Journal of Human Genetics (2013) 58, 273–278

www.nature.com/jhg

ORIGINAL ARTICLE

Molecular analysis of the genes causing recessive demyelinating Charcot–Marie–Tooth disease in Japan

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Charcot–Marie–Tooth disease (CMT), the most common hereditary neuropathy, has been classified into two types, demyelinating and axonal types. We previously analyzed the genes causing dominant demyelinating CMT in 227 Japanese patients to identify the genetic background, but could not find any mutations in 110 patients. To investigate the frequency of patients with autosomal recessive demyelinating CMT (CMT4) mutations, we analyzed the coding sequence of known causative genes of CMT4 in 103 demyelinating CMT patients, excluding seven patients owing to lack of specimens. We found one patient with a *GDAP1* mutation, one patient with an *MTMR2* mutation, two patients with *SH3TC2/KIAA1985* mutations and three patients with *FGD4* mutations. Twelve patients, including five previously detected patients with *PRX* mutations, were diagnosed as CMT4, accounting for 5.5% of demyelinating CMT. In the patient with *GDAP1* mutation, only one mutation inherited from his mother was detected by genomic sequencing. Analysis by reverse transcription polymerase chain reaction using messenger RNA (mRNA) from the patient's leukocytes revealed the absence of transcription from the allele inherited from his father, suggesting the existence of one more mutation leading to a lack or destabilization of mRNA. Most patients carrying CMT4 gene mutations present with early-onset and slowly progressive symptoms, which may be associated with the function of mutants. We could not identify the disease-causing gene in 96 patients (about 45%). Further studies including studies with next-generation sequencers will be required to identify the causative gene in Japanese CMT.
Journal of Human Genetics (2013) **58,** 273–278; doi:10.1038/jhg.2013.15; published online 7 March 2013

Keywords: Charcot–Marie–Tooth disease; CMT; CMT4; demyelinating Charcot–Marie–Tooth disease; FGD4; GDAP1; MTMR2; SH3TC2/KIAA1985

INTRODUCTION

Charcot–Marie–Tooth disease (CMT) is the most common hereditary neuromuscular disorder, with a prevalence estimated at up to 5–40 individuals in every 100 000. Motor and sensory peripheral nerves are affected, and clinical features comprise slowly progressive distal muscle weakness and atrophy and glove-stocking-type sensory disturbance.[1,2] CMT has been traditionally classified into two types by electrophysiological studies: demyelinating and axonal types. Recently, a group showing intermediate median motor nerve conduction velocities (MCV) and overlapping demyelinating and axonal CMT has been referred to as intermediate CMT.[2] More than 30 genes have been identified as CMT disease-causing genes.[1–3]

To identify the genetic background of Japanese CMT, we analyzed the disease-causing genes in about 350 patients; however, we could not identify the causative genes in about 50% of demyelinating CMT and 80% of axonal CMT. In the demyelinating type, a low prevalence of *PMP22* duplication is a feature of Japanese patients, which likely leads to the high frequency of unknown cause patients.[4] The Japanese patients carrying *PMP22* duplication seem to have mild symptoms due to genetic and or epigenetic-modifying factors and do not use medical services, reflecting a low prevalence.[4]

In the present study, we analyzed the genes causing autosomal recessive demyelinating CMT (CMT4) to further clarify the genetic background of Japanese patients.

MATERIALS AND METHODS

The Ethics Committee of Yamagata University School of Medicine approved this study. Genomic DNA and RNA were prepared from peripheral blood specimens after written informed consent was obtained from the patients and their families.

Patients

Two hundred twenty-seven patients with demyelinating CMT were recruited in this study. Of the 227 patients, 93 patients with autosomal dominant CMT (CMT1),[4–12] 19 patients with X-linked inheritance (CMTX),[8,13,14] 5 patients

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Received 29 November 2012; revised 24 January 2013; accepted 14 February 2013; published online 7 March 2013

with *PRX* mutations[4,15,16] and 7 patients who lacked DNA samples were excluded (Figure 1). The remaining 103 patients (32 with and 71 patients without family history) were unrelated, and 14 patients were from consanguineous families.

Screening by genomic DNA

Genomic DNA was extracted from peripheral blood using a standard method. The genes targeted in this study were CMT4-causing genes: *GDAP1* (ganglioside-induced differentiation-associated protein 1),[17] *MTMR2* (myotubularin-related protein 2),[18,19] *SBF2/MTMR13* (SET binding factor 2/myotubularin-related protein 13),[20] *SH3TC2/KIAA1985* (SH3 domain and tetratricopeptide repeats 2),[21] *NDRG1* (N-Myc downstream-regulated gene 1),[22] *FGD4* (FYVE, RhoGEF and PH domain containing 4),[23,24] and *FIG4* (FIG4 homolog, SAC1 lipid phosphatase domain containing).[25] We amplified all coding regions and their exon–intron boundaries using a set of PCR primers designed by Primer 3 software based on the genomic information (details available upon request) or a set of primers as described in previous reports: *GDAP1*,[26] *MTMR2*,[27] *SH3TC2/KIAA1985*,[28] *NDRG1*[22] and *FGD4*.[24] Sequencing of PCR products was directly determined using a dye-terminator reaction (BigDye Terminator v. 1.1, Applied Biosystems, Foster City, CA, USA) with an ABI 3500 × l automated genetic analyzer (Applied Biosystems). The number of nucleotides was based on the published online protein and messenger RNA sequences of the respective genes (www.ncbi.nlm.nih.gov).

Analysis by reverse transcription polymerase chain reaction

To ascertain that the mutations of *GDAP1* and *FGD4* bring about abnormal splicing, we performed reverse transcription polymerase chain reaction (RT-PCR) analysis.[29,30] Total RNA was extracted from the leukocytes of each patient who carried the mutations of *FGD4* at the splice site (patients 6 and 7) and who had a mutation in *GDAP1* in one allele (patient 1), with ISOGEN-LS (Nippon Gene Co., Tokyo, Japan). Subsequently, messenger RNA (mRNA) was prepared using an Oligotex-dT30 Super mRNA purification kit (Takara Bio, Otsu, Japan) and was reverse transcribed into cDNA with random primers using a TaKaRa RNA PCR kit (AMV) ver. 3.0 (Takara Bio). cDNAs were then amplified using each set of PCR primers; primers for *GDAP1* were: F, 5′-CAGTGTGGGAGGGAGAAGTC-3′; R, 5′-ATGCTTGGATGAGCTGCC-3′ (1178-bp fragment from wild-type mRNA); those for exons 6–8 of *FGD4* were: F, 5′-GCAAACTGTTGGAAGAAGCA-3′; R, 5′-AGGGAATCAGGAGGC AATTT-3′ (412-bp fragment from wild-type mRNA);[24] and those for exons 7–9 of *FGD4* were: F, 5′-TTGATAATGCAATGGAATTGG-3′; R, 5′-AGAA TGGCTTGCTGCTGTAG-3′ (254-bp fragment from wild-type mRNA). PCR products of *FGD4* were directly sequenced and those of *GDAP1* were subcloned into a TA vector and these sequences were determined. Relative amounts of each *GDAP1* transcript were estimated by the number of colonies.

RESULTS

We analyzed the CMT4-causing genes in 103 patients from 220 patients with demyelinating CMT who had no mutations in CMT1 or CMTX1-causing genes (Figure 1). Seven cases were found to carry CMT4-causing gene mutations: one case with a *GDAP1* mutation, one case with an *MTMR2* mutation, two cases with *SH3TC2/KIAA1985* mutations and three cases with *FGD4* mutations. Table 1 shows the type of mutation and clinical information of each patient. We detected 12 cases with mutation in CMT4 genes in all, including five cases carrying *PRX* mutations that were previously reported,[4,15,16] accounting for 5.5% of all patients with demyelinating CMT (Table 2).

Patient 1 carried a heterozygous *GDAP1* c.571C>T (p.R191*) mutation in exon 4. His mother was also a heterozygote of the mutation but was not affected. To find another mutation on the allele inherited from his father, RT-PCR analysis was performed. We extracted mRNA from leukocytes of patient 1, his mother and a healthy control. We amplified a 1178-bp fragment from c.−63 to c.*38 of *GDAP1* and found two major bands on agarose gel electrophoresis in the specimens from the control, mother and patient 1 (Figure 2). The sequence of each band was determined after subcloning and all transcripts were classified into three sizes: ~1200, 1050 and 950 bp (Figure 3). The wild allele produces three types of transcripts, 1, 2 and 3, by alternative splicing, as shown in control specimens. His mother had three types of transcripts from wild and mutant alleles, and two other alternatively spliced transcripts (4 and 5), likely derived from the mutant allele, which lacked exon 4 due to c.571C>T in exon 4. Patient 1 had four transcripts identical to the transcripts from the mutant allele of his mother; however, patient 1 lacked transcript 3 from the mutant allele and had transcript 6 transcribed from the mutant allele. All transcripts except transcript 1 from the wild allele are predicted to make premature terminal codons 5′ to the last 50 nucleotides of the penultimate exon and would be degraded by nonsense-mediated decay.[31] However, some amount of transcript 2 from a c.571C>T mutant allele was detected, suggesting that small amounts of a truncated peptide were produced at least in

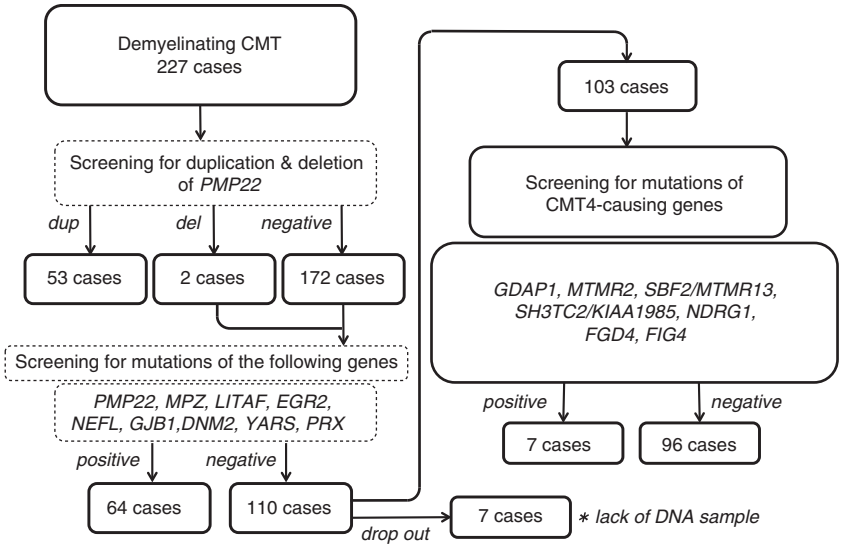

Figure 1 Genetic testing strategies for demyelinating Charcot-Marie-Tooth disease (CMT).

Table 1 Clinical and genetic information of thepatients

Patient/ sex	Gene	Mutation	Age at onset	Age at examination (years)	Consanguineous marriage/ Affected sibling	MCV ($m s^{-1}$)	Walking/additional features
1/M	*GDAP1*	c.[571C>T]+[?] (p.[R191*]+[?]) Possible compound heterozygote	<1 year	3	No/No	37.8	Developed hypotonia and foot deformities Delayed motor development, stood at 11 months Started to walk with SLB at 23 months Unsteady gait
2/F	*MTMR2*	c.1882_1885delAGAG (p.R628Pfs*18) Homozygote	13 years	19	Yes/No	30	Developed abnormal gait, steppage gait Walked with orthosis at 19 years Myelin outfoldings at nerve biopsy
3/F	*SH3TC2*	c.3379C>T (p.R1127W) Homozygote	4 years	8	No/No	23.9	Developed abnormal gait, steppage gait Progressive walking difficulty
4/M	*SH3TC2*	c.3511C>T (p.R1171C) Homozygote	Childhood	76	Unknown/Unknown	Delayed	Never ran since childhood Hearing loss, respiratory distress
5/M	*FGD4*	c.1888_1892delAAAGG (p.K630Nfs*4) Homozygote	Childhood	70	No/Yes	9.6	Developed foot deformities Walked without assistance until 65 years Severe gait disturbance from 68 years
6/M	*FGD4*	c.[837−2A>G]+[1132+1G>A] (p.[W279*fs]+[Y355Ifs*2]) Compound heterozygote	At birth	10	No/Yes	Not evoked	Developed foot deformity Started to walk at 11 months, abnormal gait from 3 years
7/F	*FGD4*	c.837−1G>A (p.E280Kfs*23) Homozygote	4 years	10	No/Unknown	8.8	Started to walk at 14 months Frequent fell at 4 years, walked with limp from 6 years Myelin outfoldings at nerve biopsy

Abbreviations: F, female; M, male; MCV, median motor nerve conduction velocities; SLB, short leg brace.

Table 2 Frequency of type in demyelinating CMT

		Our data		USA (2011)[a]	
CMT type	*Gene*	n	%	n	%
1	5 genes[b]	40	18.2	31	8.1
1A	*PMP22* dup.	53	24.1	290	76.1
X	*GJB1*	19	8.6	26	6.8
4A	*GDAP1*	1	0.5	1	0.3
4B1	*MTMR2*	1	0.5	0	0
4B2	*SBF2*	0	0	0	0
4C	*SH3TC2*	2	0.9	3	0.8
4D	*NDRG1*	0	0	0	0
4E	*EGR2*	0	0	0	0
4F	*PRX*	5	2.3	1	0.3
4H	*FGD4*	3	1.4	0	0
4J	*FIG4*	0	0	2	0.5
	Unknown	96	43.6	27	7.1
	Total	220		381	

Abbreviation: CMT, Charcot–Marie–Tooth disease.
The patients of our data had median motor nerve conduction velocities below 38 $m s^{-1}$ and the patients of USA had ulnar motor nerve conduction velocities of 35 $m s^{-1}$ or less.
[a]Saporta *et al.* (2011).[3]
[b]5 genes including *PMP22*, *MPZ*, *LITAF*, *EGR2* and *NEFL*.

the leukocytes of patient 1 and his mother. By RT-PCR analysis, we could not find any mRNA from the allele inherited from his father in the patient, indicating that patient 1 likely had another mutant allele inherited from his father leading to a lack or destabilization of mRNA.

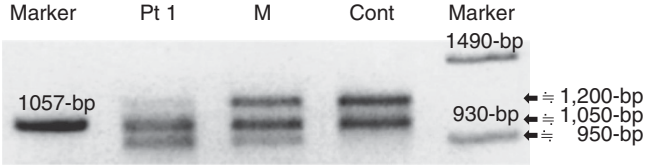

Figure 2 Agarose electrophoresis of RT-PCR products using mRNA from leukocytes. Cont, healthy control; M, mother of patient 1; Pt 1, patient 1.

Patient 2 was a homozygote of the *MTMR2* c.1882_1885delAGAG mutation. This mutation causes a frame shift and was predicted to produce a prolonged protein (p.R628Pfs*18). Her mother was a heterozygote for the mutation and no specimen from her father was available. A nerve biopsy revealed myelin outfoldings.

We found *SH3TC2/KIAA1985* mutations in two patients; patient 3 was a homozygote of the c.3379C>T (p.R1127W) mutation and patient 4 was a homozygote of the c.3511C>T (p.R1171C) mutation.

FGD4 mutations were detected in three patients: patient 5 was a homozygote of the c.1888_1892delAAAGG (p.K630Nfs*4) mutation, patient 6 was a compound heterozygote of the c.837−2A>G and c.1132+1G>A mutations and patient 7 was a homozygote carrying the c.837-1G>A mutation. The c.837−2A>G, c.1132+1G>A and c.837−1G>A mutations are predicted to cause splicing abnormalities. RT-PCR analysis confirmed that those mutations lead to frame shift, and they were predicted to produce the truncated proteins,

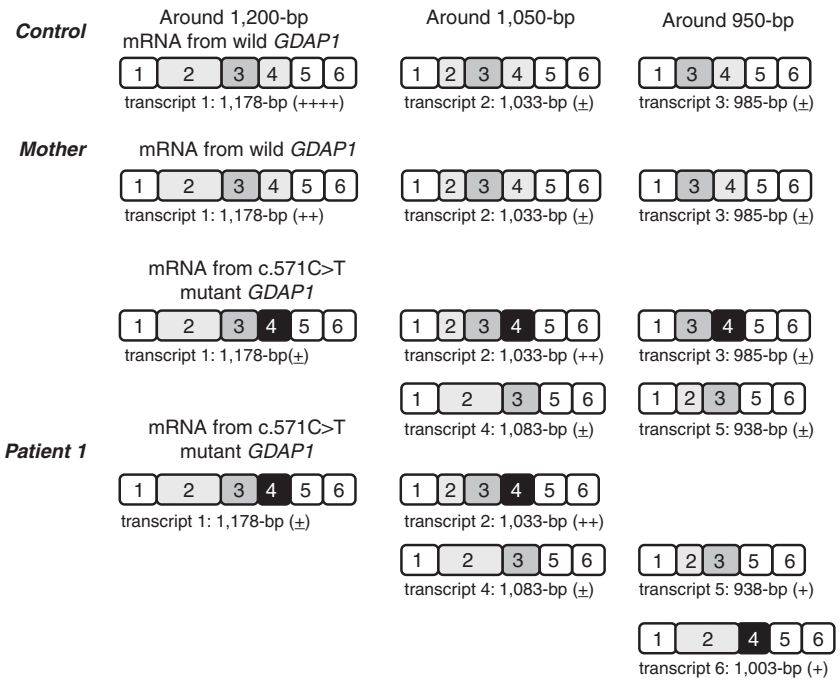

Figure 3 Splicing variations of *GDAP1* in patient 1. Transcript 1 (1178 bp) is a full length of messenger RNA (mRNA). Transcript 2 (mRNA lacks the 145 bp of the 3′ part of exon 2), transcript 3 (mRNA lacks an entire exon 2) and transcript 6 (mRNA lacks an entire exon 3) are registered as wild-type variations. Black boxes represent exon 4 carrying the c.571C>T mutation inherited from his mother. RT-PCR products of *GDAP1* were subcloned into a TA vector and these sequences were determined. Relative amounts of each transcript of *GDAP1* were estimated by the numbers of colonies and are described in parentheses.

p.W279*fs, p.Y355Ifs*2 and p.E280Kfs*23, respectively (Figures 4 and 5). Patients 5 and 6 had similarly affected siblings carrying the same mutations. Patient 7 showed myelin outfoldings in the nerve biopsy specimens.

All mutations except the *GDAP1* c.571C>T (p.R191*) mutation[32] were novel and were not detected in 100 healthy controls.

DISCUSSION

We performed a broad genetic screening in 220 patients with demyelinating CMT and detected 93 CMT1 patients (42.3%), including 53 patients with *PMP22* duplication,[4–12] 19 CMTX patients[8,13,14] (8.6%) and 12 CMT4 patients[4,15,16] (5.5%) (Table 2). As for the frequency of autosomal recessive CMT, there are few studies in the literature.[3] Sapora *et al.*[3] analyzed 381 patients with demyelinating CMT and found 7 CMT4 patients (1.8%). In the Mediterranean Sea area and the Middle East, it is reported that autosomal recessive CMT is more frequent than in North America and Europe because of a high percentage of consanguineous marriages.[33,34] We detected 12 patients with mutations in CMT4 genes and nine of them were homozygotes for the mutation. The percentage of CMT4 may reflect the percentage of consanguineous marriages (3.88%) in Japan: lower than that (20% to over 50%) in the Mediterranean Sea domain and the Middle East and higher than that (<1.0%) in the USA.[35–37] There are still 96 patients (43.6%) with unknown causes.

GDAP1 is a tail-anchored protein of the outer mitochondrial membrane and it regulates the dynamics of the mitochondrial network by inducing mitochondrial fragmentation.[17,38] Mutations in *GDAP1* are responsible for the most frequent recessive form of demyelinating and axonal CMT. We found a heterozygous p.R191* mutation in patient 1, but did not find any other mutation. The p.R191* mutation was reported as one of the mutations detected in a compound heterozygote,[32] indicating that patient 1 should have had one more mutation on an allele inherited from his father. RT-PCR analysis revealed no transcript from the allele from his father. We determined the sequence of the promoter region and poly-A additional signals (data not shown), but could not find any mutations. The patient likely has an unknown mutation in an allele inherited from his father leading to a lack or destabilization of mRNA. The patient developed hypotonia and a foot deformity within the first year of life.

MTMR2 encodes a protein that belongs to the myotubularin family, which is characterized by the presence of a phosphatase domain, and may play a significant role in neural membrane recycling, membrane trafficking and endo- and exocytic processes.[18,19] We found a homozygous c.1882_1885delAGAG mutation in patient 2. The c.1882_1885delAGAG mutation is located in the last exon, and is predicted to escape nonsense-mediated decay and produce a prolonged peptide, p.R628Pfs*18. Most patients carrying *MTMR2* mutations present symptoms in early infancy and have a subsequent severe course;[18,39] however, patient 2 presented at 13 years of age with mild clinical symptoms. The p.R628Pfs*18 mutant protein may be partly functional and therefore only cause mild symptoms. Myelin outfoldings and redundant myelin loops are characteristic findings in the mutation of *MTMR2, MTMR13* and *FGD4*.[2,18,23] Patient 2 revealed these characteristic pictures on nerve biopsy (data not shown).

SH3TC2/KIAA1985 plays a significant role in membrane trafficking and is required for proper myelination and integrity of the node of Ranvier.[21] We found homozygous missense *SH3TC2/KIAA1985* mutations in two patients: a c.3379C>T (p.R1127W) mutation in patient 3 and a c.3511C>T (p.R1171C) mutation in patient 4.

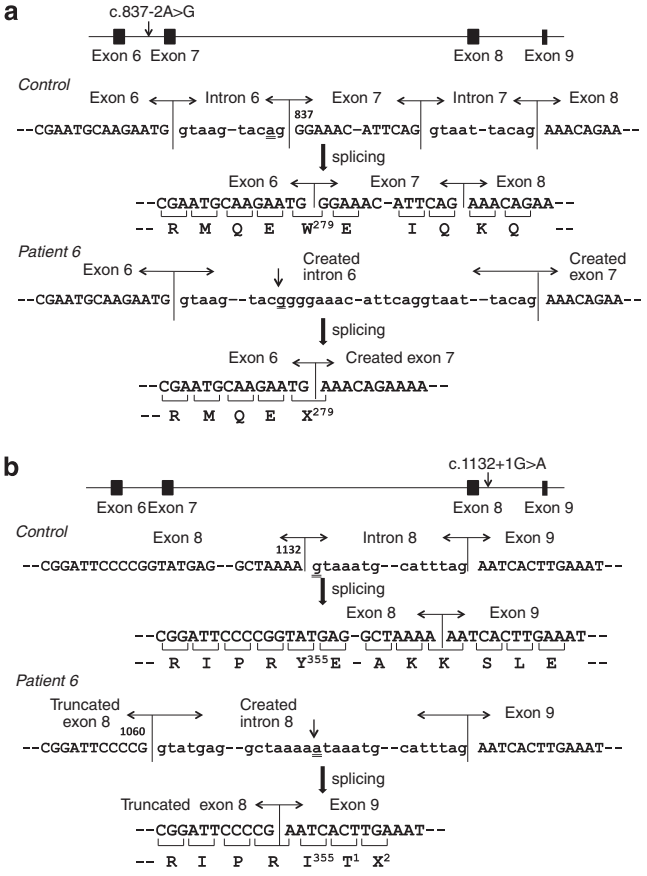

Figure 4 (**a**) *FGD4* c.837−2A>G mutation in patient 6. (**b**) *FGD4* c.1132+1G>A mutation in patient 6.

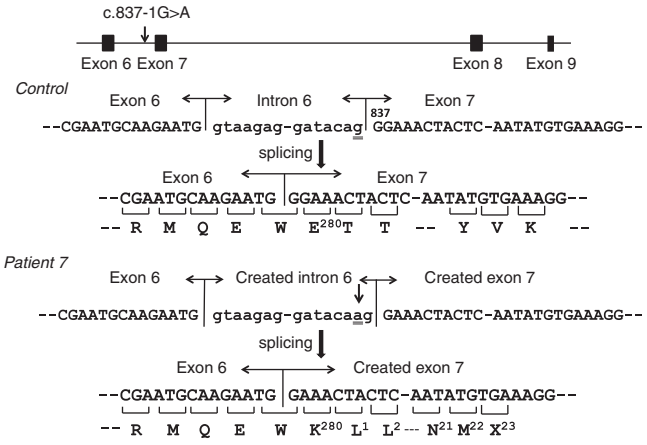

Figure 5 *FGD4* c.837−1G>A mutation in patient 7.

The specimens from both families were not available and segregation analysis could not be performed. However, c.3379C>T and c.3511C>T mutations were not detected in 100 healthy controls. R1127 and R1171 are conservative amino-acid residues, and scores from PolyPhen-2 software analysis (http://genetics.bwh.harvard.edu/pph2) to predict the functional effect of p.R1127W and p.R1171C were 1, indicating that *SH3TC2/KIAA1985* variants were probably damaging and disease-causing mutations. The clinical phenotype associated with *SH3TC2/KIAA1985* mutations is variable and is mostly characterized by early onset and frequent complication of scoliosis.[40–42] Baets *et al.*[40] reported *SH3TC2/KIAA1985* was the most commonly mutated recessive gene in CMT, presenting with symptoms within the first year of life in Western countries and the Middle East. The onset age of our cases was infancy, but they did not have scoliosis. Patient 3 showed progressive gait disturbance from 4 years of age. Patient 4 had gait disturbance in childhood and showed slowly progressive symptoms and developed hearing loss and respiratory distress in his seventies.

Frabin encoded by *FGD4* likely has a key role in proliferation, polarization, survival of Schwann cells and myelination processes.[23,24,29] We found *FGD4* mutations in three cases: a homozygous c.1888_1892delAAAGG (p.K630Nfs*4) mutation in patient 5, compound heterozygous c.837−2A>G (p.W279*fs) and c.1132+1G>A (p.Y355Ifs*2) mutations in patient 6, and a homozygous c.837−1G>A (p.E280Kfs*23) mutation in patient 7. All four mutations are predicted to make premature terminal codons 5′ to the last 50 nucleotides of the penultimate exon and should be degraded by nonsense-mediated decay.[31] In the RT-PCR analysis experiment, transcripts from c.837−2A>G and c.1132+1G>A mutant alleles in leukocytes from patient 6 were detected in one-third of transcripts from the wild allele in control leukocytes. Transcripts from the c.837−1G>A mutant allele in leukocytes from patient 7 were also decreased to two-thirds of that from wild alleles in control leukocytes. Therefore, these mutations are predicted to produce some amount of truncated peptides (Figures 4 and 5). Quantitative and qualitative abnormalities in FGD4 may be associated with the pathogenesis in the patients. Patient 7 showed myelin outfolding on nerve biopsy. Three patients showed childhood onset and moderately severe and slowly progressive symptoms as in other reported cases.[43]

We systematically analyzed 220 Japanese patients with demyelinating CMT (7 cases were excluded due to a lack of specimens) and found only 12 patients carrying autosomal recessive demyelinating CMT gene mutations. The autosomal recessive demyelinating CMT mutations likely behave in a loss-of-function manner. Therefore, we cannot exclude the possibility that the patients may have microdeletion of the causative genes, which would not be detected from ordinal analysis using PCR. Our study indicated that RT-PCR analysis is useful to confirm splicing abnormalities by mutant alleles and for the screening gene mutations, as shown in patient 1. To identify the causative gene, analysis using next-generation sequencers[44] and analysis of gene-expressing tissues derived from induced pluripotent stem cells would be powerful tools.

Most patients with CMT4 gene mutations present with early-onset and slowly progressive symptoms, in contrast to the early-onset and rapidly progressive symptoms in patients carrying the dominant gene mutation.[15,45] These differences may be associated with the type of mutant, which acts in a loss- or gain-of-function manner.

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