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Mutation screening of *mitofusin 2* in Charcot-Marie-Tooth disease type 2

Donald S. McCorquodale III,

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Gladys Montenegro,

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Ainsley Peguero,

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Nicole Carlson,

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Fiorella Speziani,

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Justin Price,

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Sean W. Taylor,

Department of Neurology, Kingston General Hospital, Kingston, ON, Canada

Michel Melanson,

Department of Neurology, Kingston General Hospital, Kingston, ON, Canada

Jeffery M. Vance, and

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Stephan Züchner

Department of Human Genetics, John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Biomedical Research Building, Room 523, LC: M-860, 1501 NW 10th Avenue, Miami, FL 33136, USA

Correspondence to: Stephan Züchner, szuchner@med.miami.edu.

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Stephan Züchner: szuchner@med.miami.edu

Abstract

Charcot-Marie-Tooth (CMT) disease is among the most common inherited neurological disorders. Mutations in the gene *mitofusin 2* (*MFN2*) cause the axonal subtype CMT2A, which has also been shown to be associated with optic atrophy, clinical signs of first motor neuron involvement, and early onset stroke. Mutations in *MFN2* account for up to 20–30% of all axonal CMT type 2 cases. To further investigate the prevalence of *MFN2* mutations and to add to the genotypic spectrum, we sequenced all exons of *MFN2* in a cohort of 39 CMT2 patients. We identified seven variants, four of which are novel. One previously described change was co-inherited with a *PMP22* duplication, which itself causes the demyelinating form CMT1A. Another mutation was a novel in frame deletion, which is a rare occurrence in the genotypic spectrum of *MFN2* characterized mainly by missense mutations. Our results confirm a *MFN2* mutation rate of ~ 15–20% in CMT2.

Keywords

Charcot-Marie-Tooth disease; CMT2; CMT2A; Mitofusin 2; MFN2; Inherited peripheral neuropathies

Introduction

Charcot-Marie-Tooth disease (CMT) is a common inherited neurological disorder with an estimated incidence of 1 in 1,214–2,500 individuals [1,13]. Classically, CMT is broadly divided into two types based on nerve conduction study findings [7]. CMT type 1 (CMT1) is associated with slowed nerve conduction velocities (<38 m/s), which correlate with histopathological observations characteristic of a demyelinating peripheral neuropathy. CMT type 2 (CMT2) is characterized by normal nerve conduction velocities and low compound muscle action potentials (CMAP) reflecting selective degeneration of axons without myelin involvement [19]. Genetic studies have revealed 40 genes associated with inherited peripheral neuropathies (Mutation Database of Inherited Peripheral Neuropathies, <http://www.molgen.ua.ac.be/cmtmutations/Home/Default.cfm>). Duplications of *PMP22*, the cause of CMT type 1A (CMT1A), account for approximately 70% of CMT type 1 [15]. Mutations in the gene *MFN2* are the most common cause of CMT type 2 with frequencies ranging from 8 to 20% [10,11,14,18]. Specific alleles in CMT type 2A (CMT2A) are associated with optic atrophy, clinical signs of first motor neuron involvement, and early onset stroke [5,16,17]. In patients with a documented family history of CMT2, *MFN2* mutations were identified in 33% of 323 CMT patients [14]. Thus, screening for mutations in *MFN2* is the first step in the molecular diagnosis of CMT2. Here we report the results of a screen of 39 CMT2 patients for mutations in *MFN2*. We describe seven variants, four of which are novel. Furthermore, we examine and discuss the clinical phenotype–genotype spectrum in seven probands and discuss evidence for pathogenicity.

Materials and methods

Patients

The 39 CMT patients screened are part of our ongoing collection of unrelated CMT families and isolated index patients. Available clinical information on these samples is given in Table 1. The sample is composed of 38 patients clinically determined to have an axonal peripheral neuropathy and one patient with a mixed axonal and demyelinating type of CMT. The mode of inheritance was autosomal dominant for 29 familial cases, six were classified as sporadic with no evidence of family history of peripheral neuropathy, and four did not have sufficient

family history to make a determination. Informed consent was obtained from all individuals, and the Institutional Review Board (IRB) at the University of Miami Miller School of Medicine approved the study.

Analysis of *MFN2*

Blood (~24 ml) was collected in either EDTA or acid citrate dextrose tubes from participating individuals by venipuncture and DNA was extracted in the Biorepository of the Hussman Institute for Human Genomics. Oligonucleotide primers flanking each exon (and neighboring intronic sequences) of *MFN2* were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/>) and are available upon request. Exons and flanking intronic sequences were amplified on the Applied Biosystems (ABI) Veriti 96-well Fast Thermal Cyclers using a touchdown protocol. PCR purification was completed with QuickStep™2 SOPE resin (Edge BioSystems). Sequencing was performed using ABI BigDye® Dye Terminator Cycle Sequencing Kit on an ABI 3730 sequencer. Sequence traces were analyzed using Sequencher® ver. 4.8 (Gene Codes Corporation). Each nucleotide variation identified was confirmed by completing PCR amplifications and subsequent bi-directional sequencing on fresh aliquots of sample DNA. All variants were screened for segregation in available family members and were absent in 200 control samples. All mutations were analyzed with the PolyPhen-2 software (<http://genetics.bwh.harvard.edu/pph2/>) that predicts the functional significance of protein changes and categorizes missense mutations as benign, possibly damaging, or probably damaging.

Results and discussion

Direct sequencing of the *MFN2* gene in 39 CMT2 patients identified seven non-synonymous coding sequence variants in seven probands (Table 2). Four mutations were novel disease variants. Available clinical information regarding the seven probands is summarized in Table 3 and family pedigrees can be found in Fig. 1. Nerve conduction studies were available for five of the seven identified patients. Four of the five nerve conduction studies revealed median and ulnar nerve conduction velocities over 38 m/s and low CAMP, which were classified as axonal type CMT (CMT2). The fifth nerve conduction study suggested a mixed axonal and demyelinating peripheral neuropathy (family 20004). In all cases, studies were performed in advanced disease, as nerve conduction studies of the leg were not recordable in all but one case (Table 3). Nerve biopsies were not performed. In our sample, the age of onset ranged from 1 to 77 years.

The most severe phenotype (family 20006) with severe disability by the age of 7 years was caused by a L92R variant. This finding is consistent with previous reports on changes in residues 92 and 94, which reside in the Ras-binding domain of *MFN2* and are also associated with increased disease severity [3,12,14]. The proband's parents reported low birth weight, *pes cavus*, and difficulty walking due to "weak ankles" by the age of three years. Nerve conduction study results were not available but were reported to demonstrate an axonal neuropathy. Problems with visual acuity were not reported. Beyond a diagnosis of Raynaud's syndrome in the mother, the family history is negative for neuropathies or related conditions. As both parents were screened negative for the same change, the mutation likely arose de novo (Fig. 1a).

Two mutations identified in separate probands occurred on adjacent residues (R250, P251) within the conserved GTPase domain of *MFN2*. The R250Q change has been previously reported as a causative variant [14]. In silico modeling of protein function rated the R250Q mutation as probably damaging. The 50-year-old proband of family 20023 was diagnosed with CMT at the age of 12 years and has a complicated medical history including

degenerative disc disease, a 10-year history of type II diabetes, and hypertension. A cranial MRI study was unremarkable. At the time of exam, the patient was able to ambulate with the aid of a cane and complained of mild to severe numbness, tingling, and neuropathic pain in hands and feet. Neurologic exam and nerve conduction studies were both consistent with an axonal peripheral neuropathy with symptoms complicated by diabetic neuropathy. The lack of a detailed history for family 20023 limits determination of the mode of inheritance, although a reported neuropathy in the aunt of the proband suggests that the change is not sporadic (Fig. 1b). Additional family members were not available for segregation analysis.

A change in the adjacent proline residue (P251R) was associated with a more severe form of CMT2A. The P251R change is predicted to be damaging but has not been previously described. A variant in the same residue (P251A) has been previously reported [18]. The 37-year-old female patient was diagnosed with a neuropathy at the age of 2 years and has been wheelchair dependent for the past 10 years. Physical exam and nerve conduction studies are consistent with CMT2 and her medical history is negative for other conditions. The patient reports no family history of CMT (Fig. 1c). Subsequent screening of the affected daughter revealed co-segregation of the change with the disease.

The M376L variant is predicted to be benign. However, changes in the same residue have been previously described in multiple reports [4,6,8,14]. The M376L change, clustering with a number of previously reported mutations outside of known conserved domains, was identified in the proband from family 20033 [6,8,14]. Family 20033 has a positive history of neuropathy symptoms in five generations consistent with an autosomal dominant inheritance pattern, though no additional family members were available for segregation analysis (Fig. 1d). At the time of exam the 48-year-old female patient, diagnosed with CMT at the age of 25 years, ambulated with the aid of ankle foot orthotics yet has difficulty walking distances over ~20 m. In addition to typical lower extremity findings, the proband reported numbness and neuropathic pain in hands and arms. Physical examination revealed significant thenar and hypothenar atrophy. A cranial MRI study was unremarkable. In addition to CMT the proband's medical history included a diagnosis of carpal tunnel syndrome.

The R468H is predicted to be possibly damaging. The proband of family 20004 had a relatively mild CMT2 phenotype consistent with a previous report by Engelfried of the R468H change [8]. Her medical history includes hereditary elliptocytosis, diagnosed at the age of 10 years, with subsequent splenectomy and cholecystectomy. She was clinically diagnosed with CMT at 40 years of age, but retrospectively reports a symptomatic history of CMT since childhood, including avoidance of running and trouble walking. The patient also reported a recent worsening of her ability to walk and balance, which she associated with the onset of menopause. Her neurological exam revealed mild *pes cavus*, hammer toe, and peroneal muscle wasting. Nerve conduction studies revealed both axonal and demyelinating features. Her father had a medical history of CMT-like symptoms described as abnormal gait and weak ankles. Interestingly, he also had hereditary elliptocytosis. Although it appears the elliptocytosis co-segregates with CMT2A in this family, we are unaware of any previous reports of this association. One of the proband's daughters showed suggestive signs of CMT at the age of 11 years, including high foot arches, hammer toe, and difficulty running. No additional family members were available for genetic tests. Nerve conduction studies revealed both axonal and demyelinating features. Interestingly, in review of additional clinical documents, we discovered she had been previously found to have a *PMP22* duplication. However, the relatively mild course and late onset of disease contrast with previously reported case studies of patients co-inheriting *PMP22* duplications with other neuromuscular disease alleles. For instance, co-inheritance of *GJB1/Connexin-32*, *DMPK*, and *ABCD1*, with *PMP22* duplications leads to more severe neuromuscular symptoms and earlier ages of onsets consistent with a "double hit" hypothesis [9]. Moreover, the R468H

variant was previously reported in a screen of 130 anonymous healthy blood donor samples [8]. However, the R468H change was recently identified in a larger screen of CMT families in a patient diagnosed with CMT1 [2]. It is thus conceivable that the R468H change is not associated with strong causality, but reflects a rare modifying allele in CMT.

In family 20037 we identified the V705I variant, which is predicted to be probably damaging and has been previously described in three CMT2 families (Fig. 1e) [2,8]. The proband was diagnosed with CMT at the age of 12 years. At age 31 years the neurological examination revealed weakness and sensory loss in the lower extremities, but also significant involvement of the upper extremity. Additionally, the patient reported extensive neuropathic pain and paraesthesia in her hands and feet. The neuropathic pain localized to her shoulders and radiated to her hands. Significant loss of sensation was noted in the innervation area of the right trigeminal nerve (V1). The family history includes three maternal generations with CMT and a diagnosis of multiple sclerosis in the sister of the proband. No family members were available for segregation analysis.

The novel deletion R707_N709del identified in the proband from family 20013 represents only the second deletion described in *MFN2* and clusters with two other known mutations in the C-terminus of *MFN2* [14]. This 9 bp deletion results in the loss of three amino acid residues with the remainder of the protein remaining in frame. The proband is currently 13 years of age and was diagnosed at the age of 10 years after a longstanding history of weakness and clumsiness in his lower limbs. His parents noted that he has always been a slow runner and, in retrospect, state that he has a mild steppage gait; however, he refuses to use AFOs to correct his foot drop. Physical examination revealed *pes cavus* and foot deformities, but no evidence of scoliosis. He had normal strength in the upper extremities with the exception of weakness in the interossei muscles and hyporeflexia. There was a moderate degree of muscle wasting in both legs with corresponding decreased strength and absent reflexes. The remainder of his motor examination in the lower limbs was normal. The proband had decreased thresholds to cold and pain sensation to the knees and slightly increased thresholds to vibration in the toes. The absence of a family history or clinical findings of neuropathy in the proband's parents and sister as well as the negative genetic testing for the change in both parents suggests that the deletion is a *de novo* event (Fig. 1g).

In summary, the identification of seven *MFN2* mutations in our cohort of 39 unrelated CMT2 families corresponds to a prevalence of 17.9% in our sample set, although questions remain regarding the R468H variant. Exclusion of the R468H variant would reduce the prevalence to 15.8%. This is in agreement with previous reports for *MFN2* mutation frequency in CMT2 [10,11]. We have provided careful phenotypic descriptions, which will be valuable additions to a catalogue of pathogenicity of *MFN2*. Mutations in *MFN2* remain the primary cause of CMT2 and are thus of great importance in the molecular diagnosis of CMT2.

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Fig. 1.

Pedigrees and corresponding sequence traces of probands (indicated with *arrowhead*) and families with identified mutations in MFN2. Individuals whose DNA was tested are indicated with a "+". *The R468H variant may be a rare benign polymorphism

Table 1

Sample characteristics

	Patients	Percent (%)
Gender		
Male	20	51.3
Female	19	48.7
Ethnicity		
European	32	82.1
Hispanic	3	7.7
Unknown	4	10.3
Age of onset		
Range in years	1–77	
Average in years	24.8	
Clinical phenotype		
Demyelinating	0	
Axonal	38	
Mixed axonal/demyelinating	1	
Inheritance pattern		
Autosomal dominant	29	54.1
Unknown	4	20.9
Sporadic	6	25.0
Patients with MFN2 variants	7 (6) ^a	17.9 (15.8) ^a

^aOne variant (R468H) may represent rare benign polymorphis

Table 2

Mutations identified in *MFN2*

Family	Exon	Mutation type	Mutation	Protein change	Consequence		Familial/sporadic	Reference/novel
					PolyPhen prediction	PolyPhen score		
20006	4	Missense	c.275T > G	p.L92R	Probably damaging	0.988	Sporadic	Novel
20023	8	Missense	c.749G > A	p.R250Q	Possibly damaging	0.359	Familial	Verhoeven et al. [14]
20035	8	Missense	c.752C > G	p.P251R	Probably damaging	0.990	Familial	Novel
20033	11	Missense	c.1126A > C	p.M376L	Benign	0.084	Familial	Novel
20004	14	Missense	c.1403G > A	p.R468H ^a	Possible damaging	0.225	Familial	Engelfried et al. [8], Nelis 2006 ^b
20013	18	Deletion	c.2120delGGGAGAAACC	p.R707_N709del	N/A	N/A	Sporadic	Novel
20037	18	Missense	c.2566C > A	p.V705I	Probably damaging	0.059	Familial	Engelfried et al. [8], Braathen et al. [2], Nelis 2006 ^b

^aVariant may represent rare benign polymorphism^bPersonal communication published online at the inherited peripheral neuropathies database (<http://www.molgen.ua.ac.be>)

Clinical characteristics of CMT2 patients with mutations in *MFN2*

Table 3

Patient	Mutation	Sex	Age of onset	Age at diagnosis	Assistive devices/orthotics	Co-morbidities	Lower extremity motor ^a	Upper extremity motor ^a	Sensory ^b	Nerve	Distal CMAP (mV)	Distal latency (ms)	NCV (m/s)
20006	L92R	M	3	7	AFO	Low-birth weight	++	–	+	Median	NA	NA	NA
										Ulnar	NA	NA	NA
										Tibial	NA	NA	NA
20023	R250Q	M	12	50	Cane	Degenerative disk disease, diabetes, hypertension	++	++	+++	Median	3.97	6.64	42.66
										Ulnar	2.34	4.1	45.61
										Tibial	NR	NR	NR
20035	P251R	M	2	37	Wheelchair		+++	++	++	Median	2.4	3.78	55
										Ulnar	7.5	2	56
										Tibial	NR	NR	NR
20033	M376L	F	26	48	AFO	Carpal tunnel	++	++	++	Median	1.8	3.6	42.4
										Ulnar	3.5	2.8	27
										Tibial	NA	NA	NA
20004	R468H ^c	F	~Childhood	40	None	Co-inherited PMP22 duplication hereditary elliptocytosis	++	+	+	Median	5.2	8.6	22
										Ulnar	3.5	17.6	20
										Tibial	NR	NR	NR
20037	p.V705I	F	12	12	None		++	++	+++	Median	N/A	N/A	N/A
										Ulnar	N/A	N/A	N/A
										Tibial	N/A	N/A	N/A
20013	R707_N709del	M	5	10	AFO prescribed		++	–	+	Median	6.3	4.3	48.5
										Ulnar	9.3	3.1	46.3
										Tibial	5	4.3	31

^aMotor strength score: “–” is normal (5/5), “+” indicates scores of 4/5, “++” indicates scores of 2/5 to 3/5, and “+++” indicates scores of 0/5 to 1/5

^bSensory score: “+” indicates sensory loss in lower extremities, “++” indicates sensory loss in upper and lower extremities, “+++” indicates upper and lower extremity sensory loss with additional sensory symptoms (paresthesia, neuropathic pain, etc)

^cVariant may represent rare benign polymorphism