increased in patients with NF1 microdeletion, although the exact mechanism remains unknown.

In patients with NF1, the expression of only one copy of OMM61 (15q38) gene plays a significant role in intellectual disability traits in these patients. Similarly, COQ10, a gene coding for cytochrome c oxidase subunit 6, may contribute to intellectual disability in patients with NF1 microdeletion. The risk of NF1 microdeletion may be increased in patients with NF1 microdeletion, although the exact mechanism remains unknown.
is associated with learning disabilities in NF1 microdeletion patients and tumorigenesis. In addition, ADAP2 gene deletion (MIM: 608635) may lead to cardiovascular abnormalities. Likewise, the deletion of the SUZ12 and UTP6 genes, which are also expressed during human heart development, maybe related to cardiomyopathy. Although all these genes were deleted in our patients, only facial dysmorphism appeared to be a discriminating feature for microdeletion in our patient group.
## TABLE 2. Clinical and Genetic Features of NF1 Microdeletion Patients

<table>
<thead>
<tr>
<th>Family</th>
<th>Case</th>
<th>Gender/Age</th>
<th>NIH Criteria</th>
<th>Molecular results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 3</td>
<td>A3-III-2</td>
<td>F/18</td>
<td>+ + + +/+</td>
<td>Hypertelorism, macrocephaly, mild micrognathia, bilateral cubitus valgus, hyperextensibility, genu recurvatum, hamartoma of the splenium of the corpus callosum, ectopia of the cerebellar tonsils, hypothyroidism, and thyroiditis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whole NF1 gene deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17q11.2 deletion (1.2 Mb), Position (29124299_3026958), LOC2071333515, SH3GLP1, SUZ12P1, CRLF3, ATAD5, TEFM, ADAP2, RNF135, DPRXP4, MIR4733, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, MIR4724, MIR193A, MIR4725, MIR365B</td>
</tr>
<tr>
<td>Family 11</td>
<td>A11-III-4</td>
<td>F/66</td>
<td>+ + + +/+</td>
<td>Hypertelorism, macrocephaly, hyperparathyroidism, hypertension, Adrenal adenoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whole NF1 gene deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17q11.2 deletion (1.4 Mb), Position (28941066_30367214), CRLF3, ATAD5, EFM, ADAP2, RNF135, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, MIR193A, SUZ12, LRRC37B, LOC2071333515, SH3GLP1, SUZ12P1, DPRXP4, LOC107984974, LOC646030, MIR4733, MIR4724, MIR4725, MIR365B, COPRS, UTP6</td>
</tr>
<tr>
<td></td>
<td>A11-IV-3</td>
<td>F/35</td>
<td>+ + + +/+</td>
<td>Hypertelorism</td>
</tr>
<tr>
<td></td>
<td>A11-V-1</td>
<td>M/8</td>
<td>+ + + +/+</td>
<td>Neurofibrosarcoma</td>
</tr>
<tr>
<td>Family 19</td>
<td>A19-III-5</td>
<td>M/40</td>
<td>+ + + +/+</td>
<td>Glaucoma, neurofibrosarcoma, malignant mesenchymal tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whole NF1 gene deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17q11.2 deletion (1.4 Mb), Position (28941066_30367214), CRLF3, ATAD5, EFM, ADAP2, RNF135, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, MIR193A, SUZ12, LRRC37B, LOC2071333515, SH3GLP1, SUZ12P1, DPRXP4, LOC107984974, LOC646030, MIR4733, MIR4724, MIR4725, MIR365B, COPRS, UTP6</td>
</tr>
</tbody>
</table>

Total, N 5 5 1 5 0 2 0 3

*CAL, cafe-au-lait spots; NF, neurofibromas; PNF, plexiform neurofibroma; AF, axillary freckling; IF, inguinal freckling; OG, optic glioma; LN, lisch nodules; MPNST, malignant peripheral nerve sheath tumors; FH, family history.
The phenotypic variation of NF1 microdeletion syndrome depends on the extent of the chromosomal rearrangements and the genes that have been deleted. However, even in cases with the same deletion, phenotypic variations may be observed. It may be suggested that the NF1-related phenotypes may be influenced by additional mechanisms such as modifier genes and regulatory proteins that interact with the NF1 gene. Although the clinical features of NF1 microdeletion patients are generally more severe, we suggest that the presence of a mild clinical phenotype should not exclude the possibility of NF1 gene deletion in a patient. Despite the various types of pathogenic variants identified in this study, the evaluation of the correlation between the genotype–phenotype of these patients was not possible due to the small patient sample size. A systematic comparative study with more patients of the same age and with overlapping clinical manifestations may provide a more reliable definition of genotype–phenotype relations. Therefore, future WES/WGS studies may provide the variants in modifying genes which may help to explain genotype–phenotype correlations in NF1. Though prenatal diagnosis of NF1 is controversial, our three-step approach presented here seems to be more effective for symptomatic/presymptomatic diagnosis and genetic counseling for NF1.

**Ethics Committee Approval:** The study was approved by the İstanbul Medical Faculty Ethics Committee (June 30 2017, 825 issues).

**Patient Consent for Publication:** Informed written consent was obtained from each patient.

**Data-sharing Statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

**Author Contributions:** Concept - S.S., K.C.; Design - S.S.; Supervision - S.S., K.C.; Data Collection and/or Processing - S.S.; Analysis and/or Interpretation - S.S.; Literature Review - S.S., K.C., T.K., Ş.P., Ş.Ö.; Writing - S.S.; Critical Review - K.C., T.K., Ş.P., Ş.Ö.

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**Conflict of Interest:** The authors have declared that no conflicts of interest exist.

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**REFERENCES**