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Genetic Survey of Autosomal Recessive Peripheral Neuropathy Cases Unravels High Genetic Heterogeneity in a Turkish Cohort

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Abstract

Background and Objectives

Inherited peripheral neuropathies (IPNs) are a group of genetic disorders of the peripheral nervous system in which neuropathy is the only or the most predominant clinical feature. The most common type of IPN is Charcot-Marie-Tooth (CMT) disease. Autosomal recessive CMT (ARCMT) is generally more severe than dominant CMT and its genetic basis is poorly understood due to high clinical and genetic diversity. Here, we report clinical and genetic findings from 56 consanguineous Turkish families initially diagnosed with CMT disease.

Methods

We initially screened the *GDAP1* gene in our cohort as it is the most commonly mutated ARCMT gene. Next, whole-exome sequencing and homozygosity mapping based on whole-exome sequencing (HOMWES) analysis was performed. To understand the molecular impact of candidate causative genes, functional analyses were performed in patient primary fibroblasts.

Results

Biallelic recurrent mutations in the *GDAP1* gene have been identified in 6 patients. Whole-exome sequencing and HOMWES analysis revealed 16 recurrent and 13 novel disease-causing alleles in known IPN-related genes and 2 novel candidate genes: 1 for a CMT-like disease and 1 for autosomal recessive cerebellar ataxia with axonal neuropathy. We have achieved a potential genetic diagnosis rate of 62.5% (35/56 families) in our cohort. Considering only the variants that meet the American College for Medical Genetics and Genomics (ACMG) classification as pathogenic or likely pathogenic, the definitive diagnosis rate was 55.35% (31/56 families).

Discussion

This study paints a genetic landscape of the Turkish ARCMT population and reports additional candidate genes that might help enlighten the mechanism of pathogenesis of the disease.

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Glossary

ACMG = American College for Medical Genetics and Genomics; **ARCMT** = autosomal recessive CMT; **CMT** = Charcot-Marie-Tooth; **FRDA** = Friedreich's ataxia; **IPN** = inherited peripheral neuropathy; **mNCV** = motor nerve conduction velocity; **NGS** = next-generation sequencing; **SIFT** = sorting intolerant from tolerant; **WES** = whole-exome sequencing.

Inherited peripheral neuropathies (IPNs) are a group of clinically and genetically diverse disorders of the peripheral nervous system in which neuropathy is the only or the most predominant clinical feature.[1] The most common type of IPN is hereditary motor and sensory neuropathy, generally referred to as Charcot-Marie-Tooth (CMT) disease, named after the 3 neurologists who first reported the clinical features.[2] Widespread population analyses are very limited to pinpoint the true prevalence of CMT; however, recent population-based studies report a prevalence between 9.7 and 82.3 in 100,000 individuals.[3] The clinical progression of the disease is characterized by prominent length-dependent muscle weakness and sensory loss with commonly observed foot deformities such as pes cavus.[1,2]

Historically, CMT is classified into 2 broad groups by evaluating the clinical features of the patient: an upper limb motor nerve conduction velocity (mNCV) less than 38 m/s suggests a demyelinating pathology (also called CMT1), whereas a velocity above 38 m/s suggests an axonal pathology (also called CMT2).[4] Later, an additional subtype was introduced into the literature as intermediate CMT (CMT-I) for individuals with an upper limb mNCV between 25 and 45 m/s.[5] As the field advanced, a further subclassification was used that assigns different letters to phenotypically classified subtypes according to the causative gene.[6] More recently, a new classification was proposed that uses abbreviations for inheritance type, phenotypical form of the disease, and the genetic cause.[7,8]

The first CMT-causing genetic locus was identified in 1982,[9] and, at the time of writing, more than 90 distinct disease-causing genes were reported.[10-12] Investigation of novel causative genes was initially performed by genetic linkage analyses in large pedigrees, positional cloning, or candidate gene approaches, whereas the Human Genome Project and subsequent advances in next-generation sequencing (NGS) technologies have led to a great acceleration in the number of CMT-causing genes and mutations.[11,13] However, even with the widespread use of advanced NGS technologies, only about 45%–60% of patients with CMT receive genetic diagnosis worldwide, suggesting that the number of CMT-causative genes will increase by time.[11,14-17]

In the current study, we evaluated 56 Turkish families likely representing an autosomal recessive CMT (ARCMT) cohort. In the strategy used, initially *GDAP1* was screened for causative variants in the cohort, followed by a combination of whole-exome sequencing and homozygosity mapping with HOMWES approach. This allowed us to reach a potential genetic diagnosis rate of 62.5% (35/56 families) and identify 2 novel candidate genes: 1 of which is likely causative for ARCMT disease with atypical features and 1 for autosomal recessive cerebellar ataxia with axonal peripheral neuropathy. The genetic data should be considered cautiously since large datasets for control individuals of Turkish origin are limited in the literature.

Methods

Patient Cohort

A total of 180 individuals including affected and unaffected members from 56 unrelated families from different regions in Turkey have been analyzed in this study. The index patients from each family were evaluated by expert neurologists and were initially diagnosed with CMT. Among these 56 families, 27 had a family history of CMT with multiple affected individuals, whereas 29 families had a single affected individual born to consanguineous parents. Age at onset was in childhood in 52 index cases and in adulthood in 4 families. Over 50% of index patients studied had a severe phenotype with additional clinical features, such as severe scoliosis, hearing loss, vocal cord involvement, and intellectual disability along with symmetrical distal weakness. The presence of CMT1A duplication or hereditary neuropathy with pressure palsies deletion was excluded in all patients using short tandem repeat markers.[18] Acquired neuropathy was excluded for all patients in the clinical setting. Therefore, the patients studied here most likely represented an ARCMT cohort.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Human Research Ethics Committees of Istanbul University (45103048) and Boğaziçi University (FMINAREK-2018/05). All participants (or guardians of participants) enrolled in the study signed an informed consent for research. STROBE cohort checklist was used when writing the report.[19]

Genetic Analyses

Peripheral blood samples from 180 individuals (56 families) were obtained, and genomic DNA was purified from these samples. All DNA samples were barcoded anonymously with a unique family identifier and kept refrigerated until further use.

The first step of the analysis was screening of the coding regions of the *GDAP1* gene using PCR and subsequent Sanger sequencing. The patients with a *GDAP1* mutation previously identified as disease-causing (a recurrent mutation) were excluded from further analyses. Next, whole-exome sequencing was performed using the Illumina NextSeq

500 device with Illumina Nextera rapid capture kit for the patients without a *GDAP1* recurrent mutation. Whole-exome sequencing (WES) data quality was confirmed by combining paired-end and single-end binary alignment map files, excluding repetitions, and excluding variants with a coverage less than 50X. An average of 20,000 different variants were observed in each index patient. Initially, WES data were filtered for variants in a data set of known causative genes for IPN: synonymous and deep intronic variants and variants with alternative allele frequency over 5% in the general population were filtered out. Recurrent disease-causing mutations identified in patients using this approach were verified in index cases using Sanger sequencing. In a number of patients, novel variants were identified in known disease-causing genes that were not previously reported in databases as disease causing. For these patients, the segregation of variants was verified in the proband and their available affected or unaffected family members using Sanger sequencing. For the variants that fit the inheritance pattern in the family, possible diagnoses were considered when the referring clinician approved the genotype/phenotype correlation.

Finally, the patients who could not be genetically diagnosed by this procedure were further analyzed for disease-causing gene discovery. For this purpose, homozygosity mapping based on whole-exome sequencing analysis (HOMWES) software (genomecomb.sourceforge.net/releases/release0.11.0.html) was used to determine the homozygous regions in patient exomes as previously described.[20] To search for novel candidate genes in these patients, variants that reside in the large homozygous regions identified by HOMWES were prioritized. Variant filtering was performed with strict parameters: variants with a read depth of less than 30, variants with alternative allele frequency over 1%, and variants that were predicted to be benign/tolerated by both sorting intolerant from tolerant (SIFT) and PolyPhen2 algorithms were excluded. Candidate variants were then verified in the proband and their affected or unaffected family members with Sanger sequencing. ToppGene (toppgene.cchmc.org/prioritization.jsp) and Endeavour (homes.esat.kuleuven.be/;bioiuser/endeavour/tool/endeavourweb.php) algorithms were used to prioritize among the multiple candidate genes. All genetic findings were analyzed for American College for Medical Genetics and Genomics (ACMG) criteria and classified according to this guideline.[21]

Data Availability

Whole-exome sequencing data of all participants are present in the Genesis Platform (tgp-foundation.org/g-e-n-e-s-i-s). All variants reported here are submitted to the ClinVar database and can be found in accession numbers SCV001548301-SCV001548332. Additional data can be made available on reasonable request.

Results

Mutations in the *GDAP1* Gene

Mutations in the *GDAP1* gene are the most common cause of ARCMT disease with a frequency of 10%–15% in ARCMT cases.[22,23] Therefore, we initially screened our cohort for mutations in this gene. As expected, 6 patients were shown to carry recurrent homozygous mutations in *GDAP1* (eTable 1, links.lww.com/NXG/A464). Families 5, 12, 26, and 42 had c.786del, p.Phe263Leufs*22 variant, family 9 had c.174_176delinsTGTG, p.Pro59Valfs*4 variant, and family 50 had c.458C>T, p.Pro153Leu variant, all in homozygous condition. These patients with recurrent GDAP1 mutations were excluded from further analyses. The clinical and genetic findings of all 56 patients enrolled in the study are given in eTable 1, links.lww.com/NXG/A464.

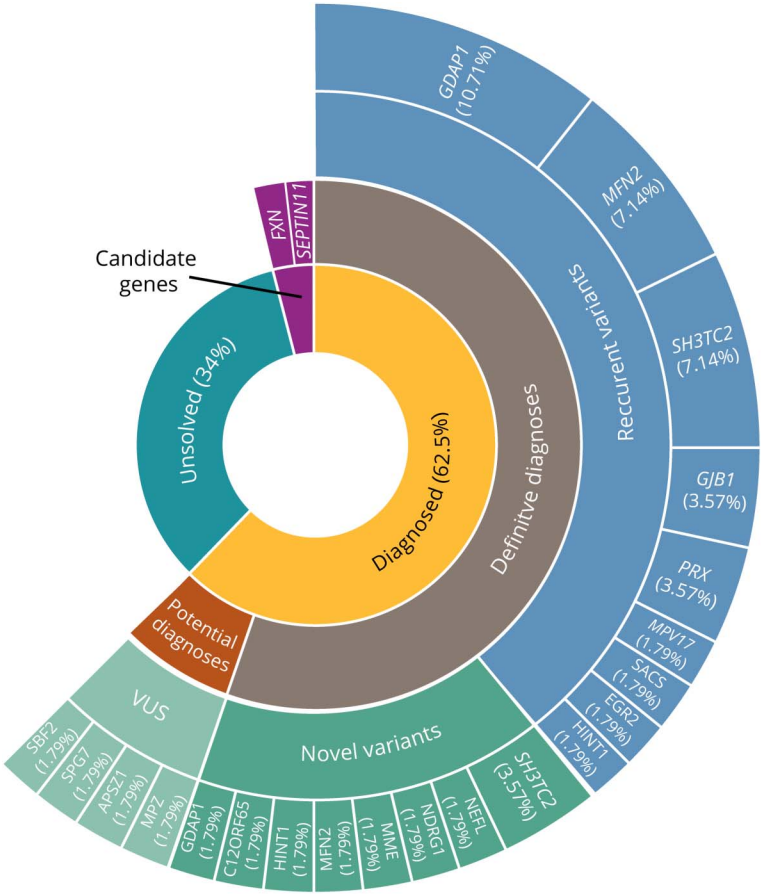
Whole-Exome Sequencing

WES was performed for 50 patients, and among those, 16 were genetically diagnosed by filtering for recurrent variants in known IPN-causing genes. Among these, 1 patient was shown to carry a recurrent mutation (c.2182C>T, p.Arg728Ter) in the *SACS* gene, which is a known causative gene for autosomal recessive spastic ataxia of Charlevoix-Saguenay.[24] The clinical re-evaluation revealed that the patient developed mild spasticity, positive Babinski sign, and cerebellar ataxia after his initial referral for genetic analysis.

We have identified 13 further candidate variants in known IPN genes that were not previously reported as disease causing. These variants were shown to fit the segregation of the disease in the pedigree, and the referring clinicians suggested that the corresponding genes could explain the clinical representation of each patient. Seven of these 13 patients carried homozygous termination or frameshift mutations in genes that were reported to be disease causing due to loss of function. These 7 mutations were in *MME* (homozygous, c.531del, p.Lys177Asnfs*15), *HINT1* (homozygous, c.99del, p.Phe33Leufs*22), *NDRG1* (homozygous, c.237C>A; p.Tyr79Ter), *NEFL* (homozygous, c.54C>A, p.Tyr18Ter), *GDAP1* (homozygous, c.112C>T, p.Gln38Ter), *C12ORF65* (homozygous, c.18_21del, p.Leu6Phefs*7), and *SH3TC2* (homozygous, c.54dup, p.Lys19Ter) genes, and they were classified as pathogenic or likely pathogenic according to the ACMG criteria. The other 6 patients were homozygous for missense mutations; thus, pathogenicity could not be assessed solely on familial segregation analysis. Still, 2 of those alleles (homozygous c.1586G>A; p.Arg529His variant in *SH3TC2* and homozygous c.271G>T; p.Val91Leu variant in *MFN2* genes) were classified as likely pathogenic according to the ACMG criteria because, in addition to other supporting evidence, these variants were observed in the same codon where a different missense change was reported as pathogenic previously (CM033080 and CM127950 for *SH3TC2*; CM117904 for *MFN2* in HGMD). The remaining 4 missense variants with unknown significance were in *SPG7* (c.454A>G, p.Met152Val), *AP5Z1* (c.1568G>A, p.Arg523His), *SBF2* (c.2549T>C, p.Met850Thr), and *MPZ* (c.362A>G, p.Asp121Gly) genes (eTable 1, links.lww.com/NXG/A464).

Although the cohort represented possible recessive inheritance based on declared parental consanguinity, pedigree

Figure 1 Summary of Diagnostic Outcome of the Study



Finding	Gene name	Total number of cases	Percentage
Diagnosed	*GDAP1*	7	12.50
	SH3TC2	6	10.71
	MFN2	5	8.92
	HINT1	2	3.57
	PRX	2	3.57
	GJB1	2	1.79
	EGR2	1	1.79
	SACS	1	1.79
	MPV17	1	1.79
	C12ORF65	1	1.79
	MME	1	1.79
	NDRG1	1	1.79
	NEFL	1	1.79
	SBF2	1	1.79
	AP5Z1	1	1.79
	MPZ	1	1.79
Candidate genes	*FXN*	1	1.79
	SEPTIN11	1	1.79
Unsolved		19	33.90

analysis, and/or severity of symptoms, pathogenic dominant mutations have also been observed. Families 32, 52, and 53 were shown to carry recurrent heterozygous mutations in the *MFN2* gene and family 43 to carry a novel heterozygous *MPZ* variant of unknown significance. Besides, family 51 was shown to have a recurrent disease-causing mutation in the *GJB1* gene.

Novel Candidate Genes

We have used homozygosity mapping on WES data to unravel the causative loci for the remaining undiagnosed 21 families. This analysis revealed 2 candidate disease-causing genes.

An isolated pediatric patient (family 39) had a biallelic frameshift variant in the *SEPTIN11* gene (c.265dup; p.Glu89Glyfs*12). Her symptoms started with walking difficulty at age 7 years. Dysmetria, dysdiadochokinesia, and truncal ataxia were her prominent findings during neurologic examination. She had additional axonal sensorimotor polyneuropathy prominent in the lower extremities and hypertrophic cardiomyopathy. Her visual evoked potential examination revealed bilateral symmetrical prolongation of latencies. The p.Glu89Glyfs*12 variant in *SEPTIN11* was not reported in population databases. Besides, MutationTaster (mutationtaster.org/) algorithm predicted the variant to cause nonsense-mediated mRNA decay. qPCR and Western blotting analyses performed on the skin fibroblasts of the proband showed significantly decreased expression of Septin11 mRNA and protein, respectively (data not shown). We could not identify an additional family with a mutation in the same gene in our patient cohort, through GeneMatcher (genematcher.org/) or Genesis Platform (tgp-foundation.org/).

In family 24, we have identified a biallelic missense variant in the *FXN* gene (c.493C>T; p.Arg165Cys), which is a known causative gene for Friedreich ataxia. Three affected siblings in this family (24) were homozygous for the variant and presented a CMT-like phenotype. The clinical features of this family and the genetic findings were reported previously.[25]

Diagnostic Outcome of the Analyses

The initial screening of the patients for founder *GDAP1* mutations in the Turkish population revealed that about 10% of the cohort has causative mutations in this gene. WES analysis identified the causative genes in 29 additional cases. Among these, 16 cases had recurrent and 13 had novel variants in known IPN-related genes. This approach for screening known disease-causing genes allowed genetic diagnosis of 62.5% (35/56) of families in our cohort. Nine of the novel deleterious variants met the ACMG variant classification as

likely pathogenic or pathogenic. The other 4 were missense variants of unknown significance and need further molecular analyses to assess pathogenicity. When these 4 cases are not considered as definitive diagnoses, the diagnostic rate remains to be 55.35% (31/56 cases). Diagnostic outcome of the study is summarized in Figure 1.

Discussion

In this study, genetic survey of 56 index patients with CMT unraveled the genetic causes of autosomal recessive subtypes in the Turkish population and allowed identification of 2 novel candidate genes. The *GDAP1* gene was the most commonly mutated gene in 12.5% of the cases. WES analysis allowed further identification of causative variants in 29 patients in known genes for CMT or other related neuronopathies. Nine of 13 novel variants were likely pathogenic or pathogenic according to the ACMG criteria, whereas 4 variants were of unknown significance. Thus, we provided genetic diagnosis in 35 patients (62.5%), 31 of which were definitive (55.35%). In family 24, we defined a new gene-disease relationship and showed that biallelic *FXN* missense mutations are not lethal, but can cause a CMT-like phenotype, rather than Friedreich's ataxia (FRDA), as reported previously.[25] In another family (39), we identified *SEPTIN11* as a novel candidate disease-causing gene for autosomal recessive cerebellar ataxia with axonal peripheral neuropathy.

The overall definitive genetic diagnosis rate in our study was 55.35% in accordance with 45%–60%, reported by previous studies.[11,14,15,17,26] In our cohort, *GDAP1* gene mutations were the most common genetic cause (12.5%, 7/56 patients), followed by mutations in *SH3TC2* (10.7%, 6/56 patients). In similar studies examining patients with ARCMT, the mutation frequency in *GDAP1* was reported to be 10%–15%, and the mutation frequency in *SH3TC2* was 7.5%.[22,23] Thus, the commonly mutated genes were also in correlation with the previously reported population frequencies. *MFN2* was the third most commonly mutated gene, with 5 families (8.9%), and *HINT1*, *PRX*, and *GJB1* mutations were observed in 2 families (3.6% each). Mutations in *AP5Z1*, *C12ORF65*, *EGR2*, *MME*, *MPV17*, *MPZ*, *NDRG1*, *NEFL*, *SACS*, *SBF2*, and *SPG7* genes were observed only once in our cohort.

We have identified recurrent heterozygous mutations in the *MFN2* gene in 3 families and a novel heterozygous *MPZ* variant in 1 family implicating dominant cases in a possible recessive inheritance cohort. Besides, 1 family was shown to have a recurrent disease-causing mutation in the *GJB1* gene. Thus, it is advisable to focus on known disease genes, but not particularly on inheritance pattern during initial variant filtering. Otherwise, we would have missed these variants in genes responsible for autosomal dominant and X-linked forms of the disease. It should also be noted that disease-causing mutations in *MFN2* and *MPZ* could occur sporadically and expressivity could be low for some individuals.[27]

The filtering criteria used to evaluate WES data generally include the nucleotide changes caused by the variant (such as substitutions, short indels, frameshifts, and changes in regulatory regions), alternative allele frequency, and pathogenicity scores predicted by SIFT and PolyPhen2.[28] Read depth is usually used as a filtering criterion to remove false positives from the data. In our study, although the variants in the known IPN genes were examined, read depth or pathogenicity predictions were not used as filtering criteria initially, and the alternative allele frequency was set to less than 5%, which could be considered as a wide range. Still, we did not encounter a high number of false-positive results and found out that the use of this initial relaxed filtering criteria allowed us to reach a relatively high genetic diagnosis rate. Furthermore, although all patients enrolled were initially diagnosed with CMT, genetic findings suggested overlapping neurologic disorders for some patients. Thus, investigating causative genes for related disorders, as well as CMT-causing genes, in data analysis also improved genetic diagnosis rate. To all our efforts, we could not identify the genetic cause in about 40% of patients, which can be attributed to disadvantages of WES,[29,30] but also underlines the genetic heterogeneity of IPN and points to the presence of unknown causative genes or perhaps to nonmendelian characteristics.[31]

Apart from providing a genetic overview of ARCMT in Turkey, we have identified 2 potential candidate genes. One of the families (24) had a homozygous missense *FXN* mutation with a CMT-like disease, instead of FRDA. To the best of our knowledge, this case was the first family reported in literature with a biallelic missense mutation in this gene, and the findings challenge the idea that these mutations cause embryonic lethality, as suggested previously.[32] This finding represents a novel phenotype in the clinical spectrum between CMT and FRDA for which the clinical findings were reported previously.[25] Another family we identified in this study (39) has a biallelic frameshift mutation in the *SEPTIN11* gene. The clinical features of the index patient revealed cerebellar ataxia, axonal sensorimotor polyneuropathy, and hypertrophic cardiomyopathy. Unfortunately, we were not able to find any additional families with similar clinical features and genetic findings through matchmaking tools including GeneMatcher and Genesis Platform. However, we found that Septin11 mRNA and protein was significantly reduced in patient skin fibroblasts (data not shown). Septin11 protein was shown to be highly expressed in intact mouse cerebellum, particularly in Purkinje cells and the knockdown of Septin11 reduced dendritic branching and spine density, while increasing the length of dendritic protrusions in cultured murine hippocampal neurons.[33] The clinical features of our patient can be explained by these alterations in the neuronal cytoarchitecture due to reduced expression of Septin11 caused by the biallelic frameshift mutation. Therefore, *SEPTIN11* should be considered as a causative gene for autosomal recessive cerebellar ataxia with axonal neuropathy, and patients with similar phenotypes should be screened for mutations in this gene.

In conclusion, we have analyzed a cohort of 56 consanguineous Turkish families with likely autosomal recessive peripheral neuropathy and provided genetic diagnoses to about 55% (31/56) of the patients. Our genetic diagnosis rate is one of the highest reported in the literature, and we believe that this is achieved by initially analyzing the data with relaxed filtering criteria and not restricting the analysis to CMT-causative genes. We have identified 22 families with 17 distinct recurrent mutations, as well as 13 families with novel alleles in known IPN-related genes, suggesting a rather high heterogeneity in this cohort. We believe that our study provides a genetic overview of the ARCMT population in Turkey and can provide a reference for genetic diagnosis strategies for populations with similar genetic background. In accordance with one of the main objectives of the study, we have identified 2 novel candidate disease-causing genes in this cohort. We suggest that biallelic *FXN* and *SEPTIN11* mutations should also be screened in patients with relevant clinical features. Based on our findings with marked genetic heterogeneity in this cohort, we suggest use of gene panels or whole-exome sequencing rather than single gene screening in populations with high consanguinity rate.

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Disclosure

The authors report no disclosures relevant to the manuscript. Go to Neurology.org/NG for full disclosures.

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