RESEARCH ARTICLE

Novel de novo POLR3B mutations responsible for demyelinating Charcot–Marie–Tooth disease in Japan

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Abstract

Background: Biallelic POLR3B mutations cause a rare hypomyelinating leukodystrophy. De novo POLR3B heterozygous mutations were recently associated with afferent ataxia, spasticity, variable intellectual disability, and epilepsy, and predominantly demyelinating sensorimotor peripheral neuropathy. Methods: We performed whole-exome sequencing (WES) of DNA samples from 804 Charcot–Marie–Tooth (CMT) cases that could not be genetically diagnosed by DNA-targeted resequencing microarray using next-generation sequencers. Using WES data, we analyzed the POLR3B mutations and confirmed their clinical features. Results: We identified de novo POLR3B heterozygous missense mutations in two patients. These patients presented with early-onset demyelinating sensorimotor neuropathy without ataxia, spasticity, or cognitive impairment. Patient 1 showed mild cerebellar atrophy and spinal cord atrophy on magnetic resonance imaging and eventually died of respiratory failure in her 50s. We classified these mutations as pathogenic based on segregation studies, comparison with control database, and in silico analysis. Conclusion: Our study is the third report on patients with demyelinating CMT harboring heterozygous POLR3B mutations and verifies the pathogenicity of POLR3B mutations in CMT. Although extremely rare in our large Japanese case series, POLR3B mutations should be added to the CMT-related gene panel for comprehensive genetic screening, particularly for patients with early-onset demyelinating CMT.

Introduction

Charcot–Marie–Tooth (CMT) disease is the most common type of inherited peripheral neuropathy characterized by clinical and genetic heterogeneity. The prevalence of CMT ranges from 9.7/100,000 to 82.3/100,000. The disease is classified by motor nerve conduction velocity (MNCV) and mode of inheritance, as determined by family history. Generally, autosomal dominant CMT can be classified as demyelinating type (CMT1; median MNCV...
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<35 m/sec), axonal type (CMT2; median MNCV >45 m/sec), and dominant intermediate type (DI-CMT; median MNCV 35–45 m/sec). To date, more than 100 genes have been associated with CMT and related disorders (https://neuromuscular.wustl.edu/). The common causative genes of CMT1 are PMP22 (CMT1A, 1E), MPZ (CMT1B), LITAF (CMT1C), EGR2 (CMT1D), NEFL (CMT1F), and PMP2 (CMT1G) (https://www.omim.org). Other genes such as FBRN5, C1orf94, and ITTP3 have also been found to cause CMT1.3–5

Polymerase III (Pol III) transcribes small untranslated RNAs (such as tRNA, 5S RNA, 7SK RNA, and U6 RNA) and is involved in the regulation of important cellular processes, such as transcription, RNA processing, and translation. POLR3B is the second largest subunit of Pol III and, together with POLR3A, forms the catalytic center of the enzyme.6 In 2011, biallelic mutations in POLR3B were reported to cause a rare hypomyelinating leukodystrophy,7 whereas in 2021, de novo missense mutations in POLR3B were associated with afferent ataxia, spasticity, variable intellectual disability, and epilepsy, and predominantly demyelinating sensorimotor peripheral neuropathy.8 Heterozygous mutations in POLR3B could result in a demyelinating CMT phenotype without any additional neurological or extra-neurological involvement.9

In this study, we re-analyzed whole-exome sequencing (WES) data targeting POLR3B from more than 800 Japanese patients with CMT and described the clinical and genetic features of two patients harboring de novo heterozygous missense mutations in POLR3B.

Materials and Methods

Patients

We analyzed 2399 unrelated Japanese patients clinically diagnosed with CMT between 2007 and 2020. All patients with the demyelinating type were confirmed negative for PMP22 duplication/deletion using fluorescence in situ hybridization or multiple ligation probe amplification. This study was approved by the institutional review board of Kagoshima University. All patients and family members provided informed consent for participation in the study.

Genomic DNA extraction

We extracted genomic DNA from peripheral blood or saliva using a Puregene Core Kit C (QIAGEN, Valencia, CA, USA) or Oragene DNA self-collection kit (DNA Genotek, Ottawa, Ontario, Canada) following the manufacturer’s instructions.

Microarray chip sequencing and gene panel sequencing

From 2007 to 2012, mutation screening of 419 patients was carried out using a customized MyGeneChip® CustomSeq® Resequencing Array (Affymetrix, Inc., Santa Clara, CA, USA), targeting 30 CMT-related genes. Since 2012, Illumina Miseq (Illumina Inc., San Diego, CA, USA) and Ion Proton (ThermoFisher Scientific, Inc.,Walther, MA, USA), have been used to screen 60 and 72 known/candidate CMT-related genes, respectively. Until 2020, 438 CMT cases were analyzed using the Illumina Miseq system, whereas Ion Proton was used for 1542 cases. The detailed procedures have been described previously.10,11 POLR3B was not involved in any of these three in-house gene panels.

Whole-exome sequencing

Among the negative cases, 804 patients were further processed for WES via the illumina Hiseq2000 platform (Illumina) or Ion Proton (ThermoFisher Scientific, Inc.). Sequencing data alignment (NCBI37/hg19) and variant calling were conducted with Burrows–Wheeler Aligner and SAM tools, or the Ion Reporter Server system. The called variants were annotated using the CLC Genomics Workbench software program (QIAGEN) and in-house R script. The details of our WES workflow have been described previously.12

Variant analysis and interpretation

We extracted all POLR3B (NM_018082.6) variants detected by WES from 804 CMT cases. These variants were checked against gnomAD (https://gnomad.broadinstitute.org), a Japanese control database (jMorp; https://jmorj.megabank.tohoku.ac.jp/202109/), and our in-house control database. Moreover, we performed six lines of computational analysis, consisting of SIFT/PROVEAN (http://provean.jcvi.org/index.php), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Assessor (http://mutationassessor.org/r3/), FATHMM (http://fathmm.biocompute.org.uk), and Condel (https://bgglab.ibibbarcelona.org/fannsdb/), to predict the pathogenicity of the variants.

Protein stability and genetic tolerance analyses

Further protein stability analysis for these mutations were carried out using DynaMut (http://biosig.unimelb.edu.au/dynamut/), iMutant (https://folding.biofold.org/i-mutant/i-mutant2.0.html), and ConSurf (https://consurf.tau.ac.il).
Otherwise, MetaDome (https://stuart.radboudumc.nl/metadome) was used to measure the genetic tolerance of the entire POLR3B gene. All variants were validated using Sanger sequencing and interpreted according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.13

Results
Clinical features
Patient 1
This was a female patient with non-consanguineous parents and no family history of peripheral neuropathy. Her father was diagnosed with amyotrophic lateral sclerosis (ALS) at the age of 80. Delayed motor milestone was noted in this patient when she was 1 year and 6 months old. At age 10 years, she was diagnosed with CMT and became wheelchair-bound at age 16. She also had scoliosis. When she was 34 years old, neurological findings showed predominant muscle weakness (manual muscle testing [MMT]: upper limb proximal 2/2, distal 0–1/0–1; and lower limb proximal 2/2, distal 0–1/0–1) and muscle atrophy in her extremities. She also showed distal dominant sensory impairment and absence of tendon reflexes. No intellectual disability, ataxia, spasticity, or seizure was noted. Her cognitive function at age 36 was normal (Hasegawa Dementia Scale–Revised, 29/30). Muscle biopsy at 10 years of age showed neurogenic changes (data not shown). Electrophysiological studies conducted at ages 36 and 46 revealed that none of the tested nerves were evoked except the musculocutaneous nerve. The MNCV of the musculocutaneous nerve was 26.5 m/sec, and the compound motor action potential was 1.84 mV. Her CMT type was not determined, but the reduced velocity of the musculocutaneous nerve suggested the possibility of demyelinating CMT. Needle electromyography revealed fibrillation potentials/positive sharp waves in her flexor digitorum profundus muscle. High-amplitude and long-duration motor unit action potentials were evident in her biceps brachii. Her electrophysiological data are shown in Table 1. Whole-body computed tomography at age 51 showed marked muscle atrophy of the upper and lower extremities. Brain magnetic resonance imaging (MRI) at ages 47 and 53 showed nonprogressive mild cerebellar atrophy and left cerebellar vermis atrophy. No evidence of cerebral atrophy was observed. Her cervical spine MRI at age 50 revealed spinal cord atrophy (Fig. 1).

The patient also developed respiratory dysfunction. Her vital capacity was 2.04 L (72%) at age 28 and 0.88 L (34%) at age 46. This patient refused mechanical respiratory support and died of acute progression of chronic respiratory failure at the age of 54.

Table 1. Electrophysiological findings of two patients with POLR3B heterozygous variants.

| Patient | Family 1 | Family 2 |
|---------|----------|----------|
| II-2    | II-2     |          |
| Exam age (y.o) | 36 | 46 | 8 | 10 |
| Median nerve | | | | |
| MNCV (m/sec) | NE | NE | 22.2 | 21 |
| CMAP (mV) | NE | NE | 2.2 | 3 |
| SCV (m/sec) | NE | NE | NE | NE |
| SNAP (µV) | NE | NE | NE | NE |
| Ulnar nerve | | | | |
| MNCV (m/sec) | n.a | NE | 25.3 | 22.8 |
| CMAP (mV) | n.a | NE | 0.9 | 1.8 |
| SCV (m/sec) | n.a | NE | NE | NE |
| SNAP (µV) | n.a | NE | NE | NE |
| Tibial nerve | | | | |
| MNCV (m/sec) | NE | NE | n.a | NE |
| CMAP (mV) | NE | NE | n.a | NE |
| SCV (m/sec) | NE | NE | NE | NE |
| SNAP (µV) | NE | NE | NE | NE |
| Peroneal nerve | | | | |
| MNCV (m/sec) | n.a | n.a | NE | NE |
| CMAP (mV) | n.a | n.a | NE | NE |
| Sural nerve | | | | |
| MNCV (m/sec) | NE | NE | NE | NE |
| CMAP (mV) | n.a | 26.5 | n.a | n.a |
| SNAP (µV) | n.a | 1.8 | n.a | n.a |
| Musculocutaneous nerve | | | | |
| MNCV (m/sec) | n.a | NE | NE | NE |
| CMAP (mV) | n.a | NE | NE | NE |
| SNAP (µV) | n.a | NE | NE | NE |
| nEMG | | | | |
| Fibs/PSW | + | – | n.a | – |
| MUP Amp | High | High | High | High |
| MUP Dur | Long | Long | Long | Long |

Normal range: median CMAP >3.1 mV; median MCV >49.6 m/sec; median SNAP >7.0 µV; median SCV >47.2 m/sec; tibial CMAP >4.4 mV; tibial MCV >41.7 m/sec; sural SNAP >5.0 µV; sural SCV >40.8 m/sec; musculocutaneous CMAP and MCV have not been determined. MNCV, motor nerve conduction velocity; CMAP, compound motor action potential; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential; NE, not evoked; n.a, not available; Fibs, fibrillation potentials; PSWs, positive sharp waves; nEMG, needle electromyography; MUP Amp, motor unit potential amplitude; MUP Dur, motor unit potential duration.

Patient 2
The patient was a 26-year-old female. Her parents were not consanguineous, and no similar symptoms were found in her family members. Her initial symptom was delayed motor milestone. At 18 months of age, she began walking independently but developed walking difficulties half a year later. She was diagnosed with CMT at the age of 8 due to marked muscle weakness and atrophy in the distal extremities, foot deformity, and loss of tendon reflexes. Her median MNCV decreased (22 m/sec) on nerve conduction study and was thus diagnosed with demyelinating CMT. Sural nerve pathology at 8 years of
age showed a complete absence of large myelinated fibers, but small myelinated fibers and normal unmyelinated fibers were observed.\textsuperscript{14} Her neurological, electrophysiological, and pathological findings at age 8 were reported by Fukuda et al.\textsuperscript{14} Nerve conduction test at age 10 showed lower median (21 m/sec) and ulnar

**Figure 1.** Radiological findings in patient 1 with \textit{POLR3B} Arg469Cys. (A) Her computed tomography (CT) at age 51 shows marked muscle atrophy in both distal and proximal lower limbs, and in upper limbs with distal predominance. (B) Brain MRI axial slice at age 47, suggesting mild cerebellar atrophy and left cerebellopontine angle meningioma. (C) Brain MRI axial slice at age 53, with no notable changes in her cerebellar volume and meningioma. (D) Brain MRI sagittal slice at age 47, showing atrophy of cerebellar but not in corpus callosum. (E) Cervical spine MRI at age 50 showing cervical spinal cord atrophy.
She exhibited significant muscle weakness in the tibialis anterior muscle, which required a brace, but she was able to walk independently at age 26. Her MMT was 3–4 in the fingers and 4–5 in the proximal muscles. No intellectual disability, ataxia, spasticity, or seizure was noted in this patient.

**Genetic findings**

From the two patients with demyelinating CMT, we identified two novel POLR3B heterozygous variants, c.1405C>T (p.Arg469Cys; NM_018082.6; chr12: 106824192C>T, GRCh37/hg19) and c.1469G>A (p.Cys490Tyr; chr12: 106826100G>A) from our WES data.
We present the pedigrees of these patients in Figure 2A. Segregation studies were carried out on both pedigrees, and these variants were not detected in their parents. The parentage of each pedigree was verified based on rare variants identified using whole-exome sequencing. These variants were thus considered as de novo (ACMG criteria; PS2). Meanwhile, we could not identify suspected variants from any other known disease-causing genes. Both variants were absent in the public databases (ACMG criteria; PM2) and our in-house control database (ACMG criteria; PS4-moderate). The Arg469 and Cys490 residues are highly conserved throughout multiple species (Fig. 2B). Computational analyses (SIFT, PROVEAN, PolyPhen-2, Mutation Assessor, FATHMM, and Condel)
using multiple tools (ACMG criteria; PP3) indicate these variants to have damaging effects.

ConSurf predicted high conservation scores for Arg469 and Cys490 of between 8 and 9 and found them located in close proximity to each other on the protein surface (Fig. 3A). The DynaMut prediction outcome of Arg469Cys (ΔΔG: −1.554 kcal/mol) showed a destabilizing effect. However, Cys490Tyr (ΔΔG: 0.866 kcal/mol) exhibited a stabilizing effect. MetaDome demonstrated that both variants were located on the RNA polymerase Rpb2, domain 3, which is highly intolerant to genomic variants (Fig. 3B). Furthermore, cases with these variants exhibited a CMT1 phenotype, which was consistent with this genetic etiology (ACMG criteria; PP4). Altogether, we classified these variants as pathogenic according to the ACMG guideline. These genetic findings are shown in Table 2. All POLR3B variants in this study identified are shown in Tables S1 and S2.

**Discussion**

Based on WES data from more than 800 Japanese CMT cases, we identified two novel de novo missense mutations in POLR3B, c.1405C>T (p.Arg469Cys) and c.1469G>A (p.Cys490Tyr), from two nonconsecutive patients with demyelinating CMT.

POLR3B is the second largest subunit of Pol III. The biallelic mutation in POLR3A, which encodes the largest Pol III subunit, is known to cause tremor-ataxia with central hypomyelination, hypodontia, hypogonadotrophic hypogonadism (4H syndrome), and leukodystrophy with oligodontia.15 In 2011, biallelic mutations in POLR3B were reported to cause a rare hypomyelinating leukodystrophy. Such types of leukodystrophy are now referred to as POLR3-related leukodystrophy, including leukodystrophy caused by biallelic mutations in POLR1C or POLR3K.16,17 The typical neurological features of patients with POLR3B-associated leukodystrophy mainly implicate the central nervous system, whereas no obvious peripheral neuropathy has been reported. In 2021, Djordjevic et al. reported that multiple de novo heterozygous variants of POLR3B, namely, c.1087G>A [p.Glu363Lys], c.1094C>T [p.Ala365Val], c.1124A>T [p.Asp375Val], c.1277T>C [p.Leu426Ser], c.1385C>G [p.Thr462Arg], and c.3137G>A [p.Arg1046His], were linked to a broad spectrum of clinical phenotypes, including demyelinating neuropathy, ataxia, spasticity, and variable intellectual disability and epilepsy. They further evaluated the assembly of specific RNA Pol III subunits in human embryonic kidney cell line 293 cells using affinity purification and mass spectrometry to define the impact of the de novo variant. They stated that the heterozygous mutations in POLR3B may cause disruption in the association of one or two enzyme subunits, possibly with a dominant-negative effect, and exert pathogenicity.

| POLR3B variant (NM_018082.6) | c.1405C>T | c.1469G>A |
|-------------------------------|-----------|-----------|
| Amino acid change             | p.Arg469Cys| p.Cys490Tyr|
| Zygosity                      | Heterozygous| Heterozygous|
| Allele frequency-gnomAD       | 0         | 0         |
| Allele frequency-jMorp        | 0         | 0         |
| Our control                   | –         | –         |
| SIFT/prediction               | 0         | Damaging  |
| PROVEAN/prediction            | −7        | −10.37    |
| Polyphen2/prediction          | 1         | Damage    |
| MutationAssesor/prediction    | 4.265     | Damage    |
| FATHMM/prediction             | −4.43     | Benign    |
| Condel/prediction             | 0.745     | Damage    |
| Consurf/prediction            | 9         | High conserved |
| Dynamut ΔΔG/prediction        | −1.554 kcal/mol | Destabilizing |
| ΔΔG (ENCOMI)/prediction       | −0.678 kcal/mol | Destabilizing |
| iMutant RI/stability          | 5         | Decrease  |
| ACMG Population data          | PS4(M), PM2 | PS4(M), PM2 |
| In silico data                | PP3       | PP3       |
| De novo data                  | PS2       | PS2       |
| Other data                    | PP4       | PP4       |
| Criteria                      | Pathogenic| Pathogenic|

Table 2. Genetic findings of POLR3B Arg469Cys and Cys490Tyr.
Xue et al.\textsuperscript{9} found a case of demyelinating CMT with a \textit{POLR3B} heterozygous mutation (c.3137G>A [p.Arg1046His]) had no additional neurological or extra-neurological involvement. This phenotypic similarity strongly reinforced the possibility that \textit{POLR3B} missense heterozygous mutations could cause demyelinating CMT. In the present study, the two cases with novel and pathogenic \textit{POLR3B} variants also shared a comparable phenotype of early-onset demyelinating CMT, as in previous reports. A segregation study of both pedigrees suggested that these variants were de novo, which is also consistent with the original report.

Although no evidence of cerebellar ataxia, spasticity, seizures, or dementia was identified in either of our patients, the MRI study of patient 1 showed mild atrophy in the cerebellum and spinal cord. This radiological finding is consistent with the broad spectrum of clinical phenotypes described by Djordjevic et al. Although the father of patient 1 was clinically suspected with ALS at an old age, their clinical phenotypes were completely different, and no suspicious variant was identified in any ALS-related genes using the WES data of patient 1 and her father. More likely, her father developed sporadic ALS incidentally.

Since we were unable to perform functional studies on these mutations due to technical and material limitations, we evaluated the pathogenicity using several in silico analyses and protein stability testing. The results showed that these mutations were located within a highly intolerant region of the protein surface, suggesting potential damaging effects. Based on all these findings, we believe that the two de novo mutations are causative for patients with demyelinating CMT.

This article is the third report on patients with demyelinating CMT associated with \textit{POLR3B} heterozygous mutations. Our findings further verify the pathogenicity of the de novo heterozygous mutations in \textit{POLR3B} and highlight the role of this gene in the genetic spectrum of CMT1. These findings also broaden the clinical spectrum of \textit{POLR3B}-related phenotypes. Although extremely rare in our large Japanese cohort, the CMT-related gene panel for comprehensive genetic screening should be performed, particularly for patients with early-onset demyelinating CMT.

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**Conflict of Interest**

All authors declare that there is no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Genomic variants of POLR3B detected by WES.
Table S2. Variant interpretation with ACMG guideline for POLR3B variants detected by WES.