ORIGINAL ARTICLE

Molecular diagnosis and clinical onset of Charcot–Marie–Tooth disease in Japan

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To study the genetic background of Japanese Charcot–Marie–Tooth disease (CMT) patients, we analyzed qualitative and quantitative changes in the disease-causing genes mainly by denaturing high performance liquid chromatography and multiplex ligation-dependent probe analysis in 227 patients with demyelinating CMT and 127 patients with axonal CMT. In demyelinating CMT, we identified 53 patients with *PMP22* duplication, 10 patients with *PMP22* mutations, 20 patients with *MPZ* mutations, eight patients with *NEFL* mutations, 19 patients with *GJB1* mutations, one patient with *EGR2* mutation, five patients with *PRX* mutations and no mutations in 111 patients. In axonal CMT, we found 14 patients with *MFN2* mutations, one patient with *GARS* mutation, five patients with *MPZ* mutations, one patient with *GDAP1* mutation, six patients with *GJB1* mutations and no mutations in 100 patients. Most of the patients carrying *PMP22*, *MPZ*, *NEFL*, *PRX* and *MFN2* mutations showed early onset, whereas half of the patients carrying *PMP22* duplication and all patients with *GJB1* or *MPZ* mutations showing axonal phenotype were adult onset. Our data showed that a low prevalence of *PMP22* duplication and high frequency of an unknown cause are features of Japanese CMT. Low prevalence of *PMP22* duplication is likely associated with the mild symptoms due to genetic and/or epigenetic modifying factors.

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INTRODUCTION

Charcot–Marie–Tooth disease (CMT) is the most common inherited peripheral neuropathy-affecting motor and sensory nerves of the peripheral nervous system. The disease is genetically highly heterogeneous and has been traditionally classified into demyelinating and axonal forms based on nerve conduction studies. More than 27 genes have been identified as disease-causing genes of CMT (<http://www.molgen.ua.ac.be/CMTMutations/Mutations>). Disease-causing genes of demyelinating forms have been identified as follows: genes encoding myelin components, genes encoding regulators of myelin gene transcription and genes encoding intracellular Schwann cell proteins that are likely associated with the synthesis, transport and degradation of myelin components.^{1–4}

Regarding the causes of axonal forms, genes encoding cytoskeletal proteins, genes associated with axonal transport or with mitochondrial dynamics and genes encoding several aminoacyl-tRNA synthetases have been identified.⁵

To identify the genetic background of Japanese CMT cases, we previously screened for gene mutations using single-strand conformation polymorphism^{6–9} or denaturing gradient gel electrophoresis^{10,11} and recently performed such screening by denaturing high performance liquid chromatography (DHPLC).^{12–14} In addition, we screened for quantitative alterations in the major causative genes

using multiplex ligation-dependent probe analysis (MLPA).¹⁵ We also studied the relationship between the genotype and age at clinical onset.

MATERIALS AND METHODS

The Ethics Committee of Yamagata University School of Medicine approved this study. Peripheral blood specimens were used for genetic analysis after written informed consent was obtained from the patients or patient's families.

Patients

We studied 227 patients with demyelinating CMT: male-to-female ratio, 1.6:1; median age, 29.5 years old (ranging from 6 months to 88 years old), and 127 patients with axonal CMT: male-to-female ratio, 1.8:1; median age, 27.5 years old (ranging from 2 to 75 years old).

Extraction of genomic DNA

We extracted genomic DNA from the peripheral blood of patients with CMT, patients' family members and healthy controls using a standard method.

Screening for PMP22 duplication/deletion and gene mutations using DHPLC

Analysis flow charts are shown in Figures 1 and 2. First, we screened for *PMP22* duplication or deletion by fluorescence *in situ* hybridization (FISH) or Southern blot hybridization methods in patients showing both phenotypes.¹⁶ Patients

Figure 1 Schema of mutation analysis of demyelinating and axonal Charcot-Marie-Tooth disease (CMT). (a) Demyelinating CMT. (b) Axonal CMT.

with no *PMP22* duplication or deletion were then screened for gene mutations by single-strand conformation polymorphism^{6–9} or denaturing gradient gel electrophoresis^{10,11} and recently by DHPLC.^{12–14} In patients with demyelinating CMT, we screened for mutations of *PMP22*, *MPZ*, *LITAF*, *NEFL*, *GJB1*, *GDAP1*, *MTMR2*, *MTMR13*, *EGR2*, *PRX*, *DNM2* and *YARS*. In patients with axonal CMT, we screened for the mutation of *MFN2*, *RAB7*, *GARS*, *NEFL*, *HSP27*, *MPZ*, *HSP22*, *GDAP1*, *GJB1*, *DNM2* and *YARS*. We amplified all coding regions and its exon–intron boundary regions using a set of PCR primers designed based on the genomic information (details available upon request) or previous reports: *NEFL*,¹⁷ *GDAP1*,¹⁸ *EGR2*,¹¹ *PRX*,¹² *MFN2*¹⁴ and *HSP27*.¹³ After a heteroduplex was introduced, we analyzed the samples by DHPLC (WAVE DHPLC System; Transgenomic, Omaha, NE, USA). The fragments showing heteroduplex were sequenced by the Dye Deoxy Terminator Cycle method on an ABI Prism Genetic Analyzer 310 (PE Applied Biosystems, Foster City, CA, USA).

Screening for quantitative alteration of the major causing genes using MLPA

We screened for quantitative alteration (microdeletion or amplification) of the major causing genes using MLPA in patients, who did not show any mutation on DHPLC. We analyzed *PMP22*, *MPZ*, *LITAF*, *GJB1*, *NEFL* and *EGR2* in 115 patients with demyelinating CMT, and *MFN2*, *RAB7*, *GARS*, *NEFL*, *HSP27*, *HSP22*, *MPZ* and *GJB1* in 100 patients with axonal CMT. Probes for MLPA were designed based on genomic information so that the length of the PCR products varied in the sets of genes analyzed at the same time (details available upon request). MLPA reaction was performed using an MLPA kit (MRC Holland, Amsterdam, The Netherlands), and PCR products were analyzed by fragment analysis using ABI Prism Genetic Analyzer 310. The obtained peak area was standardized for each gene. We calculated the mean and standard

deviation of each peak in 20 normal controls. We set threshold values for identification of abnormal results at $>+3.0$ s.d. or <-3.0 s.d. based on the mean of normal controls.¹⁹

RESULTS

Disease-causing gene mutation in the patients

As shown in Figure 1a, we analyzed 227 cases of demyelinating CMT and found 50 cases carrying *PMP22* duplication by FISH or Southern blot analysis. We also detected *PMP22* duplication in three cases by MLPA, which had not been detected by FISH or Southern blot analysis, indicating that MLPA is a more sensitive method of detecting the copy number variation of *PMP22* than FISH or Southern blot analysis. We finally found 53 cases carrying *PMP22* duplication, which were ~25% of demyelinating CMT and significantly lower than the frequency of 50–70% reported from European countries²⁰ and the USA²¹ (Table 1). By screening with single-strand conformation polymorphism or denaturing gradient gel electrophoresis or DHPLC and MLPA, we found 10 cases carrying *PMP22* mutations, 20 cases carrying *MPZ* mutations, eight cases carrying *NEFL* mutations, 19 cases carrying *GJB1* mutations, one case carrying *EGR2* mutation and five patients carrying *PRX* mutations (Table 1). Two cases carrying *PMP22* deletion on one allele demonstrated severe symptoms and were further analyzed. Single-strand conformation polymorphism and MLPA analyses detected a missense mutation and a small deletion on the other *PMP22* allele, respectively. We finally identified one patient was a compound heterozygote carrying a deletion of the whole *PMP22* on one allele and a missense Arg157Gly mutation of *PMP22* on the other allele²² and the other patient was a compound heterozygote carrying a deletion of the whole *PMP22* on one allele and a deletion of exon 5 of *PMP22* on the other allele.²³ However, we could not find any mutation in 111 patients (Table 1).

In axonal type, we analyzed 127 patients by DHPLC and detected mutations in 27 cases: 14 cases carrying *MFN2* mutations, one case carrying *GARS* mutation, five cases carrying *MPZ* mutations, one case carrying *GDAP1* mutation and six cases carrying *GJB1* mutations (Figure 1b). In 100 patients, we could not find any mutations by DHPLC analysis. We further screened for quantitative alteration of major causative genes using the MLPA method, but did not find any mutations (Figure 1b, Table 2).

Previously unreported novel mutations are described in Table 3.

Relationship between the genotype and age at clinical onset

We studied the relationship between genotype and patient age at clinical onset. In the demyelinating form, half of the patients with *PMP22* duplication presented with symptoms within the first two decades of life, whereas half of them presented with symptoms after 20 years of age (Figure 2a). Most of the patients carrying *PMP22*, *MPZ*, *NEFL* or *PRX* mutations were early onset and presented within the first decade. The patients with *GJB1* mutations showed symptoms before 40 years of age.

As for the axonal type, nearly all patients with *MFN2* mutations were early onset in childhood (Figure 2b). Most patients with *MPZ* or *GJB1* mutations were adult onset and patients carrying *MPZ* mutations were older than patients carrying *GJB1* mutations.

DISCUSSION

To identify the genetic background of Japanese CMT cases, we screened gene mutations by FISH or Southern blot analysis, DHPLC and MLPA methods. The major characteristics of demyelinating CMT in Japanese are the low prevalence of *PMP22* duplication and high frequency of unidentified gene mutations compared with the reports

Figure 2 Genotype and age at clinical onset in demyelinating and axonal Charcot-Marie-Tooth disease (CMT). Association between genotype and age at clinical onset is shown. Vertical axis corresponds to the numbers of patients and horizontal axis to the age at onset. **(a)** Demyelinating CMT. **(b)** Axonal CMT.

Table 1 Gene mutations in demyelinating CMT

	<i>PMP22</i> duplication	<i>PMP22</i>	<i>MPZ</i>	<i>LITAF</i>	<i>NEFL</i>	<i>GJB1</i>	<i>GDAP1</i>	<i>MTMR2</i>	<i>MTMR13</i>	<i>EGR2</i>	<i>PRX</i>	<i>DNM2</i>	<i>YARS</i>	Unknown	Total
Our study	53 (40) 23.3%	10 (8) 4.4%	20 (15) 8.8%	0 0%	8 (8) 3.5%	19 (15) 8.5%	0 0%	0 0%	0 0%	1 (1) 0.4%	5 (4) 2.2%	0 0%	0 0%	111 48.9%	227
Italy	98 57.6%	2 1.2%	4 2.3%	ND ND	ND ND	12 7.1%	ND ND	ND ND	ND ND	ND ND	ND ND	ND ND	ND ND	54 31.8%	170
USA	79 54.1%	5 3.4%	5 3.4%	ND ND	ND ND	8 5.5%	0 0%	ND ND	ND ND	1 0.7%	1 0.7%	ND ND	ND ND	51 34.9%	146

Abbreviations: CMT, Charcot-Marie-Tooth disease; ND, not done.

Numbers in parentheses indicate data previously published.^{6-14,16,22,23,27,33,34,41,42} *PMP22* mutations include a compound heterozygote of a *PMP22* deletion and a missense Arg157Gly mutation²² and a compound heterozygote of a *PMP22* deletion and an exon 5 deletion mutation.²³

Table 2 Gene mutations in axonal CMT

	<i>MFN2</i>	<i>RAB7</i>	<i>GARS</i>	<i>NEFL</i>	<i>HSP27</i>	<i>MPZ</i>	<i>HSP22</i>	<i>GDAP1</i>	<i>GJB1</i>	<i>DNM2</i>	<i>YARS</i>	Unknown	Total
Our study	14 (7) 11.0%	0 0%	1 (1) 0.8%	0 0%	0 0%	5 (3) 4.0%	0 0%	1 0.8%	6 (4) 4.7%	0 0%	0 0%	100 78.7%	127

Abbreviation: CMT, Charcot-Marie-Tooth disease.

Numbers in parentheses indicate data previously published.^{14,33,34}

from European countries²⁰ and the USA²¹ (Table 1). The low prevalence of *PMP22* duplication in Japanese CMT can be explained by ethnic differences. *De novo PMP22* duplication is known to arise from unequal crossing over via long repeat sequences, located at the 5' and 3' regions of the *PMP22* gene.²⁴ More than 10% of the patients

with *PMP22* duplication are reported to show *de novo* mutations.²⁵ If Japanese have a variation in the sequence of long repeats that decreases the frequency of unequal crossing over, the low prevalence of *PMP22* duplication would be explained. However, there is no report indicating any variation in the repeats. Vincristine-induced peripheral

Table 3 Novel mutations

Gene	Nucleotide change	Amino-acid change	Genotype	Phenotype
MPZ	c.235-1G>C	3'-splice site	Heterozygote	Axonal CMT
	c.278G>C	Gly93Ala	Heterozygote	Demyelinating CMT
	c.410G>C	Gly137Ala	Heterozygote	Demyelinating CMT
GJB1	c.124A>C	Ser42Cys	Hemizygote	Demyelinating CMT
	c.247_256del10	Leu83ProfsX22	Hemizygote	Axonal CMT
MFN2	c.310C>T	Arg104Trp	Heterozygote	Axonal CMT
	c.476C>T	Ser249Phe	Heterozygote	Axonal CMT
	c.2171T>C	Leu724Pro	Heterozygote	Axonal CMT
PRX	(c.875-906del32; c.915-923dup9; c.924A>T; c.927T>G)	Val292AlafsX52	Homozygote	Demyelinating CMT
GDAP	(c.740C>A)+(c.845G>A)	(Ala247Val)+(Arg282His)	Compound heterozygote	Axonal CMT

Abbreviation: CMT, Charcot-Marie-Tooth disease.

neuropathy is sometimes an initial symptom of patients with *PMP22* duplication.¹⁰ In the study of families with vincristine-induced peripheral neuropathy, we frequently found family members, who were almost or completely asymptomatic and were unaware of being affected.²⁶ Recently, modifying factors have been discussed especially in association with the phenotypic variability in *PMP22* duplication. Japanese patients with *PMP22* duplication may have mild symptoms due to genetic and or epigenetic modifying factors and may be unaware of being affected. Ascertainment bias may be associated with low prevalence of *PMP22* duplication. In addition, social and or reproductive fitness impairment of the affected individuals should also be considered.

We found *NEFL* mutations in eight patients (3.5%) with demyelinating form, but not in patients with the axonal form.²⁷ *NEFL* mutations had initially been identified as the cause of dominant axonal CMT, but these mutations were also detected in many patients with dominant demyelinating CMT. Diameter of the axon is the principal determinant of conduction velocity and conduction velocity is nearly proportional to the diameter of the axon.^{28,29} Decrease in the diameter of the axon in the patients with *NEFL* mutations is likely associated with decreased nerve conduction velocities. We found a patient carrying a homozygous Glu140Stop mutation, which may cause loss-of-function.²⁷ *NEFL* mutation is one of the major causes of dominant demyelinating CMT and also a cause of recessive phenotype.^{27,30}

In the axonal phenotype, *MFN2* mutations were the most frequent in 10% of the patients. However, its frequency was lower than 20–30% reported in other countries.^{14,31,32} Following the *MFN2* mutations, we found *GJB1* or *MPZ* mutations in 4–5% of patients with axonal CMT (Table 2). *GARS* or *GDAP1* mutations are very rare and were detected in one patient each.³³ No disease-causing mutation was identified in ~80% of patients with axonal CMT. An unknown cause in axonal CMT is more frequent than that in demyelinating CMT.

Recently copy number variations have been shown to be a widespread phenomenon associated with disease. However, we did not find any quantitative alteration in major-causative genes other than *PMP22* by MLPA analysis. Huang *et al.*³⁴ did not find any copy number variation in CMT-causing genes other than *PMP22* on comparative genomic hybridization microarrays. This suggests that quantitative alteration of disease-causing genes other than *PMP22* is not likely a major cause of CMT.

We studied the relationship between genotype and patient age at clinical onset. Half of the patients with *PMP22* duplication presented with the symptoms after the age 20, supporting our speculation that Japanese patients with *PMP22* duplication have mild symptoms

(Figure 2a). Most patients carrying *PMP22*, *MPZ*, *NEFL* or *MFN2* mutations showed early onset and severe progressive symptoms probably due to a gain-of-function mutation. Verhoeven *et al.*³¹ reported that most patients with *MFN2* mutations presented with early onset and severe disease status, but a small number of the patients showed a later onset and milder disease course. Recently, a recessive type of *MFN2* mutation was also reported.³⁵ Patients with early onset and severe disease and late onset mild disease have been described in the same family.³⁶ However, severity of the disease is typically similar in each family and the phenotype of the patients is likely associated with the genotype of the mutation.^{32,37} Patients with *PRX* mutations presented with early onset, but slowly progressive symptoms, suggesting that the mutations may cause loss-of-function.¹² All patients with *MPZ* or *GJB1* mutations showing the axonal type were late onset, probably because it takes a long time to damage the axon after the initial damage to myelin.³⁸ CMT is characterized by phenotypic variability even within patients carrying the same mutations.^{39,40} However, overall severity varies according to the causative gene and type of mutation.

Our data showed that the low prevalence of *PMP22* duplication and high frequency of unknown cause are features of Japanese patients with CMT. The peripheral nervous system seems to be vulnerable because of its characteristic structure and function, suggesting that there are more genes associated with CMT. It will be necessary to establish a high-throughput method for screening of many disease-causing genes and to resequence the whole genome of patients with unidentified mutations to detect a new disease-causing gene.

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