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Mutational Spectrum and Clinical Symptoms of Iranian Patients with Charcot-Marie-Tooth Disease: A Study of 23 Patients

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Abstract

Background: More than 80 genes are involved in the pathogenesis of the most common single gene peripheral neuropathies denoted as Charcot-Marie-Tooth (CMT). Only a few studies have investigated the pathological molecular mechanisms of Iranian patients affected with CMT. The aim of this study is to identify the clinical manifestation, mutational spectrum and phenotypic correlation of a cohort of patients with Charcot-Marie-Tooth disease (CMT) in Iran.

Methods: We conducted a comprehensive gene panel sequencing consisting of 80 genes in a cohort of 23 patients with CMT referred between January 2015 and March 2021. The recruited samples indicated almost an equal distribution of demyelinating and axonal types of CMT, with practically no difference between AD or AR patterns of inheritance.

Results: Four novel mutations, including c.271C>T in *LITAF* and c.205+1delG in *NDRG1*, c.2455A>C in *KIF1B* and c.1728A>G in FIGF were detected in four patients affected with demyelinating CMT types (CMT1C and CMT4D), and characterized phenotypically.

Conclusion: Our promising results unravel the complicated genetic architecture of Iranian CMT patients and help physicians and researchers achieve earlier diagnosis, better clinical management and recognizing high risk families. Further large-scale studies are needed to improve our understanding of CMT complex genetic architecture

Keywords: Charcot-Marie-Tooth disease, Gene, Genetic neuropathy, Mutation spectrum, Next generation sequencing, Rare disorder

Introduction

Charcot-Marie-Tooth (CMT) is a large heterogeneous group of neuromuscular disorders in the Peripheral Nervous System (PNS), which is responsible for the most common form of inherited polyneuropathies. Classically, CMT comprises hereditary disorders associated with sensory and motor dysfunction of the PNS, and is also referred to as Hereditary Motor and Sensory Neuropathy (HMSN). CMT has phenotypic overlap with two other forms of hereditary neuropathy, including distal Hereditary Motor Neuropathy (dHMN) and hereditary sensory and autonomic neuropathy (HSAN) (1).

Patients with CMT present various clinical manifestations including a slowly progressive symmetric weakness, muscle atrophy of the peroneal and distal muscles of the lower limbs, sensory loss, foot deformities (pes cavus and hammer toes), and decreased or absent tendon reflexes. Hands and forearms are affected later in life (2). This disorder has a variable prevalence, however previous epidemiological studies estimate the rate of 1 in 2500 (3). Dysfunction in different parts of nervous system such as lower motor neurons, sensory neurons and Schwann cells lead to CMT (4). CMT shows a wide range of onset, varying from severe complications in early childhood to mild symptoms in late adulthood. Furthermore, CMT demonstrates diverse inheritance patterns including autosomal dominant, autosomal recessive and X-linked. CMT is a chronic disease which disturbs many aspects of patients' life such as streaked legs syndrome, obstructive apnea vocal cord disorder, respiratory defects and laryngeal neuron dysfunction. Other symptoms reported in one large scale study include dysphagia and dyspnea (5,6). The average prevalence of CMT is 14.5 per 100 000, however the prevalance varies from 9.7 per 100 000 to 82.3 per 100 000 (7).

Different types of CMT can be distinguished by clinical and electrophysiological findings, which are classified into demyelinating types (CMT1, CMT4), with decreased median motor nerve conduction velocity (MCV; <38 *m/s*); axonal types (CMT2, AR-CMT2) with preserved median MCV (>38 *m/s*) but reduced amplitudes; and intermediate types (CMTI) with MCV of 25–45 *m/s*. The intermediate types are divided based on inheritance pattern into DI-CMT (autosomal dominant) or RI-CMT (autosomal recessive) (8). Mutation in different genes involved in biological pathways related to maintenance of PNS may be associated with CMT. To date, more than 80 genes have been reported to be linked with CMT [http://neuromuscular.wustl.edu/time/hmsn.html, accessed May 2018]. Most of the genes demonstrate definite inheritance patterns, while a minority are both dominant and recessive (*e.g. NEFL, HSPB1* and *MFN2*) (9,10). Recessive forms have generally a childhood onset and are more severe than dominant phenotypes (11).

It is worth noting that the only clinical sign which differentiates CMT1 from CMT2 is the presence of visible or palpable thickening of the nerve trunks in CMT1 (8). CMT types are further divided into numerous subtypes based on associated gene mutations. Most prevalent subtypes comprise CMT1A, CMT1B, CMT1E, CMT2A, CMT 2I/2J, DI-CMTD, CMTX1. 89% of patients with classical phenotype, without delay in the onset of walking (<15 months), AD inheritance and MCV in arm between 15 and 35 *m/s* presents CMT1A. Delay in the onset of walking occurs more often in CMT1B compared to CMT1A (12). Patients with CMT1E have more severe symptoms than those with CMT1A and usually have slower MCV. Most CMT2A patients (80%) have an early onset of symptoms (<10 years old) with a severe phenotype and will be wheelchair bound by 20 years of age. The remaining 20% have a later onset of symptoms (10-50 years old) and a milder phenotype (13). CMT2A patients with optic atrophy (CMT2A2), hearing loss, cerebral white matter abnormalities and diabetes mellitus have been described (14).

The first symptoms of CMT2I/2J appear very late (between 45 and 60) with a typical CMT2 phenotype, although there are some patients with pupillary abnormalities, deafness and sensory disturbances (15). A late onset phenotype with Adie pupil should point towards CMT2J. DI-CMTD is characterized by a variable severity, distal muscle atrophy, weakness, and sensory loss in the lower and upper limbs; MCVs are 30-40 *m/s* (16). CMTX1 may be associated with MCV within the limits established for different CMT types, the syndrome presenting with apparent vertical inheritance but no evidence of male-male transmission (17). The emergence of Next-Generation

Sequencing (NGS) leads to genome analysis of various heterogeneous diseases such as CMT (18, 19). Due to progress in sequencing technology more than 100 causative mutations have been reported to be involved in both CMT pathogenesis and clinical manifestations' development (20). The established flowchart for CMT is based on the different CMT forms that are determined by the clinical and electrophysiological evaluation of the patient and the existing frequency of causative mutations.

There is a considerable overlap between CMT and other phenotypes, and defects in different genes may cause similar phenotypes, and conversely, different clinical phenotype can be the consequence of defects in one unique gene. Aforementioned, CMT is clinically and genetically a heterogeneous disorder which makes it a challenge for genetic counsellors and clinicians (21). In the present study, we analyzed exome sequencing data for a cohort of Iranian patients clinically diagnosed with CMT, with the purpose of studying CMT mutation spectrum and its genotype-phenotype relationship in domestic population.

Materials and Methods

Patients

This study was approved by the ethics committee of Tehran University of Medical Sciences (TUMS) with number IR.TUMS.IKHC.REC.1396.4227 and all the protocols and study strategies were conducted in accordance to the approved guidelines. Informed written consent was obtained from each patient or their parents/guardians. Patients clinically diagnosed with different types of CMT, referred to Neuromuscular Clinic at different Hospital in Tehran, between 2015 and 2021, were enrolled in this study. Inclusion criteria included:

1. Clinical features of distal muscle wasting and atrophy, absent deep tendon reflex, Pes cavus presence and steppage gait, 2. Electro diagnostic findings of peripheral neuropathy. Exclusion criteria consisted of positive result of MLPA in *PMP22* gene. The final diagnosis of the CMT was confirmed by neurologists, and all the demographic data, clinical and physical evaluations, all the neurological finding and examinations, genetic counselling data and family history were recorded for each individual.

Molecular analysis

DNA extraction was performed using QIAamp DNA blood mini kit (QIAGEN, Valencia, CA). According to the clinical diagnosis, multiplex ligation-dependent probe amplification (MLPA) was requested and conducted using P405 CMT1 probe kit (MRC Holland, Amsterdam, the Netherlands). In addition, DNA samples were used for the amplification and preparation of a library by a custom Ampliseq panel (Thermo-Fisher, Waltham, MA) which covered all the genes listed in table 1. The prepared libraries were sequenced by Ion S5 sequencer (Thermo-Fisher, Waltham, MA) and the raw data was base-called, annotated and analyzed through a local validated NGS data pipeline. Identified variants were confirmed by direct sequencing and Segregation analysis was performed as well.

In silico analysis

Pathogenicity, frequency, novelty of all the detected variants were tested using different databases including 1000 Genomes (http://browser.1000genomes.org/index.html), ExAC (http://exac.broadinstitute.org) and gnomAD (gnomad.broadinstitute.org), MutationTaster (http://www.mutationtaster.org), CADD (cadd.gs. washington.edu), SIFT (sift.jcvi.org) and Poly-phen genetics.bwh.harvard.edu/pph/data) algorithms and provean. The variants' true nomenclature was checked according to the American College of Medical Genetics and Genomics (ACMG) guidelines.

Results

Patients' demographic data and clinical evaluations

A total of 23 patients including 19 males (82.6%) and 4 females (17.39%), aged between 8 months and 42 years, clinically diagnosed with CMT, were enrolled and analyzed. The onset age of patients was between 7 months and 20 years. Totally, 2 patients (8.69%) did not indicate any abnormality in the first physical examinations. 3 patients (13.04%) indicated three symptoms including the absence of knee reflection (Deep Tendon Reflex=0), Pes cavus presence and steppage. The description of all clinical symptoms is categorized in table 2. MLPA analysis revealed no

Table 1. Identified variants in our 23 recruited patients

Patient number	*Gene*	Nucleotide change	Protein change	Zygosity	Pathogenicity	Report
1	*LRSAM1*	c.1576G>C	p.Glu526Gln	Het	P	
2	*KIF1B*	c.2455A>C	p.Ser819Arg	Het	VUS	Novel
3	*NDRG1*	c.205+1delG	Splice	Homo	LP	Novel
4	*VCP*	c.G1318A	p.E440K	Het	LP	
5	*GDAP1*	c.579delG	p.L193fs	Homo	LP	
6	*MFN2*	c.281G>A	p.Arg95Gln	Het	LP	
7	*IGHMBP2*	c.138T>A	p.Cys46Term	Het	P	
8	*SH3TC2*	c.2860C>T	p.Arg954Ter	Homo	P	
9	*PMP22*	c.147-149delTAT	p.Ile50del	Het	VUS	
10	*LMNA*	c.1189C>T	p.Arg397Cys	Homo	VUS	
11	*LITAF*	c.271C>T	p.Pro91Ser	Homo	VUS	Novel
12	*SBF1*	c.3454A>G	p.Ile1152Val	Het	VUS	
13	*FGD4*	c.344C>G	p.Ser115Cys	Het	VUS	
14	*PLEKHG5*	c.1368+1G>C	Exon 12	Homo	VUS	
15	*NEFH*	c.3011-3012delCA	p.D1004EfsX58	Homo	P	
16	*PMP22*	c. 47T>G	p.L16R	Het	P	De novo
17	*GDAP1*	c.802_803delTG	p.W268GfsX22	Homo	P	
18	*MME*	c.499T>A	p.Trp167Arg	Homo	VUS	
19	*SH3TC2*	c.2552G>A	p.Arg851Gln	Homo	VUS	
20	*GDAP1*	c.118-2A>G	NA	Homo	P	
21	*FIG4*	c.1728A>G	p.Arg576Arg	Homo	P	Novel
22	*PLEKHG5*	c.1368+1G>C	Exon 12	Homo	VUS	
23	*FIG4*	c.1728A>G	p.Arg576Arg	Homo	P	

mutation in the patients, and NGS analysis detected 20 different mutations (Table 1).

Molecular analysis and patients' description

Patient #1 carries a heterozygous transversion in *LRSAM1*, a gene linked with CMT2P and AR-CMT2E forms of Charcot-Marie-Tooth disease. Thus, the inheritance can be Autosomal Recessive (AR-CMT2) or autosomal dominant (CMT2P). The first symptoms of CMT2P appear between the second to fifth decade of life (as seen in patient #1) which include distal weakness in the lower limbs and in some patients also present in the upper limbs. Other features have been reported in some patients including episodic cramps, bilateral pes cavus, foot drop, absent tendon reflexes, severe loss of sensation in feet and legs and mild loss of sensation on fingertips, sensory and motor dysfunction. MNCVs are normal to slightly decreased (22-25). Our analysis of pathogenicity classified this mutation as a Variant of Uncertain Significance (VUS).

Patient #2 is heterozygous for the c.2455A>C transversion in *KIF1B* gene. Mutation in this locus has once been reported to cause CMT2A (26), and *KIF1B*β is known to be associated with CMT2A1, a subtype presenting classical CMT2 signs (described

Table 2. Description of clinical symptoms

Patient	Marriage	Age/Onset	Pes Cavus	CPK	Atrophy	Scoliosis	Steppage	DTR	Electromyography	Speech	Motor growth and development	Foot drop	Running	Climbing	Ataxia	Walking	Muscular pain	Jumping difficulties	Imbalance/Seizure
1	Con *	38/22		126	+				Axonal			+							
2	Con	15/15		53					Axonal			+							
3	Con	6/3							Demyelinating motor and sensory neuropathy										
4	Con	6/2	+	167	+		+	Flexor	Axonal		+	+		+	+				
5	Con	6/1	+		+		+	ND	Demyelinating motor and sensory neuropathy										
6	Non	8/2	+		Severe spasticity	+	+	+4	Axonal							+			
7	Non	3/1			Generalized hypothony				Axonal		+								+
8	con	15/6	+				+	Not detected	Demyelinating motor and sensory neuropathy			+		+			+		
9	Con	16/11	+		+(Bilateral claw hallux)		+	Not detected	Mixed Axonal and demyelinating motor and sensory neuropathy			+	+						
10	Non	62/60	+				+	Not detected	Axonal										
11	Cons	13/4	+	316				Not detected	Demyelinating motor and sensory neuropathy	+	+	+		+	+	+	+	+	
12	Non	16/11	+	120	+			Not detected	Mixed axonal and demyelinating			+			+				
13	Con	3/9m			Generalized hypothony				Mixed axonal and demyelinating motor and sensory neuropathy										+
14	Non	14/7	+				+	Not detected	Axonal										
15	Con	15/13	+		+		+	Not detected	Axonal								+		
16	Non	2/8m			Generalized hypothony			Decreased	Mixed axonal and demyelinating motor and sensory neuropathy		+								
17	Con	15/1.5	+		+		+	ND	Axonal						+				
18	Con	43/39	+		Hand tremor		+	ND	Axonal										
19	Con	7/8M			Generalized hypothony			Decreased	demyelinating motor and sensory neuropathy		+								
20	Con	7/1.3	+				+	ND	Axonal			+							
21	Con	7/5			+			Not detected	Axonal			+			+				
22	Non * 1	23/14	+				+	Not detected	Axonal					+					
23	Con	25/7			+(fasicul-asion zaban)			Not detected	Axonal						+				

earlier) except nerve thickening. This variant was ranked as 'likely benign' in our analysis.

Patient #3, the second female proband in our sample, carries a homozygous splice site mutation in *NDRG1* gene. Deletion of c.205+1G is the second novel detection of our study. Patients with *NDRG1* mutations develop CMT4D, an autosomal recessive demyelinating CMT subtype characterized by distal muscle wasting and atrophy, foot and hand deformities, absent tendon reflexes, and sensory loss. The age of onset is between the first and second decade (as also observed for this patient). Deafness is an invariant feature of the phenotype and usually develops in the third decade. This mutation was identified as 'likely pathogenic' in our analysis.

A heterozygous missense mutation in the gene *VCP* of a 7-year-old male (Patient #4) replaces the negatively-charged Glu440 with the positively-charged Lys. This mutation causes a CMT2 type demonstrating diverse age of onset, and has been characterized by long, thin legs, high arches and hammer toes, problems with balance, absent Achilles deep tendon reflexes, sensory loss and weakness (symmetrical, length-dependent without weakness in the proximal leg or arm muscles) developed in fourth and fifth decade of life, and difficulties with fine movements of hands after the age of 50 (27). Our pathogenicity analyses placed this variant in the 'likely pathogenic' class.

Patient #5 is a 6-year-old male carrier of a homozygote deletion c.579delG in *GDAP1* gene. *GDAP1* is located in 8q21 region and it is associated with both demyelinating and axonal type of CMT (28,29). This type is also characterized by hand and foot wasting causative of disability (30). Patients with mutated *GDAP1* manifest symptoms early. Our patient was affected with demyelinating type and Pes cavus with atrophy were his two major symptoms. Another *GDAP1* deletion is detected in patient #17 naming c.802_803delTG leading to a frameshift at codon 268(p.W268GfsX22). This female has symptoms such as atrophy, Pes cavus and steppage and she presented symptoms since she was 1.5 years. The third identified alteration in *GDAP1* was an intronic variant naming c.118-2A>G. He had symptoms since he was 15 months and his main clinical findings were steppage, Pes cavus and foot drop. This variant was a pathogenic according to our analysis (patient #20).

Patient #6, a child with a heterozygous transition, has a mutant *MFN2* allele. This gene is known as the main cause of CMT2A, an AD-transmitted axonal CMT type whose clinical features were detailed in the introduction section. The age of onset in this patient is consistent with that described for CMT2A. This mutant was ranked as pathogenic in our bioinformatics pathogenicity analysis.

Patient #7, the youngest one in the sample, presented with two heterozygous mutations, both in *IGHMBP2* gene. Such mutations have been reported to associate with autosomal recessive axonal type AR-CMT2S. The patients present with childhood-onset distal weakness, wasting in the upper and lower limbs, areflexia and decreased sensation, but no respiratory involvement (31). One mutation in this infant (2.5 months old) is a nonsense transition affecting Cys46; the other is missense replacing a Val for Ile. Our analysis placed the former in 'pathogenic', and the latter in 'likely pathogenic' variant classes.

Mutation in *SH3TC2*, which was detected in patient #8 causes CMT4C type. This patient has homozygous inherited a nonsense mutation in this gene. CMT4C is characterized by early-onset (as observed in this patient), distal weakness, foot deformities, walking difficulty, scoliosis and occasionally facial and bulbar weakness, sensorineural deafness and respiratory insufficiency (32,33). Pathogenicity analysis identified this mutation as pathogenic. Another VUS missense mutation c.2552G>A (p. Arg851Gln) was detected in a 7-year-old homozygote patient. He manifests developmental delay, decreased DTR and generalized hypotonia.

Patient #9 is a 16-year-old male manifesting first symptoms. When he was 11 years old, was the carrier of a heterozygote deletion c.147-149delTAT in *PMP22*. *In silico* analysis categorized this variant as VUS one. His clinical symptoms were Pescavus, steppage, bilateral claw hallux and atrophy. Neuropathies caused by *PMP22* mutations is classified to three subgroups. Second group is caused by deletions leading to Hereditary Neuropathy with liability to Pressure Palsies (HNPP). 4-47% of patients with *PMP22* deletion have Pes cavus. In addition, atrophy and muscular weakness and onset in the second and third decade of life is observed. All of our patient symptoms were inconsistent with previous reported

findings (34). Another identified variant in *PMP22* is detected in patient #16 who is a 2-year-old carrier of one heterozygote variant naming c. 47T>G (p. L16R) in *PMP22*. He indicated developmental delay, generalized hypotonia and decreased DTR level.

Patient #10 is a 62-year-old man carrying homozygous mutation of *LMNA*, the causative of AR-CMT2A or CMT2B1 or CMT4C1 subtypes. Clinical symptoms usually appear in the second decade (onset between 5-25 years old) with a severe CMT phenotype including proximal muscle involvement although some have a milder phenotype. *LMNA* mutations have also been associated with other phenotypes including Emery-Dreifuss muscular dystrophy, cardiomyopathy and Dunnigan-type familial partial lipodystrophy (35). This mutation was classified as VUS in our analysis.

A homozygous missense mutation was detected in the *LITAF* gene of one of the two female patients (Patient #11). This was a novel detection. *LITAF* mutations, responsible for CMT1C subtype of Charcot-Marie-Tooth disease, account for less than 1% of CMT patients (36). The first clinical symptoms in CMT1C patients appear in the second decade with a typical CMT1 phenotype and conduction velocities around 16-25 *m/s* (12,37). Patient #11 of our study is in the eve of her second decade, almost consistent with CMT1C age of onset. This novel mutation was classified as a VUS.

Patient #12 is heterozygous for a missense transition in *SBF1*, which substitutes Val1152 for Ile. *SBF1* mutations lead to CMT4B3, a recently introduced CMT subtype (38). Generally, CMT4B types are characterized by severe CMT1 phenotype with focal myelin folding, sometimes with bulbar paralysis (CMT4B1) or glaucoma (CMT4B2) (8). This variant was ranked as VUS in our pathogenicity analysis.

Patient #13 was the carrier of a heterozygote variant c.344C>G leading to a serine substitution to Cysteine at codon115 in *FGD4* gene. *FGD4* is a vital factor for Schwann cells myelination maintenance (39). He presented the symptoms since he was 9 months and symptoms included generalized hypotonia and seizure.

Patient #14 is a homozygote carrier of a splice site mutation named c.1368+1G>C in *PLEKHG5*. Defects in *PLEKHG5* cause intermediate form of CMT. Axons and Schwann cells may be affected by this type of CMT (40). This 14-year-old patient presented symptoms since he was 7 years old and his major symptoms were absent DTR, presence of Pes cavus and steppage gait. *In silico* analysis categorized this variant as VUS. This variant was also detected in another member of this family. The second patient was a 23-year-old male and his symptoms were the same as patient 14.

C.3011-3012delCA in *NEFH* was detected in a homozygote patient #15. His clinical examination indicated that he had no DTR, presence of Pes cavus and steppage. *NEFH* mutation caused autosomal dominant axonal CMT2CC type. This type is characterized by progressive weakness in muscles and initial tibialis muscle atrophy developing into the distal muscles of the arm (41,42). Our analysis classified this alteration as a pathogenic one.

Autosomal-recessive Charcot-Marie-Tooth disease type 2 is also the consequence of defects in *MME*. *MME* encodes neprilysin (NEP) which is a key factor in degrading various types of neuropeptides (43). Previous studies demonstrated that a considerable portion of European and American patients diagnosed with autosomal dominant (AD) late-onset neuropathies have a defect in *MME* (44). Our findings also confirmed the onset age of patients with *MME* defects. One missense mutation c.499T>A (p.Trp167Arg) was detected in a 43-year-old male (patient #18) presenting CMT symptoms since he was 39 years old. He suffered from steppage, hand tremor, pes cavus and absent DTR. Our analysis categorizes this variant as a VUS.

Patient #21 who was a 7-year-old female carried a homozygous synonymous mutation in *FIG4*. Mutant forms of this gene lead to the CMT4J type of the CMT neuropathy. The clinical phenotype is severe CMT1 with early onset (childhood but sometimes adult onset) and severely decreased MCVs (<10 *m/s*) (37). This mutation was classified as VUS through our analysis. She had symptoms such as atrophy, foot drop and ataxia.

To sum up, 14 out of 23(60.86%) patients studied have developed axonal types of CMT, of which 2 or 3 demonstrated AR inheritance. AD-transmitted demyelinating form of CMT was represented by only one patient (Patient #8). The remaining patients

showed demyelinating CMT type with AR inheritance (CMT4). The present study detected four novel mutations which inherited the demyelinating types of CMT homozygous. In total, the sample included 9 heterozygous mutations and 14 homozygous ones. Most of the mutations detected in the sample do not affect the most prevalent CMT causative genes. This observation may exemplify the particularity of the genetic distribution of Charcot-Marie-Tooth disease in Iran.

Discussion

New screening methods are slowly replacing the more traditional Sanger sequencing-based screening methods. Specifically, NGS technology for whole exome and whole genome analysis provides new potential capabilities in molecular diagnostic services. NGS is a high throughput technique with low cost and enables sequencing of multiple known and unknown genes in a single run. Despite all the promising results of conducting multi gene and panel studies on CMT patients, the role of this technique as overall diagnostic yield remains unclear. In the present study, we utilized this technique to screen the CMT associated mutations in an Iranian cohort. Totally, we recruited 23 patients clinically diagnosed with CMT. Previous studies showed that more than 70% of all CMT patients have mutations in one of four genes: *PMP22* (associated with CMT1A, CMT1E), *GJB1* (causative of CMTX1), *MPZ* (associated with CMT1B, CMT 2I/2J, DI-CMTD), and *MFN2* (associated with CMT2A) (Figure 1) (14). In spite of the fact that mutations in these genes account for the majority of worldwide CMT cases, only one case in our studied samples carried such mutants (*MFN2* in patient #2). This considerable difference between the results of our study and other published data is due to the distinct genetic variation of Iranian patients which is the result of consanguinity and numerous language adoptions (45). Totally, 9 pathogenic variants were identified in our study. Among these 9 alterations, two are deletions named c.3011-3012delCA in *NEFH*

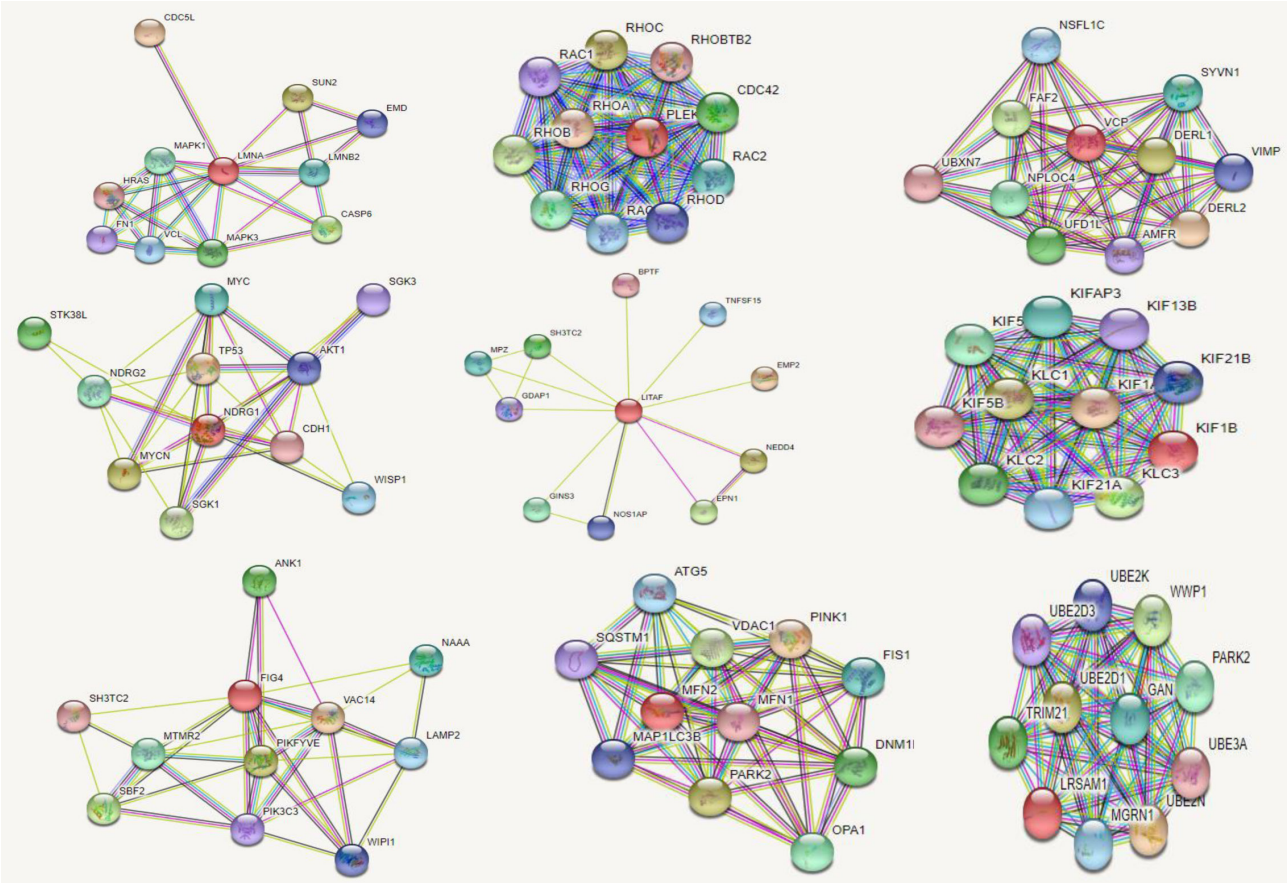

Figure 1. Interactome analysis of gene.

and the second one is c.802_803delTG in *GDAP1*.

Previous studies revealed that patients carrying a pathogenic variation in *GDAP1* showed an early-onset but among our patients, only one patient with *GDAP1* was 1-year-old and two other patients were 7 and 15 years old. *GDAP1* located in outer membrane of mitochondria is involved in various mitochondrial physiology (46). Previous studies also indicated that *GDAP1* accompanied by junctophilin-1 play a role in cells' calcium homeostasis. Any defects in mitochondrial homeostasis categorize as a causative element for CMT *GDAP1* disease. In addition to mitochondria, Golgi organ also has an association with CMT. Some mutations in *GDAP1* such as *GDAP1*-Leu239Phe alters Golgi morphology. All of these alterations are along with modifying TGN46, a protein of the TGN. Despite initial investigations showing the expression of *GDAP1* in neural cell lines. Further studies represent that *GDAP1* mRNA constitute 2.6% of human fibroblasts. Decrease in fibroblasts *GDAP1* mRNA level in CMT patients is the consequence of some mutations affecting through loss of function or even knockout mutations.

Valosin-Containing Protein (*VCP*) is associated with many ubiquitin-dependent cellular pathways (47). Most *VCP* mutations are linked to several phenotypes such as myopathy, Paget disease and ALS. CMT2 is the milder phenotype of inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD)/ALS. In a study by Michael A, the potential pathogenicity and intrinsic ATPase activity of *VCP* variants were assessed through a transfection procedure. IBMPFD mutations *VCP*-R155H and *VCP*-A232E showed 3–4-fold ATPase activity. This result was in contrast to *VCP*- E185K which was a novel variant identified by Gonzalez *et al* in 2014 (27). In our study, one CMT2 patient had one likely pathogenic mutation c.1318G>A (p.E440K). However, the patient had many severe clinical symptoms such as no knee reflex, pes cavus, steppage and muscular atrophy. CMT2 type demonstrated diverse ages of onset, and has been reportedly characterized by long, thin legs, high arches and hammer toes, problems with balance, absent Achilles deep tendon reflexes, sensory loss and weakness (symmetrical, length-dependent without weakness in the proximal leg or arm muscles) developed in fourth and fifth decades of life, and difficulties with fine movements of hands after the age of 50. One of our studied patients was the carrier of one variant in *FGD4*. *FGD4* encode 766-aa protein named FRABIN composed of five functional domains which is an F-actin Rho GEF specialized for Cdc42. Mutations in this gene are associated with autosomal recessive form of demyelinating CMT named CMT4H. These five domains include one cysteine-rich FYVE domain, Dbl Homology (DH) domain, N-terminal F-Actin Binding (FAB) domain and two Pleckstrin Homology (PH) domains. Among the mentioned domains, DH domains were identified in Dbl protein involved in the process of GDP-to-GTP exchange. In addition, PH and FYVE domains play a role in the interactions between phosphoinositides. FRABIN activated JNK pathway leading to early aberrant activation of myelination. This result is in consistent with early-onset type of CMT with slow progression (49).

Our patient manifested mild phenotype which was consistent with a study by Herminia Argente-Escrig *et al* in 2019. In this study, two novel mutations named c.514delG (p.Ala172 Glnfs*28) and c.2211dupA (p.Ala738Serfs*5) in siblings born to a healthy nonconsanguineous Spanish parent were reported. Among this sibling, one was asymptomatic, displaying no difficulties in activities such as jumping or handling small objects. The second individual showed no symptoms; however, he began to complain about dorsal kyphoscoliosis and increase in plantar arch since he was 11 years old. Our patient did not manifest these symptoms; however, the CMT severity of these patients was categorized as a mild type (48). Two of the patients were the carriers of one homozygote variant in *FIG4*. Three clinical phenotypes including YunisVaron syndrome, familial epilepsy combined with polymicrogyria and CMT4J are the results of *FIG4* mutations with recessive inheritance mode. Among these three distinct pheno-types, patients with familial epilepsy and Yunis-Varon syndrome have an early onset and characterized with abnormality in CNS development (50,51). Patients affected with CMT4J usually do not present con-siderable CNS symptoms (52). One of the investigations expanding the clinical spectrum of CMT4J affected patients was conducted by James

P Orengo *et al* in 2018. In this study, two mutations c.122T>C (p.I41T) and c.1949-10T>G were reported. The affected patient symptoms were foot drop and Ataxia. One deletion and one substitution were reported in *PMP22*. As mentioned before, alterations such as duplications, deletions and point mutations in *PMP22* result in two distinctive phenotypes including Charcot-Marie-Tooth disease type 1A (CMT1A) and Hereditary Neuropathy with liability to Pressure Palsies (HNPP), respectively (53,54). CMT1A patients present symptoms typically in the first two decades of patients' life accompanied with walking or running difficulties. In our genetic study, the diagnosis of two *PMP22* carriers were in the age of 11 and 8 months. Distal symmetrical weakness of muscle, loss of sensory and severe affected legs all witness in CMT1A patients. In this study, foot drop and running problems were the main complaints of patients with *PMP22* defects (55). In our study one substitution was reported for *LRSAM1*.The patient had CPK level 126 and suffers from foot drop. Defects in the same gene can lead to both CMT's recessive and dominant forms. *LRSAM1* is a E3 ubiquitin-protein ligase with high conservation highly expressed in spinal cord motor neurons and dorsal root ganglia sensory neurons (56-58). *LRSAM1* has an interaction with mediate monoubiquitination of the tumour suscepti-bility gene 101 protein (TSG101) as the subunit of Endosomal Sorting Complexes Required for Transport)-1 complex (59).

In a study by Paulius Pa-laima *et al* in 2021, *LRSAM1* mechanistic dysfunction was investigated (60). Previous studies suggested that patients carrying *LRSAM1* mutation have been witnessed in various regions including Asia, Europe and also North-America (25,61-65). According to this study, totally CMT-causing mutations were identified in *LRSAM1*. Patients affected with do-minant form manifest slowly progressive axonal neuropathy mainly in the second or third decade of life. Our patient was the carrier of one heterozygote substitution named c.1576G>C in *LRSAM1*. He was diagnosed with this disorder when he was 22 years old and his main clinical symptom was foot drop. *LRSAM1* located on chromosome 9q33.3 consisting of 26 exons mainly expressed in foetal and adult nervous system (64). *LRSAM1* belongs to a group of Ubiquitin Proteasome System (UPS). This group consists of enzymes which play a role in several mechanisms including ubiquitin activation (E1), conjugation (E2) and finally target ligation (E3). RING-type E3 ligase comprising RING-motif conserved cysteines and histidine. This ring has a vital role for ubiquitin-conjugating enzymes (E2) interaction and ubiquitin activation (66). Ubiquitin-ation is involved in protein activity regulation and targeting some proteins for degradation.

A study by Amit I *et al* provides a better insight for the role of *LRSAM1* mutations. In this study, mutation named Cys675Ala leads to production of a stable protein. However, this protein has an effect on one of the RING domains of critical residue. Cys675Ala expression in HeLa cells containing *LRSAM1*WT change the retroviral Gag protein localization and consequently affect circular cytosolic structures. In addition, previous studies provide evidence that CMT mutants including Pro707Leu, Cys694Tyr, and Leu708Argfx28 lose the interaction with E2-conjugating enzyme Ubc13 in an experiment with yeast-2-hybrid (62). Kinesin superfamily proteins (KIFs) are microtubule molecular motors involved in transportation of vital cellular materials to targeted destinations through synaptotagmin, synaptophysin, SV2 and regulatory adaptors such as DENN/MADD and Rab3-GTP (67). *KIF1B*β is a kinesin-3 family member which has a key role in various cellular mechanisms such as morphogenesis and neuronal survival (68). One study showed that the brains volume of *KIF1B*−/− mice were 10% reduced and commissural fibers and brain stem nuclei also did not develop sufficiently (26). In this study, axonal outgrowth mediated with *KIF1B*β in combination with IGF-I signaling play a role. Signal transduction through IGF-mediated MAPK and PI3K–Akt are a vital element for axonal development and neuronal survival (69-71). *NDRG1* located on 8q24.3 is responsible for CMT4D.

This gene is highly conserved in various organisms and belongs to α/β hydrolase superfamily without hydrolytic catalytic site (72,73). The most considerable clinical phenotype in *NDRG1* null carriers is demyelinating neuropathy (74). *NDRG1* binds to intracellular vesicle functioning through Rab4a effector. It also plays a role in low-density lipoprotein receptor trafficking (75,76). CMT4D

patients are more frequent in Roma communities. The affected patients demonstrate sensory neuropathy and severe distal motor in the first decade of life (77,78). Patients with defects in mitofusin-2 (*MFN2*) show semi-dominant and recessive mode of inheritance. *MFN2* carriers manifest pes cavus and progressive sensory loss (79,80). Our patient was the carrier of one heterozygous mutation c.281G>A(p.Arg95Gln) which was a likely pathogenic variant. In addition, the mentioned patient had various symptoms such as scoliosis, step page and increased DTR. Fibroblasts and nervous tissue of CMT2A patients indicated that there exist changes in mitochondrial morphology. These alterations include degeneration, mitochondria distribution and number of mitochondria (81).

IGHMBP2 has been also involved in other neuromuscular disorders with muscle weakness and atrophy such as spinal muscular atrophy with respiratory distress type 1 (SMARD1) (82-84). Individuals with *IGHMBP2* mutations had symptoms such as sensory and mild motor axonal polyneuropathy. *SH3TC2* gene pathogenic variants lead to the autosomal recessive demyelinating form of CMT named (CMT4C, or ARCMTde-*SH3TC2*). The main complaint of CMT4C patients is early severe neuropathy (33). Two of our patients were the carries of *SH3TC2* defects. One manifests symptoms at the age of six and the other manifests at the age of six months. Previous investigations indicated the role of three loci with autosomal recessive form of CMT. Defects in *LMNA* are associated with CMT autosomal recessive inheritance mode (85). In our *LMNA* defected patient, also the results of electromyography showed Axonal pattern. Lipopolysaccharide-induced tumor necrosis factor-alpha factor (*LITAF)* encode a 17 kDa protein targeting endocytic structures and contained an N-terminal proline-rich domain. Our patient was the carrier of one homozygote substitution named c.271 C>T(p.Pro91Ser). He indicated various symptoms such as speech problems, Ataxia, muscular pain and climbing difficulties. *LITAF* is a necessary element for ESCRT recruitment to endosomal membranes and regulation of endosomal trafficking and ErbB receptors (86). Defects in *LITAF* inhibit ErbB trafficking regulation results in loss-of-function mechanism and finally longer ERK1/2 signaling activation (87). In conclusion, in this study, 9 patients were carrier of one variant with unknown pathogenicity. This variant may be effective in unravelling the complicated genetic etiologies for CMT patients. Our interactome analysis also revealed that many genes have an interaction with each other. This fact highlights the role of other genes in CMT pathogenesis. Our study also highlights the role of NGS in CMT detection. This technique has several advantages in identifying the causative variants of heterogeneous disorders (12).

Conclusion

CMT is categorized as a rare genetic disorder and we recruited only a few numbers of patients for genetic analysis; therefore, we could not define and recognize the frequent mutation and the major gene involved in the pathogenesis of Iranian patients with CMT. Nine variants were categorized as a VUS, and further large-scale studies and genetic engineered animal models should be conducted in order to evaluate the pathogenicity of variants

Data availability statement

No other data were used.

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Conflict of Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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