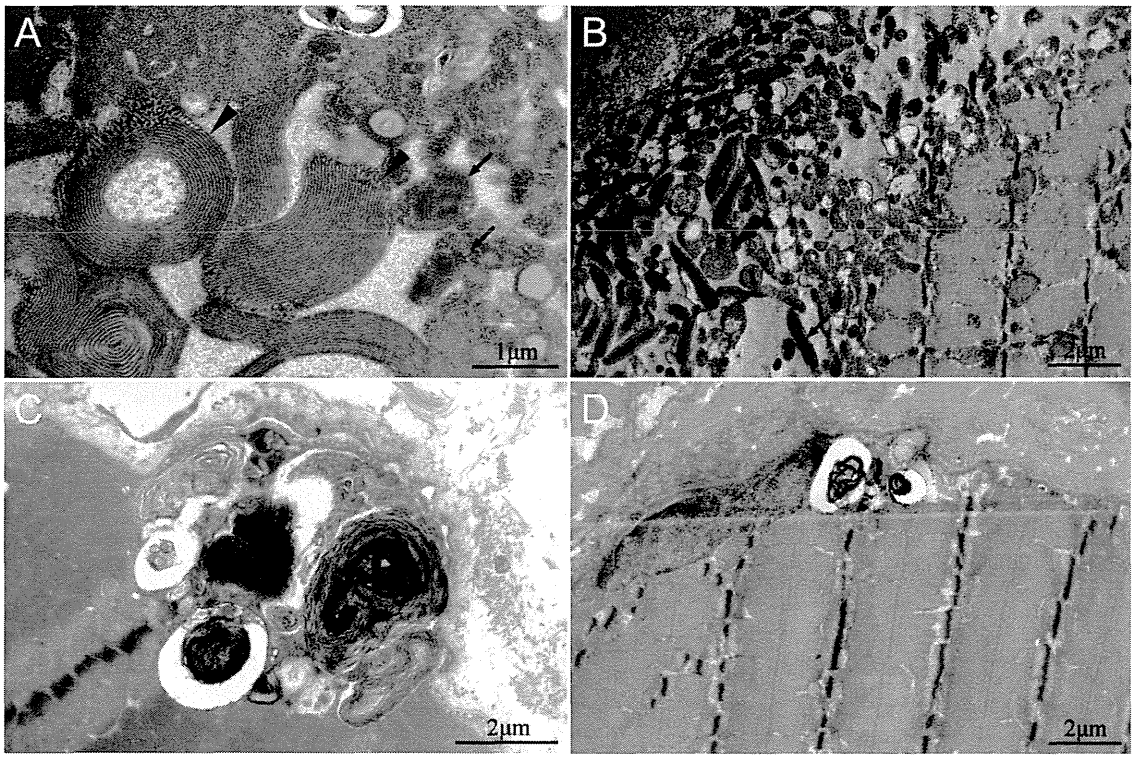

Figure 4 Electron microscopy of abnormal mitochondria and autophagic vacuoles in skeletal muscle tissue from patient 1 (A and C) and patient 2 (B and D). Significant abnormal mitochondrial proliferation is seen, with paracrystalline inclusions (↙) and circular arrangements of cristae (▲) (A and B). Some autophagic vacuoles have membranous whorls and myelin-like structures (C and D).

It has been reported that the $tRNA^{Lys}$ gene with the m.8344A>G mutation particularly lacks post-transcriptional modification of uridine at the first letter of the anticodon (the wobble position)[28]; mutant tRNAs without the wobble modification cannot fulfil their normal role as acceptor molecules in the translation process, which results in respiratory chain defects and also in impaired global protein synthesis and compromised mitochondrial translation products.[29 30] Cells with the m.8344A>G mutation were recently shown to have increased autophagic activity.[31] We therefore postulate that the singular devastating effect of the m.8344A>G mutation in the $tRNA^{Lys}$ gene is to induce active autophagy for the purpose of abnormal mitochondria removal.

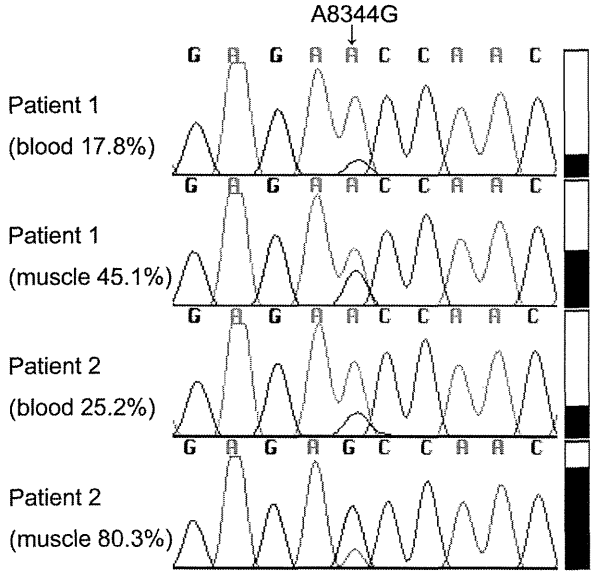

Figure 5 Sequence chromatograms and block diagrams of m.8344A>G mutation heteroplasmy in both blood and skeletal muscle DNA from the two patients. The mutation rate in blood and skeletal muscle was 17.8% and 45.1% in patient 1, respectively, and 25.2% and 80.3%, respectively, in patient 2. The arrow indicates the mutation site.

Mutations in mtDNA including m.8344A>G are always heteroplasmic, meaning that mutant and wild-type genomes coexist. The autophagic targeting of mitochondria adds to this heterogeneity.[32] The threshold for biochemical expression of a mtDNA mutation varies, depending on the mutation and the tissue involved. In the muscle fibres containing isolated RVs, the heteroplasmy of mutated G may be below the threshold required to cause a RRF, but trigger the autophagy pathway first. The GS Junior platform was used to confirm the heteroplasmy of 8344G, and the relatively low heteroplasmy level in blood may explain the predominantly skeletal muscle phenotype seen in the two patients. We recommend that patients suspected of having a mitochondrial disorder undergo genetic analysis of mtDNA, using muscle as the preferred tissue type.

In summary, we focused on a rare pathological phenomenon of coexistence of RRFs and isolated RVs, and identified the m.8344A>G mutation in two patients with atypical MERRF syndrome. This finding suggests that the distinctive pathogenesis results from the m.8344A>G mutation in both patients. The autophagy, always considered to be a secondary process after mitochondrial dysfunction, may work earlier than expected. Although the mechanism of detailed interaction between the m.8344A>G mutation and autophagy requires further investigation, these findings broaden the pathological phenotype of patients with the m.8344A>G mutation.

Take-home messages

- The interaction between autophagy and mitochondrial dysfunction remains unclear. The finding of isolated autophagic vacuoles in muscle fibres of patients with the m.8344A>G mutation, might be a starting point.
- The autophagic vacuoles, found in muscle fibres, might be associated with the atypical phenotype for a m.8344A>G mutation.
- The next-generation sequencing system might be reliable for detecting the heteroplasmy level of a mitochondrial DNA mutation.

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Contributors J-HY carried out genetic experiments and drafted the manuscript. YS, YH and AY participated in genetic experiments and revised the manuscript. IH designed the study, revised the manuscript and obtained funding. YI, KH and AH carried out pathological and electron microscopy study. HT designed and supervised the study, revised the manuscript and obtained funding.

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Competing interests None.

Ethics approval Institutional review board of Kagoshima University.

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CASE REPORT

Novel mutation in the replication focus targeting sequence domain of *DNMT1* causes hereditary sensory and autonomic neuropathy IE

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Abstract *DNMT1*, encoding DNA methyltransferase 1 (Dnmt1), is a critical enzyme which is mainly responsible for conversion of unmethylated DNA into hemimethylated DNA. To date, two phenotypes produced by *DNMT1* mutations have been reported, including hereditary sensory and autonomic neuropathy (HSAN) type IE with mutations in exon 20, and autosomal dominant cerebellar ataxia, deafness, and narcolepsy caused by mutations in exon 21. We report a sporadic case in a Japanese patient with loss of pain and vibration sense, chronic osteomyelitis, autonomic system dysfunctions, hearing loss, and mild dementia, but without definite cerebellar ataxia. Electrophysiological studies revealed absent sensory nerve action potential with nearly normal motor nerve conduction studies. Brain magnetic resonance imaging revealed mild diffuse cerebral and cerebellar atrophy. Using a next-generation sequencing system, 16 candidate genes were analyzed and a novel missense mutation, c.1706A>G (p.His569Arg), was identified in exon 21 of *DNMT1*. Our findings suggest that mutation in exon 21 of *DNMT1* may also produce a HSAN phenotype. Because all reported mutations of *DNMT1* are concentrated in exons 20 and 21, which encode the replication focus targeting sequence (RFTS) domain of Dnmt1, the RFTS domain could be a mutation hot spot.

Key words: DNA methyltransferase 1, *DNMT1*, hereditary sensory and autonomic neuropathy, missense mutation, next-generation sequencing

Introduction

DNA methyltransferase 1 (Dnmt1), encoded by *DNMT1*, is the principal enzyme responsible for the maintenance of cytosine methylation at cytosine–phosphate–guanine dinucleotides in the mammalian genome *(Feng and Fan, 2009)*, and is also crucial for gene regulation and chromatin stability *(Tohgi et al., 1999; Chen et al., 2003)*. Human Dnmt1 consists of a conserved C-terminal catalytic core and a large N-terminal region harboring multiple globular conserved domains, including the DNA methyltransferase-associated protein 1-binding domain, the proliferating cell nuclear antigen-binding domain, the replication focus targeting sequence (RFTS) domain, the CXXC domain, and two bromo-adjacent homology domains *(Syeda et al., 2011)*.

To date, eight kindreds with *DNMT1* mutations have been reported, half of which were characterized

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by sensory neuropathy, sensorineural hearing loss, and dementia, caused by mutations in exon 20 *(Klein et al., 2011)*; the other half presented with autosomal dominant cerebellar ataxia, deafness, and narcolepsy, and all mutations were located in exon 21 *(Winkelmann et al., 2012)*. It is noteworthy that all peptides coded by exon 20 and 21 belong to the RFTS domain of Dnmt1.

Using a next-generation sequencing (NGS) system, we screened a panel of candidate genes in a Japanese patient with sensory neuropathy, autonomic nervous system dysfunctions, sensorineural hearing loss, and slight dementia. This screen identified a novel missense mutation in exon 21 of *DNMT1*. We also reviewed all reported cases with *DNMT1* mutation and investigated the pathogenesis of various *DNMT1*-related phenotypes.

Case Report

The patient was a 41-year-old Japanese male from a non-consanguineous family (Fig. 1A). No neurological disorders were found in other family members. Pain perception began to decrease in his distal lower limbs after high school, and this condition progressed slowly. At the age of 30 and 32, after local infection, he had osteomyelitis in his first right toe and fifth left toe, respectively, and amputations were performed. Meanwhile, he began to experience hearing loss, and a hearing aid was used in the right ear. After his gait became unsteady he was referred to a department of neurology. Physical examination revealed foot ulcers and mutilations (Fig. 1B). His muscle strength was normal; however, a marked decrease was observed in his sense of pain and touch (1/10) in the lower limbs. Vibration perception was present in the fingers but absent in the lower limbs, and Romberg test was positive. Mild mental retardation was noted. Examination of the cranial nerves was normal except for bilateral hearing loss. Cerebellar function examination showed no abnormalities on finger-to-nose or heel-to-knee testing, or rapidly alternating pronation and supination of hands. Muscle tone was normal, without speech abnormality. The deep tendon reflexes of the lower limbs were absent. The pure tone audiometry test suggested moderate to severe bilateral sensorineural hearing loss. A 24-h Holter monitor indicated sinus bradycardia (43/min on average). Plain radiographs showed an amputation stump of the right hallux and left little toe, accompanied by bone destruction, cortical bone thickness or sclerosis, and an irregular articular surface (Figs. 1C and 1D). Brain magnetic resonance imaging (MRI) revealed mild diffuse cerebral and cerebellar atrophy (Fig. 1E). Clinical and radiological examinations revealed several saprodontia, bronchiectasis in the middle and lower lobe of the right lung, and accessory sinusitis.

In the electrophysiological study, except for the slight slowing of motor nerve conduction velocity in the right tibial nerve, motor nerve conduction studies were almost normal in the median, ulnar, and posterior tibial nerves. However, the sensory nerve action potential could not be evoked in the right median, ulnar, and sural nerves.

The protocol of the studies described below was reviewed and approved by the Institutional Review Board of Kagoshima University. The patient provided his written informed consent to participate in this study.

Methods and Results

Sixteen candidate genes, including 11 genes related to hereditary sensory and autonomic neuropathies (HSAN) and another 5 genes (Table 1) associated with sensory and autonomic dysfunctions were screened on the MiSeq sequencing system Illumina, San Diego, CA, USA).

After one run for 28 h, 400,122 (150×2) reads were generated for this patient on the NGS; 93.7% of the reads could be mapped to the reference genome and 98.1% of the target regions were covered at least 10 times. In 27 high-confidence variants, 24 known single nucleotide polymorphisms (SNPs) were coincident with the dbSNP (http://www.ncbi.nlm.nih.gov/snp/) or 1000 Genomes data (http://browser.1000genomes.org). Of the remaining three non-synonymous variants, c.3248A>C in *KIF1A* and c.3448T>C in *SCN9A* were also found in the normal control, and were thus considered SNPs. Besides, a heterozygous missense mutation, c.1706A>G (p.His569Arg) in exon 21 of the *DNMT1* gene (NM_001130823.1, NP_001124295.1) remained and was confirmed by Sanger sequencing (Figs. 2A and 2B). This mutation is located in a highly conserved domain among different species (Fig. 2C). Using the web-based programs, this His569Arg alteration was predicted to be pathogenic in POLYPHEN2 (0.982) and SIFT (0.00).

This mutation was not observed in 100 Japanese control samples, nor did we find it on the 1000 Genomes web site, which catalogs human genetic variations using 2,500 samples, including 500 East Asian (100 Japanese) samples.

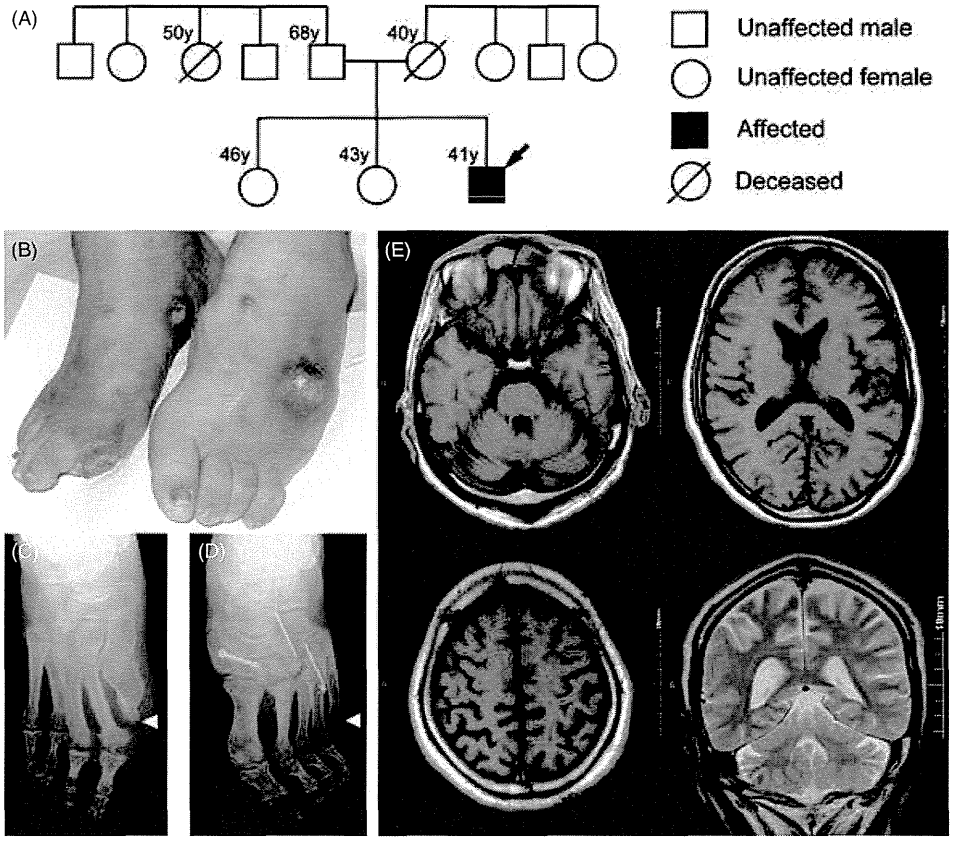

Figure 1. Pedigree and clinical photographs. (A) Pedigree of the patient. The arrow (→) indicates the index case. (B) Foot ulcers and mutilations. (C, D) Radiographs showing the amputation stump of the right hallux and left little toe (□) accompanied by chronic osteomyelitis. (E) Brain magnetic resonance imaging showing mild and diffuse cerebral and cerebellar atrophy.

Table 1. Candidate genes screened using Miseq sequencing system.

Gene symbol	Locus	Coding exons	Reference sequences
SPTLC1	9q22.31	15	ENST00000262554
SPTLC2	14q24.3	12	ENST00000216484
ATL1	14q11	14	ENST00000441560
DNMT1	19p13.2	41	ENST00000359526
WNK1	12p13.33	28	ENST00000537687
FAM134B	5p15.1	9	ENST00000306320
KIF1A	2q37.3	49	ENST00000498729
IKBKAP	9q31.3	37	ENST00000374647
NTRK1	1q23.1	16	ENST00000368196
NGF	1p13.2	3	ENST00000369512
DST	6p12.1	84	ENST00000244364
SCN9A	2q24.3	27	ENST00000303354
CCT5	5p15.2	11	ENST00000280326
PRNP	20p13	2	ENST00000379440
FLVCR1	1q32.3	10	ENST00000366971
RNF170	8p11.21	7	ENST00000527424

Discussion

We report a Japanese patient with suspected HSAN. Using a high-throughput NGS system, we established a diagnostic procedure involving screening of 16 candidate genes in one run, and identified a novel missense mutation in exon 21 of *DNMT1*.

In 2011, *DNMT1*-related dementia, deafness, and sensory neuropathy was demonstrated in four kindreds from America, Europe, and Japan and was designated HSAN IE. It is an autosomal dominant degenerative disorder of the central and peripheral nervous systems characterized by sensory impairment, sudomotor dysfunction (loss of sweating), dementia, and sensorineural hearing loss. Affected individuals are normal in their youth but begin to manifest progressive sensorineural deafness and sensory neuropathy by the age of 20–35 *(Klein et al., 2011)*. In 2012, another four kindreds from Europe were found to have early onset (18–44 years) of a narcolepsy/cataplexy syndrome followed by ataxia, deafness, sensory neuropathy, and memory loss, which was reported to be associated with *DNMT1* mutations *(Winkelmann et al., 2012)*.

The present patient was normal until graduation from senior high school, but began to manifest progressive inability to perceive pain and experienced painless osteomyelitis. Deafness, as the second symptom, started after the age of 30, and an examination

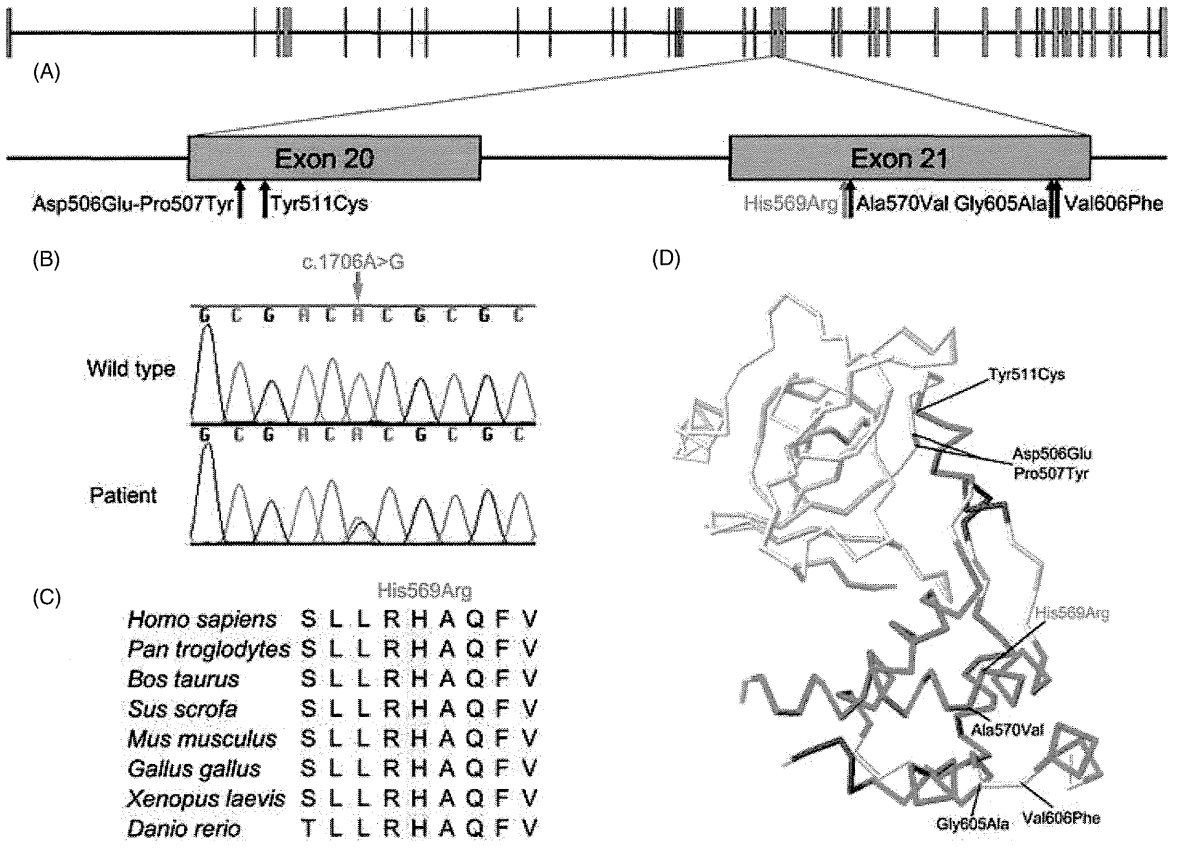

Figure 2. Genetic studies and mutations review. (A) Schematic overview and all mutations in exons 20 and 21 of *DNMT1*. (B) Sequencing chromatogram of the c.1706A>G mutation. Red arrow indicates the mutation site in present patient. (C) Amino acid sequence at the mutation site in homologs of DNA methyltransferase 1 aligned by CLUSTALW. A yellow bar indicates the highly conserved histidine at position 569. (D) Location of mutated residues in the crystal structure of the replication focus targeting sequence domain (Protein Data Bank accession number 3EPZ, showing residues 367–616 in NP_001124295.1).

revealed bilateral sensorineural hearing loss. His gait became ataxic and there was no vibration perception in the lower limbs. Although cerebellar atrophy was revealed by the brain MRI, no definite cerebellar dysfunction was identified. After examination, his ataxia was considered mainly due to loss of deep sensation. The electrophysiological studies revealed sensory dominant axonal polyneuropathy. In addition, mental retardation was observed by the neurologist and diffuse cerebral and cerebellar atrophy was noted in the brain MRI. All these findings were consistent with HSAN IE. The patient's sinus bradycardia and other dysfunctions of the respiratory system might have resulted from the autonomic nervous system dysfunction, but no reliable test was performed to check his autonomic nervous function.

Using the MiSeq sequencing system, a heterozygous missense mutation, c.1706A>G (p.His569Arg), was identified in exon 21 of *DNMT1*. As the present patient showed a definite HSAN phenotype, our findings indicated that the variable *DNMT1*-related phenotype was unlikely to have been determined by the location of the mutation. Although no narcolepsy/cataplexy was noted either in our case or the original four kindreds, the mechanism underlying the varied phenotypes requires further investigation.

It is noteworthy that all reported mutations of *DNMT1* were located in exon 20 (*Klein et al., 2011*: p.Asp506Glu-Pro507Tyr, p.Tyr511Cys; NP_001124295.1) and exon 21 (*Winkelmann et al., 2012*: p.Ala570Val, p.Gly605Ala, p.Val606Phe; NP_001124295.1), and that the original presentations were sensory neuropathy and narcolepsy/cataplexy syndrome, respectively. In Dnmt1, all the peptides encoded by exons 20 and 21 belong to the RFTS domain (Fig. 2D). Previous research indicated that this RFTS domain, inserted deeply into the DNA-binding pocket *(Takeshita et al., 2011)*, contributes to the inhibition of Dnmt1 binding to naked DNA oligonucleotides and native polynucleosomes *(Syeda et al., 2011)*. The RFTS domain also contains a binding site for Uhrf1 *(Achour et al., 2008)*, which recognizes and binds to the hemimethylation sites of DNA and recruits Dnmt1 *(Bostick et al., 2007; Arita et al., 2008;*

Avvakumov et al., 2008). Mutations in exon 20 and 21 of *DNMT1* would transform the structure of the RFTS domain and affect the recognition and binding procedure of hemimethylated DNA, creating abnormal methylation and gene silencing. On the basis of our findings and previous studies, we surmise that the RFTS domain is a mutation hot spot compared with the other Dnmt1 domains. The other possibility is that mutation in other functional domains might cause global genome demethylation and embryonic lethality.

In conclusion, using a MiSeq sequencing system we identified a novel missense mutation in exon 21 of *DNMT1* in a Japanese patient with the typical HSAN IE phenotype. We also reviewed all eight of the kindreds with *DNMT1* mutations in previous reports, and excluded the presumption that varied phenotypes were generated by mutations in different exons. However, further research is required to elucidate the mechanisms of alterations in the RFTS domain and their influence on the DNA methylation procedure.

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ORIGINAL ARTICLE

Vincristine exacerbates asymptomatic Charcot–Marie–Tooth disease with a novel *EGR2* mutation

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Abstract Neurotoxicity is a common side effect of vincristine (VCR) treatment. Severe exacerbations of neuropathy have been reported in patients with Charcot–Marie–Tooth disease (CMT) 1A with duplication of the *peripheral myelin protein 22* (*PMP22*) gene. However, whether or not VCR exacerbates neuropathies through mutations in other CMT-associated genes besides *PMP22* duplication has not been well studied. The purpose of this study was to identify mutations in any CMT-associated genes in a patient with hypersensitivity to VCR. We performed clinical, electrophysiological, and genetic examinations of a 23-year-old woman, who was hypersensitive to low-dose VCR, and her healthy mother. DNA analysis was performed using our specially designed resequencing array that simultaneously screens for 28 CMT-associated genes. Electrophysiological studies revealed that the patient and her healthy mother had demyelinating polyneuropathy. Furthermore, they showed the same novel mutation in the *early growth response 2* (*EGR2*) gene. Recognizing pre-existing asymptomatic CMT by electrophysiological studies and genetic analysis before VCR treatment allowed us to prevent severe VCR-induced neuropathy.

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Introduction

Vincristine (VCR) is a vinca alkaloid drug that is an essential part of the chemotherapeutic regimens used to treat Hodgkin's and non-Hodgkin's lymphoma, acute lymphocytic leukemia, and several types of solid tumors. Neurotoxicity, the most frequently predominant distal axonal sensorimotor neuropathy, is a well-known dose-limiting side effect of VCR [1]. VCR disrupts microtubule functions in axons and inhibits axonal transport by binding and inactivating tubulin, thereby leading to axonal degeneration. VCR-induced neuropathy is usually observed after cumulative doses of 6–8 mg of VCR, and significant toxicity occurs at doses greater than 15–20 mg in neurologically normal individuals [2]. The symptoms of toxicity usually include paresthesia and muscle weakness in the distal extremities. Deep tendon reflexes often diminish or disappear. In most cases, neuropathy gradually improves as VCR is discontinued, but neuropathy can persist in some cases of severe sensorimotor dysfunction. Patients with pre-existing neuropathy are generally at increased risk of developing severe neuropathy after chemotherapy [2, 3]. Charcot–Marie–Tooth disease (CMT), a hereditary motor and sensory neuropathy, is one of the most common types of inherited neuropathies, with a prevalence rate of 1 in 2,500 [4], and it is clinically and genetically heterogeneous [5]. Until date, at least 30 genes are known to be associated with CMT and related inherited neuropathies (http://www.molgen.ua.ac.be/CMTMutations/Mutations). The most common type is CMT1A, which is an autosomal dominant demyelinating neuropathy associated with duplication of the *peripheral*

myelin protein 22 (*PMP22*) gene. Some anticancer drugs such as vinca alkaloids, platinum agents, taxanes, and thalidomide are potentially toxic to patients with CMT [3, 6]. There are many reports of cases of CMT1A that deteriorated or were revealed after VCR treatment [7–12]. However, whether or not VCR exacerbates neuropathies in other types of CMT besides CMT1A is unclear. There is insufficient data to comment on the neurotoxicity of VCR in less common subtypes of CMT that affect other genes [13–15]. In order to identify the genetic risk of severe VCR-induced neuropathy, we screened for mutations in 28 CMT disease-causing genes using a custom resequencing DNA chip. Our DNA chip can screen 28 genes in 2 days and is relatively cost-effective. Using this chip, we identified a mutation in the *early growth response 2* (*EGR2*) gene in a 23-year-old woman with hypersensitivity to low-dose VCR. *EGR2* encodes a transcription factor that regulates the expression of peripheral myelin protein genes [16]. Although the risk of VCR-induced neuropathy in patients with an *EGR2* mutation is unknown, our high-throughput mutation screening method revealed a novel risk of developing drug-induced neuropathy.

Materials and methods

Patient

A 23-year-old woman was referred to our hospital with primary mediastinal large B-cell lymphoma. She presented no subjective clinical symptoms except mediastinal lymphadenopathies and was diagnosed with clinical stage IA (Ann Arbor Classification). At that time, she had not developed any neurological abnormalities. Her family seemed healthy and had no history of inherited or acquired neuropathies. She was treated with chemotherapy following the administration of rituximab, cyclophosphamide, doxorubicin, VCR, and prednisolone (day 1, 750 mg/m^2 cyclophosphamide, 50 mg/m^2 adriamycin, 1.4 mg/m^2 VCR; days 1–5, 100 mg prednisolone; and day 5, 375 mg/m^2 rituximab). After two courses (total VCR administered, 3.9 mg), she developed muscular weakness and paresthesia with pain in the distal extremities and was hardly able to walk. On day 49, she demonstrated distal predominant muscular weakness and paresthesia on neurological examination. No obvious muscular atrophy or pes cavus was evident. In addition, she had developed areflexia. Her Babinski reflex was negative, and there were no signs of cerebellar or cranial nerve disturbances.

Electrophysiological studies

On day 54, nerve conduction studies were performed using the standard procedure. Skin temperature was maintained above 32°C.

DNA analysis

Genomic DNA was extracted from the peripheral blood leukocytes of the patient using the Gentra Puregene Blood Kit (Qiagen, Tokyo, Japan). The purpose-built GeneChip® CustomSeq® Resequencing Array (Affymetrix, Santa Clara, CA) was designed to screen for CMT and related diseases such as ataxia with oculomotor apraxia type 1, ataxia with oculomotor apraxia type 2, spinocerebellar ataxia with axonal neuropathy type 1, and hereditary motor neuropathies. The resequencing array was designed to screen for the following 28 genes: *EGR2*, *PMP22*, *myelin protein zero* (*MPZ*), *gap junction protein beta 1* (*GJB1*), *periaxin* (*PRX*), *lipopolysaccharide-induced TNF factor* (*LITAF*), *neurofilament light polypeptide* (*NEFL*), *ganglioside-induced differentiation associated protein 1* (*GDAP1*), *myotubularin-related protein 2* (*MTMR2*), *SH3 domain and tetratricopeptide repeats 2* (*SH3TC2*), *SET-binding factor 2* (*SBF2*), *N-myc downstream regulated 1* (*NDRG1*), *mitofusin 2* (*MFN2*), *rab-protein 7* (*RAB7*), *glycyl-tRNA synthetase* (*GARS*), *heat shock 27 kDa protein 1* (*HSPB1*), *heat shock 22 kDa protein 8* (*HSPB8*), *lamin A/C* (*LMNA*), *dynamin 2* (*DNM2*), *tyrosyl-tRNA synthetase* (*YARS*), *alanyl-tRNA synthetase* (*AARS*), *lysyl-tRNA synthetase* (*KARS*), *aprataxin* (*APTX*), *senataxin* (*SETX*), *tyrosyl-DNA phosphodiesterase 1* (*TDP1*), *desert hedgehog* (*DHH*), *gigaxonin 1* (*GAN1*), and *K-Cl cotransporter family 3* (*KCC3*). We designed 363 primer sets to cover all the coding exons and splice sites. The 363 polymerase chain reactions (PCRs) were amplified in 32 multiplex reactions using the Qiagen Multiplex PCR system (Qiagen). Each reaction used 120 ng of genomic DNA, 10 pmol of the primer set, dNTP, and the Qiagen Multiplex PCR reaction mix (Qiagen). We generated each multiplex PCR product using the following conditions: 15 min at 95°C; 42 cycles of amplification (94°C for 30 s, 60°C for 3 min, and 72°C for 1 min 30 s); and 15 min at 68°C. Pooling, DNA fragmentation, labeling, and chip hybridization were performed using the Affymetrix CustomSeq Resequencing protocol instructions. The chips were washed using the Affymetrix fluidics station using the Customseq Resequencing wash protocols. Analysis of microarray data was performed using the GeneChip sequence Analysis Software version 4.0 (Affymetrix).

The mutations detected by our DNA chip method were confirmed by conventional DNA Sanger sequencing. Briefly, we amplified 50 ng of the patient's genomic DNA using primers and the hot start PCR method. Using a presequencing kit (USB, Cleveland, OH), we purified the patient's PCR products detected using our resequencing array method and sequenced them by dye–primer chemistry using an ABI Prism 377 Sequencer (Applied Biosystems, Foster City, CA). We then aligned the resulting sequences and evaluated the mutations using the Sequencher sequence alignment program (Gene Codes, Ann Arbor, MI).

Results

Electrophysiological studies

The motor nerve conduction studies revealed moderately slow motor nerve conduction velocities (MCV) with reduced compound muscle action potential (CMAP) amplitude in all examined nerves. The sensory nerve conduction studies showed moderately slow sensory nerve conduction velocities (SCV) with slightly reduced sensory nerve action potential (SNAP) amplitude (Table 1). No temporal dispersions or conduction blocks were observed. These results suggest demyelinating polyneuropathy complicated by axonal sensorimotor polyneuropathy. Because the patient showed hypersensitivity to low-dose VCR (total VCR administered, 3.9 mg), we suspected a pre-existing, inherited neuropathy. Furthermore, electrophysiological studies were performed on her healthy, 51-year-old mother. MCV of the mother was slower in the lower extremities than the upper extremities. CMAP amplitudes were within normal limits. Median nerve distal latency was slightly prolonged. SCV was moderately slow, but this finding was uniform in all examined nerves. SNAP amplitudes were moderately reduced in the upper extremities; SNAP amplitude of the sural nerve was at the lower limit of our normal control data. Temporal dispersions, conduction blocks, and entrapment neuropathies were not observed. These results indicate an electrophysiologically mild demyelinating polyneuropathy (Table 1). These findings suggest that this family may have an inherited demyelinating polyneuropathy.

Resequencing analysis of this family and a control study

The DNA chip resequencing analysis detected a novel c.1057 C>G (p.R353G) missense mutation in the *EGR2* gene. In contrast, the analysis was negative for mutations involving the other 27 CMT or related disease-causing genes. The patient was heterozygous for the c.1057 C>G mutation that substitutes an arginine for glycine at amino acid 353 (p.R353G) in exon 2 of *EGR2* by conceptual translation (Fig. 1a). The mother had the same mutation as the patient (Fig. 1a). We did not observe R353G in 200 control chromosomes or in the 850 chromosomes from 425 patients with inherited neuropathy. In addition, we did not find the R353G mutation in the 1000 Genomes website (http://browser.1000genomes.org), which catalogs human genetic variations using 1,197 samples including 300 East Asian (100 Japanese) samples.

Clinical course of the patient

We changed the chemotherapy regimen after we suspected that the patient had CMT. We chose radiotherapy and rituximab for the treatment of B-cell lymphoma. After 2 months, her symptoms had almost recovered, and she walked normally with only mild numbness in her distal lower limbs.

However, it was difficult to trace the causal agent because she was treated with a combination of chemotherapy agents. According to a previous report [3], there is uncertainty about the neurotoxicity of cyclophosphamide, prednisolone, and rituximab in patients with CMT, while VCR is classified as high risk for such patients. Furthermore, she and her mother's electrophysiological findings were consistent with inherited demyelinating polyneuropathy without the presence of conduction block or temporal dispersion. There were no findings indicated other inherited demyelinating polyneuropathy such as disturbance of lipid metabolism, peroxisomal disorders, hepatic porphyria and amyloidosis besides CMT. The results of her laboratory studies, including liver function tests, renal function tests, serum electrolyte and fasting blood glucose were normal. Her mother was healthy in the past periodic medical checkup, but laboratory

Table 1 Results of the nerve conduction studies

	Nerve	DL (ms)	CMAP amplitude (mV)	MCV (m/s)	SNAP amplitude (μV)	SCV (m/s)
Patient	Median	4.3	1.5	26.9	6.7	45.1
	Ulnar	3.9	2.7	31.8	7.3	45.8
	Tibial	8.2	3.9	23.0	–	–
	Sural	–	–	–	4.2	33.3
Patient's mother	Median	5.0	11.2	44.6	3.9	39.7
	Ulnar	3.3	9.1	50.1	3.1	38.7
	Tibial	4.7	23.6	37.9	–	–
	Sural	–	–	–	5.2	37.6
Control	Median	<4.5	>3.1	>49.6	>7.0	>47.2
	Ulnar	<3.6	>6.0	>50.1	>6.9	>46.9
	Tibial	<5.7	>4.4	>41.7	–	–
	Sural	–	–	–	>5.0	>40.8

DL distal latency, *CMAP* compound muscle action potential, *MCV* motor conduction velocity *SNAP* sensory nerve action potential, *SCV* sensory conduction velocity

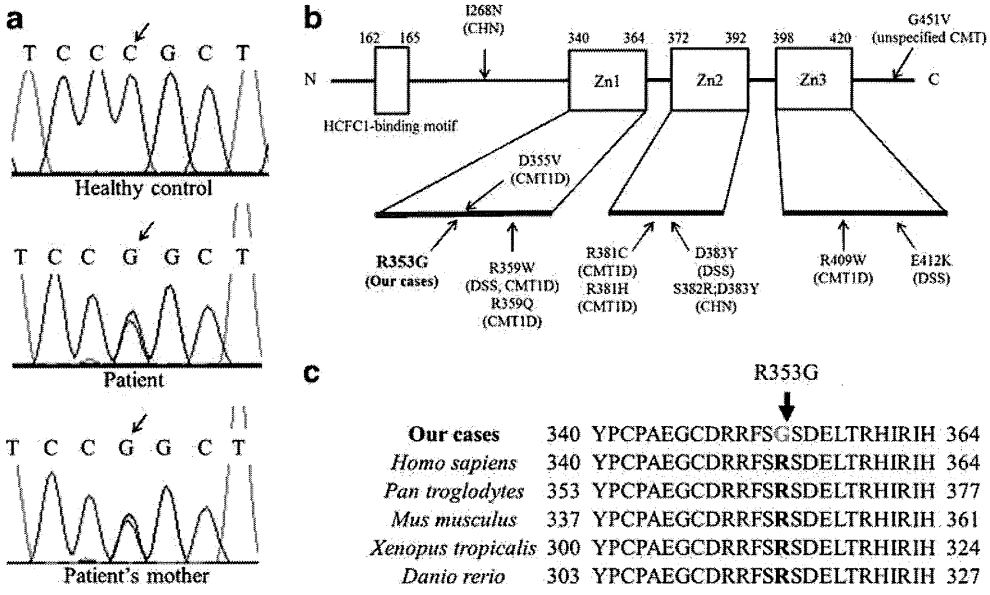

Fig. 1 **a** Chromatograms of the alterations in the *early growth response 2* (*EGR2*) gene that was identified in the patient and her mother, both of whom had the heterozygous transition c.1057 C>G that resulted in R353G. **b** Schematic diagram of the *EGR2* showing previously reported mutations and the R353G alteration. *CHN* congenital hypomyelination neuropathy, *DSS* Dejerine–Sottas disease, *Zn* zinc-finger domains. **c** Comparison of *EGR2* mutations in different species

screening tests were not examined in this report. We strongly suspected VCR-induced neuropathy in CMT with the *EGR2* mutation.

Discussion

This is the first report to describe an *EGR2* mutation that induced VCR hypersensitivity, similar to *PMP22* duplication. The *EGR2* gene located on human chromosome 10q21.1 has two exons that encode a 476 amino acid protein with three zinc finger domains, which is believed to be a transcription factor that regulates myelinogenesis [17, 18]. *EGR2* knockout mice exhibit severe hypomyelination of peripheral nerves due to the blocking of Schwann cell differentiation [19, 20]. Heterozygous mutations in *EGR2* cause myelinopathies, including congenital hypomyelinating neuropathy, Dejerine–Sottas disease, and mild to severe CMT1 [21–26]. Until date, 17 types of *EGR2* mutation have been found (http://www.molgen.ua.ac.be/CMTMutations/Mutations). *EGR2* induces high expression levels of myelin protein components such as *PMP22*, *MPZ*, *DHH*, and *PRX* in Schwann cells [27–30]. Vincristine inhibits axonal transport; thus, an insufficient supply of the myelin protein component necessary for the increased demand created by vincristine may induce a large degree of neurotoxicity. In the present study, we showed a novel R353G mutation in the first zinc finger domain of *EGR2* in a patient with late onset CMT1 who presented with

Table 2 Computational predictions of the pathogenicity on EGR2 mutation within the zinc finger domain

	Mutation	MUPro (SVM score[a])	PolyPhen[b]	PolyPhen2[c]	SIFT[d]
Our patients	R353G	−0.43[e]	2.57[e]	0.90[e]	0.00[e]
Reported mutations	D355V	1.00	2.75[e]	0.97[e]	0.00[e]
	R359W	−0.64[e]	2.79[e]	1.00[e]	0.00[e]
	R359Q	−1.00[e]	1.89[e]	0.92[e]	0.00[e]
	R381C	−0.11[e]	2.79[e]	0.99[e]	0.00[e]
	R381H	−0.24[e]	2.12[e]	0.99[e]	0.00[e]
	S382R	0.35	2.06[e]	0.81[e]	0.00[e]
	D383Y	0.09	2.75[e]	0.99[e]	0.00[e]
	R409W	−0.98[e]	2.69[e]	1.00[e]	0.00[e]
	E412K	−1.00[e]	1.69[e]	0.77[e]	0.00[e]

[a] Support Vector Machine (SVM) scores <0 indicate a decrease in protein stability

[b] PolyPhen scores ≥1.5 indicates a prediction of pathogenic

[c] PolyPhen2 scores of ~1 indicate a prediction of pathogenic

[d] SIFT scores ≤0.05 indicate a prediction of pathogenic

[e] Denotes a pathogenic prediction

a very mild phenotypic expression. Most *EGR2* mutations within the first zinc finger domain cause Dejerine–Sottas disease or severe CMT1 phenotypes (Fig. 1b) [22, 24]. A sequence homology search was performed, which aligned protein sequences from multiple species, using a Constraint-based, Multiple-Alignment tool (COBALT) (http://www.ncbi.nlm.nih.gov/tools/cobalt/). Arginine 353 was conserved among all of the species analyzed (Fig. 1c). It was found that the R353G mutation identified in our patients was located in a remarkably well-conserved sequence of amino acids, suggesting that it may have a potential impact on *EGR2* function. Furthermore, we computationally predicted the effect of the R353G mutation on protein function using the MUpro (http://www.ics.uci.edu/~baldig/mutation.html), PolyPhen (http://genetics.bwh.harvard.edu/pph/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html) algorithms. The algorithms in these programs use evolutionarily conserved species as well as reference sequence alignments, physiochemical differences, and the proximity of various substitutions to predict functional domains and/or structural features. All these programs predicted that the R353G mutation is most likely pathogen-based on the degree of conservation of the affected residues (Table 2). Therefore, the R353G mutation could possibly disrupt various functions. Furthermore, different mutations in the same codon result in divergent CMT phenotypes [26]. The electrophysiological findings were the only abnormal results for the patient's asymptomatic mother with the same *EGR2* mutation. Her neurological findings were normal, including a normal handgrip, the absence of foot deformities, normal and prompt deep tendon reflexes, and normal sensations. It is difficult to diagnose late onset mild CMT based on clinical findings and family history because the disease is heterogeneous. Although we did not perform in vitro functional analysis of the R353G mutation in this study, such further functional studies would illuminate the details of the pathomechanism of the *EGR2* mutation and its relationship with vincristine toxicity in this patient. In order to clarify the pathogenic nature of the *EGR2* mutation and vincristine neurotoxicity, we need to continue the genetic analysis of vincristine-induced neuropathy patients who do not show the CMT phenotype.

VCR-induced neuropathy is a dose-limiting side effect observed in neurologically normal individuals, but it sometimes results in severe neuropathy in patients with CMT. Early recognition of CMT before VCR treatment can prevent severe neurotoxicity. It is very important to use electrophysiological studies to recognize pre-existing CMT before VCR treatment, even if there is no family history or neurological abnormalities. Moreover, the labor and reagent costs of molecular genetic testing have significantly increased along with the increase in the number of genes associated with CMT and related neuropathies that must be screened for mutations. Realistically, it is difficult to perform nerve conduction studies or genetic testing in all patients who receive chemotherapy because of the costs and effort. Because of recent progress in the development of a new generation of genomic sequencing technologies, it will be possible to screen the entire genome/exome sequence for potential risks in all patients before they undergo chemotherapy.

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CHARCOT-MARIE-TOOTH DISEASE TYPE 4C IN JAPAN: REPORT OF A CASE

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ABSTRACT: *Introduction:* The distribution of documented cases of Charcot-Marie-Tooth disease type 4C (CMT4C) is mainly limited to the Mediterranean region. We report the first documented case of CMT4C in East Asia. Furthermore, we estimate the proportion of CMT4C in Japan and compare the same with that in European countries. *Case Report:* A 72-year-old Japanese woman presented with early-onset motor and sensory neuropathy associated with scoliosis, deformities of the hands and feet, and carpal tunnel syndrome. A genetic screen detected a homozygous p.R529Q mutation in *SH3TC2*, the causative gene of CMT4C. The *SH3TC2* mutation identified here is unique among 426 unrelated Japanese CMT patients, excluding those with CMT1A. *Conclusions:* Although CMT4C also occurs in Japan, it is less common than in European countries.

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Charcot-Marie-Tooth (CMT) disease is the most common inherited motor and sensory neuropathy.[1] CMT is usually inherited as an autosomal dominant trait and is subdivided into a demyelinating form (CMT type 1) and an axonal form (CMT type 2).[2] At present, gene analysis can identify mutations in approximately 50% of CMT cases.[3,4] The most common type of CMT, CMT type 1A (CMT1A), is an autosomal dominant demyelinating neuropathy with duplication of peripheral myelin protein 22 (PMP22).[4,5] The proportion of CMT1A cases relative to the total CMT or CMT1 population reported in East Asia is estimated to be lower than that reported in Europe and the United States.[3,6]

CMT type 4 (CMT4) is an autosomal recessive demyelinating form of CMT. One genetic subtype of CMT4, CMT type 4C (CMT4C), is linked to a locus on chromosome 5q23–33[7–9] and is characterized clinically by demyelinating peripheral neuropathy frequently associated with spinal deformities.[10]

Abbreviations: ABR, auditory brainstem response; CMT, Charcot-Marie-Tooth disease; CMT1A, Charcot-Marie-Tooth disease type 1A; CMT4, Charcot-Marie-Tooth disease type 4; CMT4C, Charcot-Marie-Tooth disease type 4C; MUP, motor unit potential; NCS, nerve conduction study; NEE, needle electromyographic examination; PMP22, peripheral myelin protein 22; SNP, single nucleotide polymorphism

Key words: autosomal recessive inheritance; elderly; hereditary neuropathy; Charcot-Marie-Tooth disease type 4C; SH3TC2 gene

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The gene implicated as the cause of CMT4C, *SH3TC2* (*KIAA1985*), was identified in 2003.[11] The gene product, SH3TC2, is a small protein with 2 Src homology 3 domains and 10 tetratricopeptide repeat motifs and is expressed specifically in Schwann cells. *SH3TC2* knockout mice develop progressive peripheral neuropathy with reduced nerve conduction velocity and hypomyelination.[12]

Of interest, the distribution of documented cases of CMT4C is limited mainly to Europe,[8–11,13–20] French Canada,[21] North Africa,[7,9,10] and West Asia.[8,11,14,18,22] Although variable prevalence of CMT4C in different ethnic groups may explain this distribution, the evidence for this proposal is inconclusive.

Here, we report an elderly Japanese patient with CMT4C. We also estimate the proportion of CMT4C in Japanese CMT, in comparison with other regions mentioned above.

CASE REPORT

A 72-year-old Japanese woman of consanguineous parentage complained of nocturnal numbness in her hands for several years and was examined in our hospital. Her parents were first cousins. Her history included hepatitis C, hypertension, and ovarian cystoma. She consumed neither alcohol nor any drug that could have potentially caused the neurological symptoms. Furthermore, she had no family history associated with peripheral neuropathy. She had been living in a small village in the Chiba prefecture (near Tokyo) where she could trace her ancestry for over 13 generations.

Careful historical analysis revealed several unique events. She was a slower runner than her peers in childhood, and she developed scoliosis in high school. She observed a deformity in her hands at the age of 20 years. Because of gait instability, she developed bone fractures in her ankles at the ages of 56 (on the right) and 67 years (on the left). It became challenging to walk up a flight of stairs without a banister by her middle sixties, and she has required a cane for walking since her

Table 1. Nerve conduction studies.

Nerve stimulated (left side)	Stimulation site	Recording site	Amplitude* (NL)	Latency (ms) (NL)	Conduction velocity (m/s) (NL)	F-Wave latency (ms) (NL)
Median (m)	Wrist	APB	2.1 (≥ 6.0)	17.0 (≤ 4.0)		n.e.
	Elbow	APB	2.0 (≥ 6.0)	24.4	27 (≥ 50)	
Ulnar (m)	Wrist	ADM	8.3 (≥ 8.0)	7.4 (≤ 3.4)		n.e.
	Below elbow	ADM	7.6 (≥ 8.0)	13.0	32 (≥ 50)	
	Above elbow	ADM	7.3 (≥ 8.0)	15.0	25 (≥ 36)	
Tibial (m)	Ankle	AH	0.68 (≥ 10.0)	9.6 (≤ 5.0)		84.2 (≤ 51.0)
	Popliteal space	AH	0.71 (≥ 8.0)	22.0	28 (≥ 45)	
Median (s)	Index finger	Wrist	0.70 (≥ 10.0)	8.74 (≤ 2.40)	14 (≥ 50)	
Ulnar (s)	Small finger	Wrist	1.30 (≥ 8.0)	3.52 (≤ 2.30)	30 (≥ 48)	
Sural (s)	Calf	Posterior ankle	2.10 (≥ 8.0)	4.32 (≤ 3.30)	35 (≥ 45)	
Facial (m)	Stylomastoid foramen	Nasalis	1.0 (≥ 1.0)	4.6 (≤ 3.0)		

The F-wave latency represents the minimum F-wave latency.
**Amplitude: motor (in millivolts); sensory (in microvolts).*
m, motor study; s, sensory study; NL, normal; APB, abductor pollicis brevis; ADM, abductor digiti minimi; AH, abductor hallucis; n.e., not elicited.

late sixties. She observed foot deformities at the age of 69 years. She had observed nocturnal numbness of the hands.

Neurological examination revealed the following: hypoacusia; mild, symmetrical distal muscle weakness and atrophy of limbs; positive bilateral Tinel and Phalen signs at the wrists; areflexia; distal dominant hyperalgesia; diminished vibratory sensation (especially prominent in the legs); Romberg sign; and gait instability. Scoliosis and deformities of the hands (claw hand) and feet (pes planus) were also observed.

Electrophysiological studies yielded several significant findings. In a nerve conduction study (NCS), relatively diffuse and homogeneous slowing of conduction velocity without conduction block, so called "uniform conduction slowing," was observed (Table 1). In median NCS, distal latencies of motor and sensory nerves were more prolonged than the distal latencies of other nerves. This focal conduction delay was presumably caused by concomitant carpal tunnel syndrome. Needle electromyographic examination (NEE) was performed in the biceps brachii, extensor carpi ulnaris, vastus medialis, and tibialis anterior muscles. All muscles showed marked high-amplitude and long-duration motor unit potentials (MUPs) with reduced MUP recruitment. Fibrillation potentials were observed in the tibialis anterior muscle. These NEE findings reflected chronic denervation and reinnervation. The blink reflex showed prolonged R1 and R2 responses, and auditory brainstem response (ABR) analysis revealed diminished wave I with delayed wave III (latencies were 5.2 ms on the left and 5.3 ms on the right; normal < 4.3 ms) and wave V (latencies were 7.0 ms on the left and 7.6 ms on the right; normal < 6.4 ms) bilaterally, suggesting cranial nerve involvement.

Because uniform slowing of conduction velocities in NCS suggested the existence of an inherited peripheral neuropathy,[23–25] we analyzed mutations associated with CMT after obtaining informed consent. First, we confirmed that the subject was negative for *PMP22* duplication or deletion by fluorescence *in situ* hybridization, eliminating CMT1A or hereditary neuropathy with liability to pressure palsies as the relevant condition. Subsequently, a re-sequencing method was performed, using GeneChip® CustomSeq® Resequencing Array (Affymetrix, Santa Clara, CA) equipped with 28 genes, including *SH3TC2*, as we previously reported.[26]

Variants c.1586G>A and c.1587T>G were observed in the *SH3TC2* gene (Fig. 1). The former variant is a rare mutation reported previously in Turkish children[11] and a middle-aged woman in France,[20] and the latter variant is a single nucleotide polymorphism (SNP). These variants caused a homozygous p.R529Q mutation, therefore, the patient was diagnosed with CMT4C. Pathological investigation was not performed, because the subject refused nerve biopsy.

Intriguingly, the *SH3TC2* mutation identified here is unique among 427 unrelated Japanese CMT cases previously investigated. The details of the 427 cases are as follows: (1) duplication of *PMP22* causing CMT1A was previously excluded; (2) the number of subjects with autosomal inherited demyelinating CMT (motor nerve conduction velocity < 38 m/s), autosomal inherited axonal CMT, X-linked CMT, and unclassified CMT was 125, 182, 26, and 94, respectively; (3) 100 subjects with CMT1 and 25 subjects with CMT4 were included in the autosomal inherited demyelinating CMT category; (4) 1 subject was CMT1A due to missense mutation of *PMP22*.

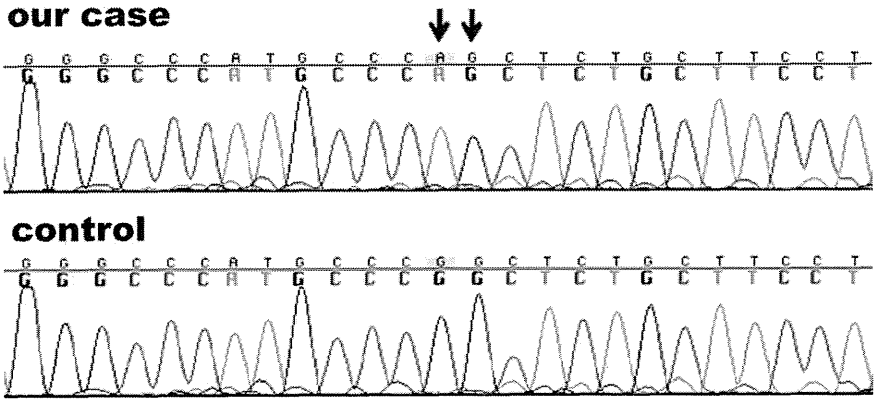

FIGURE 1. Sequence analysis showing c.1586G>A and c.1587T>G (p.R529Q, exon 11, arrow). Note that control data also show a single nucleotide polymorphism (SNP) at c.1587T>G. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

This report describes the first documented case of CMT4C in East Asia in a patient with an inherited peripheral neuropathy concurrent with scoliosis, hand and foot deformities, and carpal tunnel syndrome. DNA chip screening for the CMT causative gene detected a rare p.R529Q homozygous variant, resulting from mutations in the *SH3TC2* gene.

Mutations in *SH3TC2* tend to differ among ethnic groups, suggesting a founder effect. The mutation p.R954X appears most frequently in the Czech population[19] and in a French-Canadian cluster.[21] In contrast, the p.R1109X variant predominates in Gypsies.[13–15] Before the case of our Japanese patient, the p.R529Q mutation had been identified only in Turkish children[11] and a patient in France (the national origin was not described in the report).[20] Because the p.R529Q mutations arose from these entirely distinct ethnic and racial populations, the mutations may have distinct ancestral origins. On the other hand, we cannot deny the possibility that CMT4C in Turkish children and our Japanese patient share the same ancestry by means of the Silk Road.

The clinical aspects of CMT4C are early-onset demyelinating neuropathy with spinal deformity.[10,17] While only 8–29% of CMT1A patients develop spinal deformity,[22,27] most patients with CMT4C show spinal deformity.[10,20] As with other types of CMT,[17,28,29] foot deformity is often observed in patients with CMT4C. Usually, the deformities progress faster than the motor deficits.[10] Our patient showed early-onset spinal and hand deformities with slowly progressive motor deficits. Because her motor deficit was mild, the existence of CMT4C was less noticeable in our patient. Cranial nerves are broadly involved in CMT4C cases, with hearing loss being the most common manifestation.[10,11,13,14,16,17,19–22] Additionally, asymmetrical pupil size,[16] abnormality of pupillary reflexes,[14,16] nystagmus,[8,11] facial paresis,[10,16,17,20,21] tongue fasciculations,[14] atrophy[16,20] and weakness,[16] dysphagia,[20] vocal cord involvement,[20,22] and accessory nerve palsy[16] have been reported. In our patient, hypoacousia with diminished wave I in ABR indicated acoustic nerve involvement. Finally, combined with prolonged latency of the facial nerve in NCS, delayed R1 and R2 responses in the blink reflex suggest subclinical facial nerve involvement.

Carpal tunnel syndrome in CMT4C, which was also observed in our patient, is reported infrequently; 2 reports have been published regarding the p.R954X mutation of *SH3TC2* and carpal tunnel syndrome.[21,30] Gosselin et al.[21] reported that 2 of 17 CMT4C cases possessing the p.R954X mutation of *SH3TC2* had carpal tunnel syndrome. Recently, Lupski et al.[30] revealed by whole-genome sequencing in CMT patients that the p.R954X nonsense mutation—itself or accompanied by the missense variant (p.Y169H)—is associated with carpal tunnel syndrome and suggested that presumed loss-of-function nonsense variants of *SH3TC2* are associated with carpal tunnel syndrome. However, it is uncertain whether mutation of *SH3TC2* is related to carpal tunnel syndrome.

Our mutation analysis also revealed that *SH3TC2* mutation was detected in 1 of 426 Japanese CMT patients excluding those with CMT1A. As the proportion of CMT1A in Japanese CMT is approximately 18%,[3] the estimated proportion of CMT4C in total CMT is approximately 0.2% in Japan. Furthermore, we found that, in Japan, CMT4C accounts for approximately 4% of CMT4, an autosomal recessive demyelinating CMT.

Finally, we compared the proportion of CMT4C among different regions. Laššuthová et al.[19] detected *SH3TC2* mutation in 13 of 60 autosomal recessive demyelinating CMT patients and at least 8 of 412 CMT patients who were negative for

PMP22 duplication/deletion in Czechoslovakia. In addition, Baets et al.[17] examined 77 unrelated patients who developed motor and sensory neuropathy within the first year of life in various European countries, the Middle East, and United States. The *SH3TC2* mutation accounted for 12% of the identified mutations. Recently, Yger et al.[20] detected *SH3TC2* mutation in 16 of 102 unrelated autosomal recessive demyelinating or intermediate CMT cases in France. Although direct comparison is impossible because of varying experimental designs, these studies indicate that CMT4C in Japan is actually less common than in European countries.

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ORIGINAL ARTICLE

Late-onset Charcot–Marie–Tooth disease 4F caused by periaxin gene mutation

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Abstract We identified the main features of Charcot–Marie–Tooth (CMT) disease, type 4F, caused by a periaxin gene (*PRX*) mutation in Japanese patients. Periaxin is known as one of the key myelination molecules, forming tight junction between myelin loop and axon. We collected 427 DNA samples from individuals with CMT or CMT-related neuropathy, negative for *PMP22* duplication. We investigated *PRX* mutations using a purpose-built resequencing array screen during the period 2006–2012. We detected two types of *PRX* mutations in three patients; one patient showed a novel homozygous p.D651N mutation and the other two showed homozygous p.R1070X mutation. All *PRX* mutations reported so far have been of nonsense or frameshift type. In this study, we found homozygous missense mutation p.D651N. Aspartate 651 is located in a repeat domain; its position might indicate an important function. *PRX* mutations usually lead to early-onset, autosomal-recessive demyelinating CMT neuropathy 4F (CMT4F) or Dejerine–Sottas disease; their clinical phenotypes are severe. In our three patients, the onset of the disease was at the age of 27 years or later, and their clinical phenotypes were milder compared with those reported in previous studies. We showed a variation of clinical phenotypes for CMT4F caused by a novel, nonsense *PRX* mutation.

Keywords Charcot–Marie–Tooth disease · Periaxin gene mutation · Adult onset · Demyelinating neuropathy

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Introduction

Charcot–Marie–Tooth disease (CMT) is one of the most common inherited neurological disorders with peripheral neuropathies, with an estimated prevalence of 1 in 2,500 [1]. These progressive motor and sensory neuropathies include distal leg degeneration, pes cavus, hammer toes, and intrinsic muscle weakness and wasting. Electrophysiological or pathological studies are necessary for a proper diagnosis [2]. CMT incidents are usually classified as CMT1, the demyelinating form, or as CMT2, the axonal form, distinguished by nerve conduction velocities (NCV) of 38 m/s or less in the median nerve.

The periaxin gene (*PRX*) is located on chromosome 19 and encodes a protein involved in the maintenance of peripheral nerve myelin. *PRX* mutations cause early-onset demyelinating neuropathy, CMT4F, and Dejerine–Sottas

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neuropathy [3, 4]. We extended the phenotypes associated with mutations in the *PRX* gene by the identification of two families with novel *PRX* mutations (C715X and R82fs) [5]. The patients with homozygous C715X mutation showed much more severe sensory than motor impairment and a relatively slow disease progression despite an early disease onset. In contrast, in case of the patient with homozygous R82fs mutation, the disease course was consistent with that of Dejerine–Sottas disease, with early onset, severe muscle weakness, and substantially diminished nerve conduction. Pathological analysis of sural nerve in patients in both families showed remarkable demyelination, onion bulb, and occasional tomacula formation with focal myelin thickening, abnormalities of the paranodal myelin loops, and focal absence of paranodal septate-like junctions between the terminal loops and axon [5]. Until date, 27 such patients have been reported, and most mutations were of nonsense or frameshift type [6].

In this study, we screened for *PRX* mutations in 427 CMT or CMT-related neuropathy patients and found three Japanese patients with *PRX* mutations. One of the three patients showed a homozygous missense mutation and two had nonsense mutations. We have described the clinical features of these CMT4F patients; all three patients showed an adult-onset, mild phenotype.

Materials and methods

Standard protocol approvals, registrations, and patient consent

All patients were referred by their primary physician or neurologist and had signed an informed consent forms approved by the Institutional Review Board of Kagoshima University.

We isolated DNA from the peripheral blood of each patient. Patients negative for *PMP22* (n=427) were selected and investigated for *PRX* mutations during the period 2006–2012. All patients showing demyelinating neuropathy were negative for CMT1A *PMP22* duplication, as shown by fluorescence in situ hybridization analysis. A total of 292 (192 American and 100 Japanese) control chromosomes were also examined.

Sural nerve biopsies, performed at the age of 40 years in patient 1 and 47 years in patient 3, were analyzed according to standard morphological procedures for light and electron microscopy.

Nerve conduction studies were performed using surface electrodes and percutaneous electrical stimulation. Motor nerve conduction studies (MNCS) were conducted on the median, ulnar, and tibial nerves. Sensory nerve conduction studies (SNCS) were performed on the median, ulnar, and sural nerves. We measured motor conduction velocity (MCV) and compound muscle action potential (CMAP) amplitude during the MNCS, and sensory conduction velocity (SCV) and sensory nerve action potential (SNAP) amplitude during the SNCS.

Genomic DNA was extracted from the peripheral blood leukocytes by conventional methods using the Gentra Puregene Blood Kit (Qiagen, Tokyo, Japan). A purpose-built GeneChip® CustomSeq® Custom Resequencing Array (Affymetrix, Santa Clara, CA) was designed to screen for CMT and related diseases such as ataxia with oculomotor apraxia type 1, ataxia with oculomotor apraxia type 2, spinocerebellar ataxia with axonal neuropathy type 1, and hereditary motor neuropathies. The designed resequencing array included the following 28 genes: *EGR2*, *PMP22*, *myelin protein zero* (*MPZ*), *gap junction protein beta 1*, *PRX*, *lipopolysaccharide-induced TNF factor*, *neurofilament light polypeptide* (*NEFL*), *ganglioside-induced differentiation associated protein 1* (*GDAP1*), *myotubularin-related protein 2*, *SH3 domain and tetratricopeptide*, *SET binding factor 2*, *N-myc downstream regulated 1*, *mitofusin 2*, *rab-protein 7*, *glycyl-tRNA synthetase*, *heat shock 27 kDa protein 1*, *heat shock 22 kDa protein 8*, *lamin A/C*, *dynamin 2*, *tyrosyl-tRNA synthetase*, *aranyl-tRNA synthetase*, *lysyl-tRNA synthetase*, *aprataxin*, *senataxin*, *tyrosyl-DNA phosphodiesterase 1*, *desert hedgehog*, *gigaxonin 1*, and *K-Cl cotransporter family 3*. We designed 363 primer sets to cover all coding exons and splice sites. The 363 polymerase chain reactions (PCRs) were amplified in 32 multiplex PCR reactions using the Qiagen Multiplex PCR system (Qiagen). Each reaction used 120 ng of genomic DNA, 10 pmol of the primer set, dNTP, and the Qiagen Multiplex PCR reaction mix (Qiagen). We generated each multiplex PCR product using the following conditions: 15 min at 95 °C, 42 cycles of amplification (94 °C for 30 s, 60 °C for 3 min, and 72 °C for 1 min 30 s), and 15 min at 68 °C. Pooling, DNA fragmentation, labeling, and chip hybridization were performed using the Affymetrix CustomSeq Resequencing protocol instructions [7]. The chips were washed in the Affymetrix Fluidics Station using CustomSeq Resequencing wash protocols. Analysis of microarray data was done using Gene Chip Sequence Analysis software v 4.0 (Affymetrix).

The mutations detected by our DNA Chip method were confirmed by conventional DNA Sanger sequencing. Briefly, we amplified 50 ng of genomic DNA using primers and the hot-start PCR method. Using a pre-sequencing kit (USB, Cleveland, OH), we purified the patient PCR products detected by our resequencing array method and sequenced them with dye-primer chemistry using the ABI Prism 3130 Sequencer (Applied

Biosystems, Foster City, CA). We then aligned the resulting sequences and evaluated the mutations using the Sequencher sequence alignment program (Gene Codes, Ann Arbor, MI).

Results

Patient 1

This patient was a 65-year-old woman who was the third child of healthy, non-consanguineous parents. She had contracted infantile paralysis at the age of 18 months; as a result, her ability to walk independently was delayed. She noticed a mild distal lower limbs weakness and sensory impairment at the age of 30. Upper limbs wasting was observed at the age of 44 years and she also had vocal cord paralysis. The symptoms progressed very slowly. At the age of 65 years, a neurogenic examination revealed mild (upper proximal limbs), moderate (upper distal limbs and proximal lower limbs), and severe (distal lower limbs) muscle weakness, distal dominant sensory disturbance and areflexia. At that time, she had to use short leg braces and drive an electric wheelchair. Electrophysiological analysis showed that CMAPs and SNAPs were not detectable.

Patient 2

This patient was a 63-year-old woman who was the second child of healthy, non-consanguineous parents. At the age of around 50, she gradually acquired a mild wasting and a sensory disturbance in all extremities. At the age of 63 years, she had pes cavus and bilateral limb weakness, mild in upper and lower proximal limbs and moderate in lower distal limbs. Sensory examination showed a moderate reduction in touch and pinprick responses in hands and lower limbs. She could however walk without aid. Electrophysiology showed reduced MCV (20.5 m/s) in the median nerve.

Patient 3

This patient was a 47-year-old man who was the first child of healthy, non-consanguineous parents and had a younger healthy sister. His lower limbs weakness appeared at the age of 37 and progressed gradually. A neurological examination revealed moderate S-shaped scoliosis and severe lower motor impairment; his deep tendon reflexes were absent but he did not have pes cavus. At the age of 47 years, he could walk using a walking vehicle. Electrophysiological studies showed diminished motor NCVs and very prolonged distal latencies (Table 1).

Histopathology

Sural nerve biopsy was performed in patients 1, 2, and 3

Patient 1

Light microscopy indicated a significant loss of all types of fibers. When examined under electron microscope, Schwann cell projections were often scattered.

Patient 2

Light microscopy showed moderate loss of large myelinated fibers with onion bulb formations.

Patient 3

Light microscopy demonstrated a severe deficiency of both myelinated fibers. Many axons had onion bulb formations, but the remaining axons had thin myelin sheaths.

Genetic analysis

We tested each coding exon of *PRX* for mutations by DNA sequencing. Patient 1 carried a homozygous D651N mutation (Fig. 1a) and patients 2 and 3 were homozygous for R1070X mutation (Fig. 1b) [8]. The mutations D651N and R1070X were not found in 292 (192 American and 100 Japanese) control chromosomes or the chromosomes of 427 inherited neuropathy patients. However, we found a D651N mutation in the 1000-Genomes database cataloging human genetic variations using 1,197 samples (including 300 East Asian samples; http://browser.1000genomes.org). The analysis showed the allele frequency of 1 out of 2,188.

Discussion

Here, we report three patients with mutation. To our knowledge, 27 patients with *PRX* mutations have been described so far (Fig. 2) [3–5, 9–15]. All reported mutations were of nonsense or frameshift type. In this study, we report a patient with the homozygous missense mutation D651N.

Periaxin is a membrane-associated protein with a PDZ domain that is involved in mediating protein–protein interactions. Periaxin is mainly expressed in myelinating Schwann cells [18]. In adults, periaxin interacts with the dystroglycan complex (DGC) through dystrophin-related protein 2 and links the basal lamina to the Schwann cell cytoskeleton [19]. *Prx*-null mice develop grossly normal myelin fibers, however, cytoplasmic bands (Cajal bands) are disrupted and Schwann cell elongation during nerve