RESEARCH REPORT

Clinical and genetic diversities of Charcot-Marie-Tooth disease with *MFN2* mutations in a large case study

Masahiro Ando[1,†], Akihiro Hashiguchi[1,†], Yuji Okamoto[1], Akiko Yoshimura[1], Yu Hiramatsu[1], Junhui Yuan[1], Yujiro Higuchi[1], Jun Mitsui[2], Hiroyuki Ishiura[2], Ayako Umemura[3], Koichi Maruyama[3], Takeshi Matsushige[4], Shinichi Morishita[5], Masanori Nakagawa[6], Shoji Tsuji[2], and Hiroshi Takashima[1]

[1]*Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan;* [2]*Department of Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;* [3]*Department of Pediatric Neurology, Aichi Prefectural Colony Central Hospital, Aichi, Japan;* [4]*Department of Pediatrics, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan;* [5]*Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan; and* [6]*Department of Neurology, North Medical Center, Kyoto Prefectural University of Medicine, Kyoto, Japan*

Abstract Charcot-Marie-Tooth disease (CMT) constitutes a heterogeneous group affecting motor and sensory neurons in the peripheral nervous system. *MFN2* mutations are the most common cause of axonal CMT. We describe the clinical and mutational spectra of CMT patients harboring *MFN2* mutations in Japan. We analyzed 1,334 unrelated patients with clinically suspected CMT referred by neurological and neuropediatric departments throughout Japan. We conducted mutation screening using a DNA microarray, targeted resequencing, and whole-exome sequencing. We identified pathogenic or likely pathogenic *MFN2* variants from 79 CMT patients, comprising 44 heterozygous and 1 compound heterozygous variants. A total of 15 novel variants were detected. An autosomal dominant family history was determined in 43 cases, and the remaining 36 cases were reported as sporadic with no family history. The mean onset age of CMT in these patients was 12 ± 14 (range 0–59) years. We observed neuropathic symptoms in all patients. Some had optic atrophy, vocal cord paralysis, or spasticity. We detected a compound heterozygous *MFN2* mutation in a patient with a severe phenotype and the co-occurrence of *MFN2* and *PMP22* mutations in a patient with an uncommon phenotype. *MFN2* is the most frequent causative gene of CMT2 in Japan. We present 15 novel variants and broad clinical and mutational spectra of Japanese *MFN2*-related CMT patients. Regardless of the onset age and inheritance pattern, *MFN2* gene analysis should be performed. Combinations of causative genes should be considered to explain the phenotypic diversity.

Key words: Charcot-Marie-Tooth disease, clinical features, Japanese, *MFN2*, simultaneous mutation

Introduction

Charcot-Marie-Tooth disease (CMT) is one of the most common inherited peripheral neuropathies. The prevalence of CMT was reported to be 1 per 1,215–10,300 persons *(Barreto et al., 2016)*. To date, more than 80 causative genes have been reported to be associated with CMT *(Timmerman et al., 2014)*.

Address correspondence to: Hiroshi Takashima, Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima City, Kagoshima 890-8520, Japan. Tel: +(81)99-275-5332; Fax: +(81)99-265-7164; E-mail: thiroshi@m3.kufm.kagoshima-u.ac.jp

[†]These authors contributed equally.

The clinical features of CMT can significantly vary among patients, even among those sharing the same mutation. Generally, CMT cases are classified into a demyelinating type [median MNCV (motor nerve conduction velocity) <38 m/s], an axonal type (median MNCV >38 m/s), and an intermediate type based on the MNCV of the median nerve. Most frequently, the demyelinating type is associated with a mutation in the gene *PMP22*, while a mutation in *MFN2* is linked to the axonal type. MFN2 is a protein present in the mitochondrial outer membrane, which responds to the mitochondrial dynamics through a mitochondrial GTPase. The frequency of *MFN2* mutations in CMT2 patients has been reported to be in the range of 17%–23% in Spanish, French, Korean, and Chinese populations *(Calvo et al., 2009; Casasnovas et al., 2010; Choi et al., 2015; Xie et al., 2016)*. However, its frequency was reported to be lower, between 8.6% and 11%, in previous Japanese reports *(Kijima et al., 2005; Abe et al., 2011)*.

MFN2 mutation causes typical CMT2, which is called CMT2A2, and can also present different clinical phenotypes, including hereditary motor sensory neuropathy (HMSN) with pyramidal features (HMSN V), HMSN with optic atrophy (HMSN *VIA*), AR-CMT, severe early onset axonal neuropathy, early onset stroke without neuropathy, HMSN with cognitive impairment, and brain mitochondrial dysfunction *(Mostacciuolo et al., 2000; Zuchner et al., 2006; Chung et al., 2008; Del Bo et al., 2008; Nicholson et al., 2008; Polke et al., 2011)*. In this case series, we identified novel pathogenic mutations and investigated variations in the clinical features of CMT patients due to *MFN2* variants.

Methods and Materials

Patients

We analyzed 1,334 unrelated patients/families with clinically suspected CMT. The clinical data and DNA samples were collected from neurological and neuropediatric departments throughout Japan between 2007 and 2016. All the demyelinating patients were enrolled in this study after confirming them to be negative for *PMP22* duplication/deletion as identified using fluorescence *in situ* hybridization and multiplex ligation probe amplification. We extracted genomic DNA using QIAGEN's Puregene Core Kit C (Qiagen, Valencia, CA, USA) or Oragene DNA self-collection kit (DNA Genotech, Ottawa, Ontario, Canada) and carried out mutation screening tests using DNA microarray, targeted resequencing, and whole-exome sequencing. Candidate variants detected by these methods were validated using Sanger sequencing.

If available, segregation analysis was performed for those cases.

The study protocol was reviewed and approved by the institutional review board of Kagoshima University. All patients and family members provided written informed consent to participate in this study, including for the genetic analyses.

Microarray chip sequencing

We designed a customized MyGeneChip® CustomSeq® Resequencing Array (Affymetrix, Inc., Santa Clara, CA, USA) to screen 30 disease-causing genes for CMT and related diseases. We performed mutation screening using Microarray chip sequencing in patients who were enrolled from 2007 to 2012. The detailed methodology has been described elsewhere *(Hashiguchi et al., 2014)*. Table S1, Supporting Information includes the sequences of 30 target genes.

Targeted resequencing

We performed mutation screening of 60 or 72 known/candidate CMT-related genes using two methods: the Illumina Miseq platform (Illumina Inc., San Diego, CA, USA) and the Ion Proton using a custom Ion Ampliseq^M panel and the Ion PI Chip kit v2 BC (ThermoFisher Scientific, Inc., Waltham, MA, USA). We performed mutation screening using Illumina Miseq platform from 2012 to 2014 and Ion Proton platform from 2014 to 2016. After aligning and mapping variant calling, we annotated and filtered variants using the CLC genomics Workbench software program (Qiagen, Hilden, Germany). We filtered out the variants with low read depth (<10) and low quality (<20). The detail methods have been previously described *(Maeda et al., 2014; Higuchi et al., 2016)*. The symbols of the 60 and 72 target genes are shown in Table S1.

Whole-exome sequencing

Exome sequences were enriched using a SureSelect V4+UTRs or v5+UTRs Kit (Agilent Technologies, Santa Clara, CA, USA) and were subsequently subjected to sequencing on a hiseq2000 platform (Illumina). Sequence data was aligned to the human genome data (NCBI37/hg19), and variant calling was performed using Burrows Wheeler Aligner and SAM tools *(Higuchi et al., 2016; Li and Durbin, 2009; Li et al., 2009)*. The called variants were annotated using the CLC genomics Workbench software program and in-house script, and the variants with low read depth (<10) and low quality (<20) were filtered out.

Data analysis for the determination of pathogenic mutations

We confirmed the previously reported pathogenic mutations by reference to the Human Gene Mutation

Database Professional 2016.3 (https://portal.biobase-international.com/hgmd/pro). Moreover, we extracted variants that were not observed in global control databases [dbSNP (https://www.ncbi.nlm.nih.gov/SNP), 1000genome (http://browser.1000genome.org), Exome Sequencing Project (http://evs.gs.washington.edu/EVS), and Exome Aggeregation Consortium (http://exac.broadinstitute.org/)] and Japanese control database [iJGVD; integrative Japanese Genome Variation Database (https://ijgvd.megabank.tohoku.ac.jp), HGVD; Human genetic variation (http://www.hgvd.genome.med.kyoto-u.ac.jp)] and in-house not CMT database. Moreover, we performed *in silico* analysis using SIFT (http://sift.jcvi.org), POLYPHEN2 (http://genetics.bwh.harvard.edu/pph2), PROVEAN (http://provean.jcvi.org/index.php), Mutation Assessor (http://mutationassessor.org), and Condel (http://bg.upf.edu/fannsdb). We evaluated the detected variants using the American College of Medical Genetics and Genomics (ACMG) standards and guidelines *(Richards et al., 2015)*.

Results

Epidemiology of *MFN2* in Japan

We analyzed 1,334 unrelated patients with clinically suspected CMT. We detected 30 known pathogenic mutations, 1 novel pathogenic mutation, 14 novel likely pathogenic variants of *MFN2*, from which 44 were heterozygous mutations and 1 was compound heterozygous mutation. Of the CMT patients without *PMP22* duplication/deletion, 801 patients (60%) presented the axonal phenotype, and 367 patients (28%) were classified as having the demyelinating type. We could not classify 166 patients due to a non-recordable median compound motor action potential (CMAP) or no electrophysiological data. These CMT patients with axonal phenotype showed an *MFN2* mutation rate of 8%, 63/801 (Fig. 1A).

Clinical features

Data regarding previously reported mutations are included in Table S2. Table 1 shows the clinical data
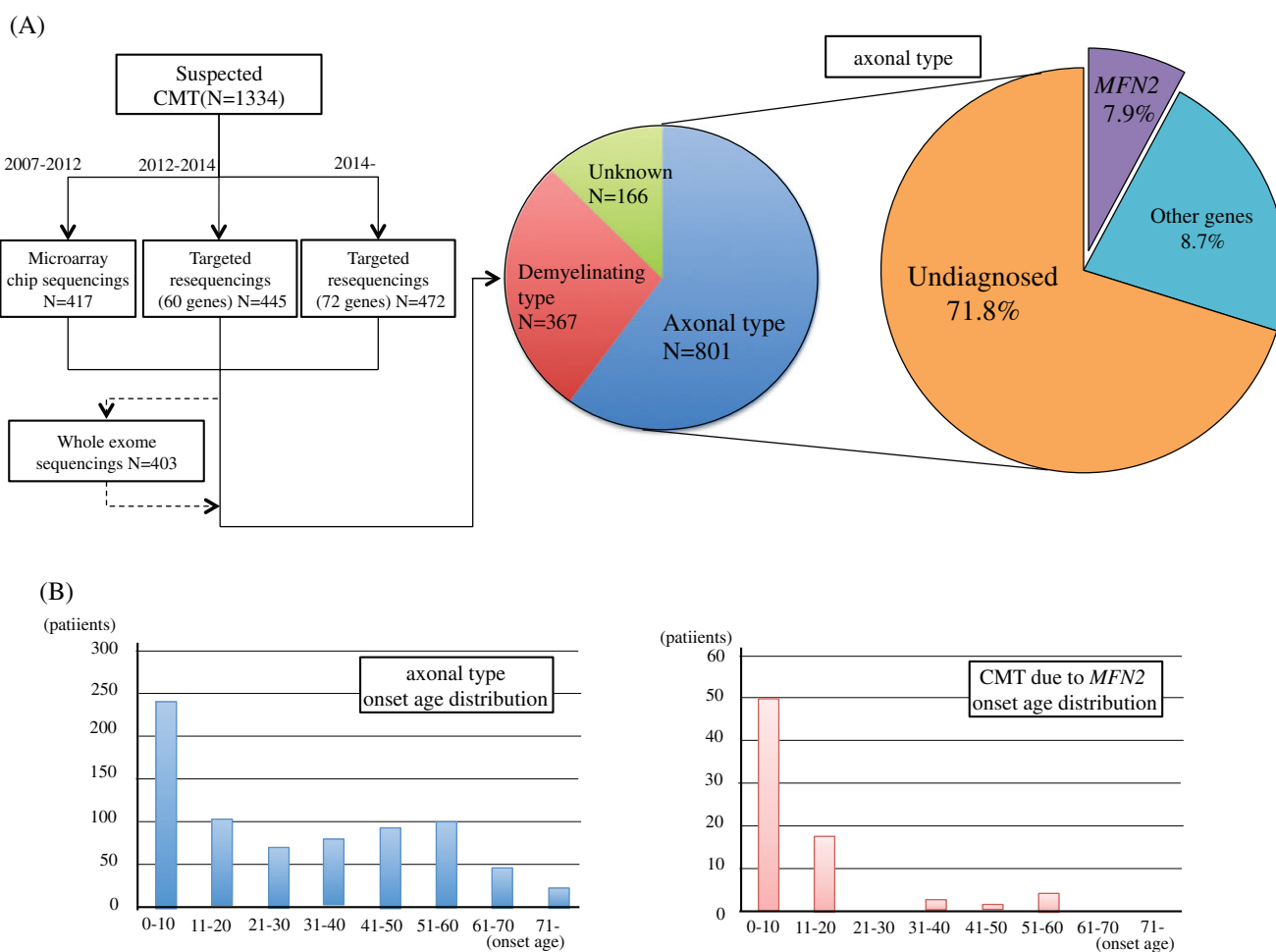

Figure 1. Study flow chart of this and onset age distribution. (A) Study flow chart and the rate of *MFN2* mutations in axonal Charcot-Marie-Tooth disease (CMT). Onset age distribution for the axonal type and cases with *MFN2* mutation.

Table 1. Clinical information of the cases with novel pathogenic mutations and likely pathogenic variants.

Nucleotide change	Amino acid change	Segregation	Gender	Exam age	Onset age	Inherited pattern	Initially symptom	Distal leg weakness	Distal leg MMT	Distal leg atrophy	Sensory disturbance	DTR	Median N MNCV (m/s)	Median N CMAP (mV)	Median N SCV (m/s)	Median N SNAP (μV)	Tibial N MNCV (m/s)	Tibial N CMAP (mV)	Sural N SCV (m/s)	Sural N SNAP (μV)	Other
c.154G>A	p.Glu52Lys	+	F	74	/		/	+	2	+	–	D	60.3	6.7	52.1	8	43.5	0.5	51.7	2	Parkinson disease
c.313A>T	p.Thr105Ser	n.d.	M	47	52	AD	Gait instability	+	3	+	+	A	55	8.4	39	6.6	34	0.2	36	4	–
c.326A>G	p.Lys109Arg	n.d	M	53	51	AD	Distal leg weakness	+	3	+	–	A	56	4.1	49	10	NE	NE	NE	NE	–
c.382C>T	p.His128Tyr	+	F	26	10	S	Impossible to run	+	3	+	/	D	51.5	11.5	55	20.9	NE	NE	42.5	3.7	–
c.382C>T	p.His128Tyr	n.d.	M	29	10	S	Impossible to run	+	3	+	+	D	>38	/	>38	8.5	NE	NE	>38	8.8	–
c.382C>T	p.His128Tyr	n.d.	M	37	15	S	Gait instability	+	3	+	+	A	51	10.4	46.4	/	32.9	0.3	31.2	/	–
c.389C>T	p.Thr130Ile	n.d.	M	48	10	AD	/	/	/	/	/	/	>38	/	>38	/	/	/	/	/	/
c.658G>A	p.Ala220Thr	n.d.	F	53	2	S	Distal leg weakness	+	/	+	+	/	55.8	0.15	/	/	/	/	/	/	Dysphagia, Tongue atrophy
c.733A>C	p.Ser245Arg	n.d.	F	33	6	AD	Gait instability	+	/	+	+	A	41.3	3	46.2	4.9	/	/	/	/	–
c.1078C>G	p.Gln360Glu	n.d	F	31	12	AD	Distal leg weakness	+	1	+	+	A	46.2	1.3	43.4	1.6	NE	NE	36.7	3.5	–
c.1147_1149delGCT	p.Ala383del	n.d.	F	30	4	AD	Impossible to run	+	0	+	–	D	51.5	2	56.5	4.6	NE	NE	NE	NE	–
c.1427G>C	p.Arg476Pro	+	M	69	54	AD	Distal leg weakness	+	0	+	+	/	/	/	/	/	33	/	45.2	/	–
c.1636T>G	p.Ser546Ala	n.d.	F	15	12	S	Gait instability	+	2	–	–	A	60.8	7.1	60.7	29	44.2	9.5	48.6	4.3	–
c.2075T>G	p.Leu692Arg	n.d.	F	39	11	S	Gait instability	+	2	+	+	A	55.2	9.3	44.2	4.8	NE	NE	33	2.2	–
c.2222T>C	p.Leu741Ser	+	M	8	6	AD	Gait instability	+	/	+	–	D	55.2	6	/	/	37.2	0.19	/	/	–
c.2240T>G	p.Met747Arg	n.d.	M	22	11	AD	Foot deformity	+	/	/	/	D	/	/	/	/	<38	/	/	/	–
c.2240T>G	p.Met747Arg	n.d.	M	8	4	AD	Impossible to run	+	/	+	+	/	57.3	4.5	/	/	38.9	0.18	/	/	–
c.2240T>G	p.Met747Arg	+	M	45	8	AD	Foot deformity	+	/	+	/	/	45.7	5.4	/	/	/	/	/	/	–
c.2243_2244insT	p.Thr749Hisfs*14	n.d.	M	39	6	AD	Gait instability	+	0	+	–	A	49.3	/	/	/	/	/	/	/	–

Segregation: +, confirmed de novo mutation or co-segregation with disease in affected family members; n.d, not done. Inherited pattern: AD, autosomal dominant form; S, sporadic form. Distal leg weakness and atrophy, sensory disturbance: +, presence of weakness/atrophy/sensory disturbance; –, absent. Distal leg MMT (manual muscle testing): ankle dorsiflexion or ankle plantarflexion); /, not available. DTR (deep tendon reflex): D, decreased; A, absent; /, normal; –, not available. MNCV, motor nerve conduction velocity; CMAP, compound motor action potential; SCV, sensory nerve conduction velocity; SNAP (sensory nerve action potential): NE, not evoked; /, not available. F, female; M, male.

Table 2. Genetic information of the novel pathogenic and likely pathogenic variants.

Nucleotide change	Amino acid change	SIFT	PP2	PROVEAN	MA	Condel	Same codon reference	Reference AA change	ACMG			Classification
									Strong	Moderate	Support	
c.154G>A	p.Glu52Lys	0.003	0.901	−2.97	2.58	0.64	No	–	–	PS4-modarate, PM2	PP1, 3, 4	Likely pathogenic
c.313A>T	p.Thr105Ser	0	1	−3.68	3.53	0.57	Kijima et al. (2005) Sitarz et al. (2012) Brozkova et al. (2013)	Thr105Met Thr105Ala Thr105Arg	–	PS4-modarate, PM2, 5	PP3, 4	Likely pathogenic
c.326A>G	p.Lys109Arg	0	1	−2.66	4.065	0.78	No	–	PS2	PS4-modarate, PM2	PP3	Pathogenic
c.382C>T	p.His128Tyr	0	0.987	−5.52	3.375	0.64	Calvo et al. (2009)	His128Arg	–	PS4-modarate, PM2, 5, 6	PP3	Likely pathogenic
c.389C>T	p.Thr130Ile	0	0.975	−5.42	3.94	0.68	No	–	–	PS4-modarate, PM1, 2	PP3, 4	Likely pathogenic
c.658G>A	p.Ala220Thr	0	0.975	−3.49	2.575	0.59	No	–	–	PS4-modarate, PM2, 6	PP3	Likely pathogenic
c.733A>C	p.Ser245Arg	0.003	0.57	−3.18	2.23	0.55	No	–	–	PS4-modarate, PM1, 2	PP3, 4	Likely pathogenic
c.1078C>G	p.Gln360Glu	0.003	0.81	−2.44	3.07	0.62	No	–	–	PS4-modarate, PM1, 2	PP3, 4	Likely pathogenic
c.1147_1149 delGCT	p.Ala383del	–	–	−6.62	–	–	Muglia et al. (2007)	Ala383Val	–	PS4-modarate, PM1, 2	PP4	Likely pathogenic
c.1427G>C	p.Arg476Pro	0.006	0.94	−2.83	2.34	0.58	No	–	–	PS4-modarate, PM2	PP1, 3, 4	Likely pathogenic
c.1636T>G	p.Ser546Ala	0	0.999	−2.64	3.08	0.58	No	–	–	PS4-modarate, PM2, 6	PP3	Likely pathogenic
c.2075T>G	p.Leu692Arg	0	1	−4.9	2.375	0.62	No	–	–	PS4-modarate, PM2, 6	PP3	Likely pathogenic
c.2222T>C	p.Leu741Ser	0	1	−4.45	2.455	0.63	No	–	–	PS4-modarate, PM1, 2	PP3, 4	Likely pathogenic
c.2240T>G	p.Met747Arg	0.53	0.001	−0.72	−0.05	0.48	Calvo et al. (2009)	Met747Thr	–	PS4-modarate, PM2, 5	PP1, 4	Likely pathogenic
c.2243_4insT	p.Thr749Hisfs*14	–	–	–	–	–	No	–	–	PS4-modarate, PM1, 2, 4	PP4	Likely pathogenic

AA, amino acid; ACMG, American College of Medical Genetics and Genomics; MA, mutation assessor; PP2, PolyPhen2.
In silico analysis cut off: SIFT <0.05, PP2 >0.9, PROVEAN <−2.5, MA >1.9, and Condel >0.47.

for novel pathogenic variants and likely pathogenic variants. The mutations appeared to be sporadic in 36 patients (46%) and presented an autosomal dominant inherited pattern in 43 patients (54%). The average age of onset was 12 ± 14 (range, 0–59) years and lower than that of the patients presenting the axonal phenotype [30 ± 22 (range, 0–79) years; $p < 0.01$]. The tendency for juvenile onset is shown in Fig. 1B. The mean onset age of sporadic cases was lower than that of autosomal dominant cases [age of onset for sporadic cases = 7.6 ± 10 years (range 0–59) years; age of onset for autosomal dominant cases = 15 ± 16 (range, 1–57); p value = 0.017].

Most patients had developed motor symptoms such as distal leg weakness, foot deformity, gait instability, impossibility to run, or delayed motor milestone by the time of recruitment. Most patients presented with distal weakness, distal atrophy, and hyporeflexia [99% (75/76), 96% (72/75), and 98% (60/61), respectively]. In addition, the frequency of sensory disturbance was lower than that of any other symptoms (61% 33/54). We could evaluate sensory modality in 35 patients of these; 19 showed a decrease in sensory detection only regarding vibration, 8 showed a decrease in both vibration and superficial sensation, and 6 did not show any decrease in either of these parameters.

Only one patient with *MFN2* p.Leu710Pro mutation showed HMSN with significant spasticity and increased patellar tendon reflex, which is the HMSN V phenotype, while four patients with *MFN2* p.Arg104Trp, p.Arg104Leu, and p.Arg364Trp mutations showed HMSN with optic atrophy. Two patients with *MFN2* p.Arg364Trp mutation had vocal cord paralysis. The patient with p.Arg280His and p.Arg250Trp compound heterozygous mutations had an earlier onset than the patients with heterozygous p.Arg280His mutation.

Electrophysiological findings

In the nerve conduction study, the MNCV of the median nerve was 51 ± 7.5 (range, 23–64) m/s and the CMAP was 4.9 ± 4.0 (range, 0–11.7) mV. In addition, the MNCV value for the tibial nerve was 38 ± 8.2 (range, 13–51) m/s and the CMAP was 0.5 ± 1.8 (range, 0–12) mV. Four patients had <38 m/s MNCV and were classified as having demyelinating type; two of them had mild MNCV reduction, 35 and 38 m/s. One patient had significantly decreased MNCV, 23 m/s. The median MNCV of four patients was unknown, and two of them had <38 m/s tibial MNCV. In nine patients, the absence of CMAP did not allow MNCV measurement.

Genetic findings

We detected 1 novel pathogenic variant and 14 novel likely pathogenic variants in 20 patients (Table 2). The novel pathogenic variant was p.Lys109Arg (one patient). The novel likely pathogenic variants were p.Glu52Lys (one patient), p.Thr105Ser (two patients), p.His128Tyr (two patients), p.Thr130Ile (one patient), p.Ala220Thr (one patient), p.Ser245Arg (two patients), p.Gln360Glu (one patient), p.Ala383del (one patient), p.Arg476Pro (one patient), p.Ser546Ala (one patient), p.Leu692Arg (one patient), p.Leu741Ser (one patient), p.Met747Arg (three patients), and p.Thr749Hisfs*14 (one patient). Furthermore, we identified 30 previously reported mutations in 59 unrelated patients. Fig. 2 shows the five pedigrees of segregated novel variants as well as the family with co-occurrence of a *de novo* *MFN2* mutation and a maternal *PMP22* mutation in the proband. All variants of uncertain significance are listed in Table S3.

Simultaneous mutations

The presence of simultaneously heterozygous mutations of different CMT disease-causing genes is noteworthy. We detected the mutations in *MFN2* p.Thr105Met and *PMP22* p.Arg159Cys. The patient (a female) developed gait disturbances at the age of 1 year and later a mild mental retardation and an IQ of 69. No parental consanguinity or obvious family history was recorded (Fig. 2). Her parents had no neuropathic symptoms and normal cognitive function, but we could not evaluate the electrophysiological findings of her parents. At age 9 years, she had limited dorsiflexion of both feet joints and clubfoot. Due to a deformity in one foot and distal weakness, she could not walk unaided at age 11 years. Her nerve conduction study was normal for the median nerve and presented low CMAP for the tibial nerve. She was diagnosed with CMT2. The genetic analysis showed a p.Thr105Met mutation in *MFN2* and a p.Arg159Cys mutation in *PMP22*. Both mutations had been previously described in other patients; however, none of the parents showed the p.Thr105Met mutation in *MFN2*, being thus confirmed as a *de novo* mutation. Her asymptomatic mother presented the p.Arg159Cys mutation in *PMP22*.

Discussion

In this Japanese case series, we detected *MFN2* mutations in 79 of 1,334 CMT patients without a *PMP22* deletion/duplication. The *MFN2* mutation accounted for 16% of the CMT2 patients in a Spanish cohort study, and 18% of CMT2 without *MPZ* and *GJB1* mutations in French patients *(Calvo et al.,*
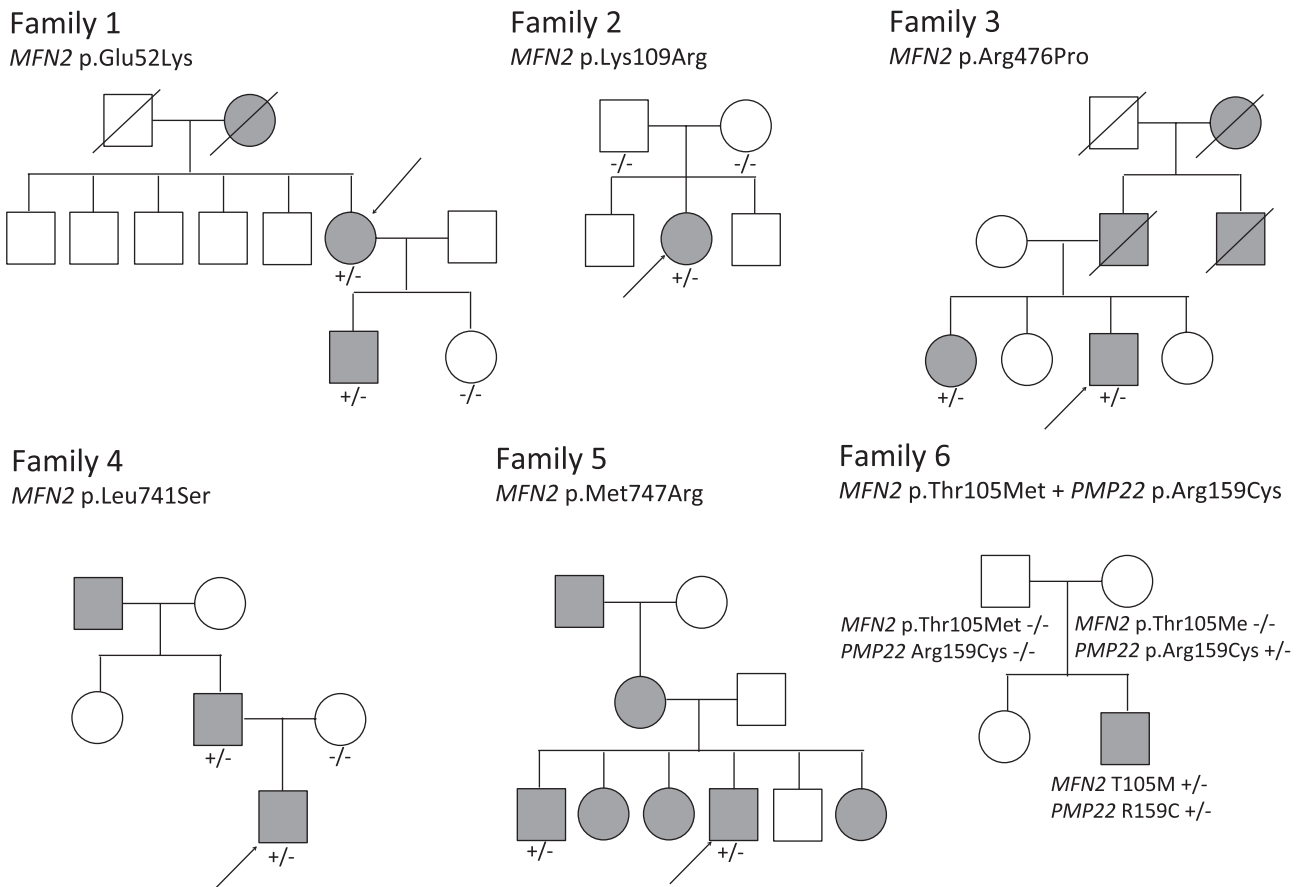
Figure 2. The pedigree of novel variants and simultaneously variants with segregation study. Families 1–5 indicate pedigree chart with novel variants and Family 6 indicate family with simultaneously *MFN2* and *PMP22* mutation. Arrow indicates probands.

2009; Casasnovas et al., 2010). In Asia, *MFN2* was the cause of CMT2 in 23% of Korean CMT2 patients and 18% of Chinese CMT2 patients *(Choi et al., 2015; Xie et al., 2016)*. The frequency of *MFN2* mutations is low, between 9% and 11%, as previously reported in a Japanese population study *(Kijima et al., 2005; Abe et al., 2011)*. Here, we show a frequency of the *MFN2* mutation of 8% (63/801) in Japanese CMT2 patients. A frequency of 28%–34% for sporadic or *de novo* mutations has been previously reported *(Verhoeven et al., 2006; Choi et al., 2015)*. However, sporadic cases reached a frequency of 46% in our study. This incidence rate is higher than that reported from other countries. The low frequency of *MFN2* mutations and high frequency of sporadic cases might indicate the influence of geographical and social distribution, although there is a possibility of an incomplete family history.

We showed an earlier onset of CMT due to *MFN2* mutation than of CMT2. Onset age for CMT2 often show a bimodal distribution with a peak at 0–20 years and another peak at 40–60 years. Onset in *MFN2*

patients tended to be earlier than the average onset for all CMT patients considered together (Fig. 1B). Some patients showed the characteristic symptoms, for example, spasticity, optic atrophy, and vocal cord paralysis. One of our patients with *MFN2* p.Leu710Pro mutation showed spasticity, which has not been previously reported *(Verhoeven et al., 2006)*. We found a novel HMSN V phenotype for CMT2 with MFN2 p.Leu710Pro mutation. Patients with *MFN2* p.Arg364Trp showed an early onset of the severe phenotype with optic atrophy. In our study, only the patients with this specific mutation presented vocal cord paralysis, similar to that previously reported for this mutation *(Zuchner et al., 2006)*. The patient with the novel p.Arg220Thr variant showed the characteristic symptoms of dysphagia and tongue atrophy. Furthermore, we described a case of earlier-onset CMT phenotype associated with p.Arg280His and p.Arg250Trp compound heterozygous mutation rather than a p.Arg280His heterozygous mutation. This proband was a sporadic case. The p.Arg250Trp heterozygous mutation was reported as causative

compound heterozygous mutations with p.Arg400X *(Verhoeven et al., 2006)*. This p.Arg250Trp variant might be a genetic burden and make the phenotype more severe.

We presented the electrophysiological findings of CMT patients related to *MFN2* mutations. We observed a tendency toward more severe axonal neuropathies in the lower extremities, and the characteristic electrophysiological findings were demyelinating phenotype in some cases. CMT due to *MFN2* mutation was known to be mainly axonal phenotype, but some cases classified as demyelinating type or intermediate type were reported *(Kijima et al., 2005; Braathen et al., 2010)*. We need to consider the possibility that *MFN2* mutation will be classified as demyelinating type.

In addition, we describe 1 novel pathogenic variant and 14 likely pathogenic variants. The novel p.Lys109Arg variant was absent in global, Japanese, and in-house databases (ACMG standards and guidelines; PS4-Moderate, PM2). This variant was validated as a *de novo* variant *via* segregation analysis (PS2), and we classified novel p.Lys109Arg variants as pathogenic. The other novel variants were classified as likely pathogenic variants according to the ACMG standards and guidelines. It is difficult to judge the novel *MFN2* variants as pathogenic variants. Without previously reported same amino acid change variant or functional study, novel *MFN2* variants with a family history could not be classified as pathogenic variants. Therefore, we reported novel pathogenic and likely pathogenic variants. Eventually, we will need to clarify the level pathogenicity of these variants based on functional or population studies.

We described characteristic cases simultaneously presenting mutations of different CMT disease-causing genes. Recent advances in genetic techniques, including next-generation sequencing, have enabled the possibility to analyze a large number of genes from a large number of patients. These data have thus helped in identifying the combined effect of rare variants, their expression, and their contribution to disease burden, included *MFN2* and *MED25*, *MFN2* and *HSPB1*, and *MFN2* and *WNK1*. *(Gonzaga-Jauregui et al., 2015)*. To this end, our study also shows how simultaneous heterozygous mutation may contribute to the level of clinical variability previously described. We presented simultaneous heterozygous mutations in *MFN2* p.Thr105Met and *PMP22* p.Arg159Cys. Both of these mutations had been reported before, and the mother of the proband appeared to have a mutation in *PMP22*. The reported onset age for patients with *PMP22* p.Arg159Cys mutation is 46 years *(Gess et al., 2011)*. Therefore, it is likely that onset had not happened yet for the mother of the proband, and the mutation may present incomplete penetrance. The patient with simultaneous mutations presented with an earlier onset age and mental retardation, which was a novel symptom in a patient with *MFN2* p.Thr105Met mutation. Similarly, *MFN2* and *PMP22* simultaneous mutations may result in a severe phenotype because of a double-dose effect or genetic burden effect.

Acknowledgements

References

Abe A, Numakura C, Kijima K, Hayashi M, Hashimoto T, Hayasaka K (2011). Molecular diagnosis and clinical onset of Charcot-Marie-Tooth disease in Japan. J Hum Genet 56:364–368.

Barreto LC, Oliveira FS, Nunes PS, de Franca Costa IM, Garcez CA, Goes GM, Neves EL, de Souza Siqueira Quintans J, de Souza Araujo AA (2016). Epidemiologic study of Charcot-Marie-tooth disease: a systematic review. Neuroepidemiology 46:157–165.

Braathen GJ, Sand JC, Lobato A, Hoyer H, Russell MB (2010). MFN2 point mutations occur in 3.4% of Charcot-Marie-Tooth families. An investigation of 232 Norwegian CMT families. BMC Med Genet 11:48.

Brozkova DS, Posadka J, Lassuthova P, Mazanec R, Haberlova J, Siskova D, Sakmaryova I, Neupauerova J, Seeman P (2013). Spectrum and frequencies of mutations in the MFN2 gene and its phenotypical expression in Czech hereditary motor and sensory neuropathy type II patients. Mol Med Rep 8:1779–1784.

Calvo J, Funalot B, Ouvrier RA, Lazaro L, Toutain A, De Mas P, Bouche P, Gilbert-Dussardier B, Arne-Bes MC, Carriere JP, Journel H, Minot-Myhie MC, Guillou C, Ghorab K, Magy L, Sturtz F, Vallat JM, Magdelaine C (2009). Genotype-phenotype correlations in Charcot-Marie-Tooth disease type 2 caused by mitofusin 2 mutations. Arch Neurol 66:1511–1516.

Casasnovas C, Banchs I, Cassereau J, Gueguen N, Chevrollier A, Martinez-Matos JA, Bonneau D, Volpini V (2010). Phenotypic spectrum of MFN2 mutations in the Spanish population. J Med Genet 47:249–256.

Choi BO, Nakhro K, Park HJ, Hyun YS, Lee JH, Kanwal S, Jung SC, Chung KW (2015). A cohort study of MFN2 mutations and phenotypic spectrums in Charcot-Marie-Tooth disease 2A patients. Clin Genet 87:594–598.

Chung KW, Cho SY, Hwang SJ, Kim KH, Yoo JH, Kwon O, Kim SM, Sunwoo IN, Zuchner S, Choi BO (2008). Early-onset stroke associated with a mutation in mitofusin 2. Neurology 70:2010–2011.

Del Bo R, Moggio M, Rango M, Bonato S, D'Angelo MG, Ghezzi S, Airoldi G, Bassi MT, Guglieri M, Napoli L, Lamperti C, Corti S, Federico A, Bresolin N, Comi GP (2008). Mutated mitofusin 2 presents with intrafamilial variability and brain mitochondrial dysfunction. Neurology 71:1959–1966.

Gess B, Jeibmann A, Schirmacher A, Kleffner I, Schilling M, Young P (2011). Report of a novel mutation in the PMP22 gene causing an axonal neuropathy. Muscle Nerve 43:605–609.

Gonzaga-Jauregui C, Harel T, Gambin T, Kousi M, Griffin LB, Francescatto L, Ozes B, Karaca E, Jhangiani SN, Bainbridge MN, Lawson KS, Pehlivan D, Okamoto Y, Withers M, Mancias P, Slavotinek A, Reitnauer PJ, Goksungur MT, Shy M, Crawford TO, Koenig M, Willer J, Flores BN, Pediaditrakis I, Us O, Wiszniewski W, Parman Y, Antonellis A, Muzny DM, Katsanis N, Battaloglu E, Boerwinkle E, Gibbs RA, Lupski JR (2015). Exome sequence analysis suggests that genetic burden contributes to phenotypic variability and complex neuropathy. Cell Rep 12:1169–1183.

Hashiguchi A, Higuchi Y, Nomura M, Nakamura T, Arata H, Yuan J, Yoshimura A, Okamoto Y, Matsuura E, Takashima H (2014). Neurofilament light mutation causes hereditary motor and sensory neuropathy with pyramidal signs. J Peripher Nerv Syst 19:311–316.

Higuchi Y, Hashiguchi A, Yuan J, Yoshimura A, Mitsui J, Ishiura H, Tanaka M, Ishihara S, Tanabe H, Nozuma S, Okamoto Y, Matsuura E, Ohkubo R, Inamizu S, Shiraishi W, Yamasaki R, Ohyagi Y, Kira J, Oya Y, Yabe H, Nishikawa N, Tobisawa S, Matsuda N, Masuda M, Kugimoto C, Fukushima K, Yano S, Yoshimura J, Doi K, Nakagawa M, Morishita S, Tsuji S, Takashima H (2016). Mutations in MME cause an autosomal-recessive Charcot-Marie-Tooth disease type 2. Ann Neurol 79:659–672.

Kijima K, Numakura C, Izumino H, Umetsu K, Nezu A, Shiiki T, Ogawa M, Ishizaki Y, Kitamura T, Shozawa Y, Hayasaka K (2005). Mitochondrial GTPase mitofusin 2 mutation in Charcot-Marie-Tooth neuropathy type 2A. Hum Genet 116:23–27.

Li H, Durbin R (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.

Maeda K, Idehara R, Hashiguchi A, Takashima H (2014). A family with distal hereditary motor neuropathy and a K141Q mutation of small heat shock protein HSPB1. Intern Med 53:1655–1658.

Mostacciuolo ML, Rampoldi L, Righetti E, Vazza G, Schiavon F, Angelini C (2000). Hereditary spastic paraplegia associated with peripheral neuropathy: a distinct clinical and genetic entity. Neuromuscul Disord 10:497–502.

Muglia M, Vazza G, Patitucci A, Milani M, Pareyson D, Taroni F, Quattrone A, Mostacciuolo ML (2007). A novel founder mutation in the MFN2 gene associated with variable Charcot-Marie-Tooth type 2 phenotype in two families from southern Italy. J Neurol Neurosurg Psychiatry 78:1286–1287.

Nicholson GA, Magdelaine C, Zhu D, Grew S, Ryan MM, Sturtz F, Vallat JM, Ouvrier RA (2008). Severe early-onset axonal neuropathy with homozygous and compound heterozygous MFN2 mutations. Neurology 70:1678–1681.

Polke JM, Laura M, Pareyson D, Taroni F, Milani M, Bergamin G, Gibbons VS, Houlden H, Chamley SC, Blake J, Devile C, Sandford R, Sweeney MG, Davis MB, Reilly MM (2011). Recessive axonal Charcot-Marie-Tooth disease due to compound heterozygous mitofusin 2 mutations. Neurology 77:168–173.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17:405–424.

Sitarz KS, Yu-Wai-Man P, Pyle A, Stewart JD, Rautenstrauss B, Seeman P, Reilly MM, Horvath R, Chinnery PF (2012). MFN2 mutations cause compensatory mitochondrial DNA proliferation. Brain 135 e219, 1-3.

Timmerman V, Strickland AV, Zuchner S (2014). Genetics of Charcot-Marie-Tooth (CMT) disease within the frame of the human genome project success. Genes 5:13–32.

Verhoeven K, Claeys KG, Zuchner S, Schroder JM, Weis J, Ceuterick C, Jordanova A, Nelis E, De Vriendt E, Van Hul M, Seeman P, Mazanec R, Saifi GM, Szigeti K, Mancias P, Butler IJ, Kochanski A, Ryniewicz B, De Bleecker J, Van den Bergh P, Verellen C, Van Coster R, Goemans N, Auer-Grumbach M, Robberecht W, Milic Rasic V, Nevo Y, Tournev I, Guergueltcheva V, Roelens F, Vieregge P, Vinci P, Moreno MT, Christen HJ, Shy ME, Lupski JR, Vance JM, De Jonghe P, Timmerman V (2006). MFN2 mutation distribution and genotype/phenotype correlation in Charcot-Marie-Tooth type 2. Brain 129:2093–2102.

Xie Y, Li X, Liu L, Hu Z, Huang S, Zhan Y, Zi X, Xia K, Tang B, Zhang R (2016). MFN2-related genetic and clinical features in a cohort of Chinese CMT2 patients. J Peripher Nerv Syst 21:38–44.

Zuchner S, De Jonghe P, Jordanova A, Claeys KG, Guergueltcheva V, Cherninkova S, Hamilton SR, Van Stavern G, Krajewski KM, Stajich J, Tournev I, Verhoeven K, Langerhorst CT, de Visser M, Baas F, Bird T, Timmerman V, Shy M, Vance JM (2006). Axonal neuropathy with optic atrophy is caused by mutations in mitofusin 2. Ann Neurol 59:276–281.

Supporting Information

Additional supporting information may be found online in the supporting information tab for this article.