Background and Purpose  Pathogenic variants in B4GALNT1 have been reported to cause hereditary spastic paraplegia 26. This study has revealed that a novel compound heterozygous pathogenic variant in B4GALNT1 is associated with axonal Charcot-Marie-Tooth disease (CMT).

Methods  Whole-exome sequencing (WES) was used to identify the causative factors and characterize the clinical features of a Korean family with sensorimotor polyneuropathy. Functional assessment of the mutant genes was performed using a motor neuron cell line.

Results  The WES revealed a compound heterozygous pathogenic variant (c.128dupC and c.451G>A) in B4GALNT1 as the causative of the present patient, a 53-year-old male who presented with axonal sensorimotor polyneuropathy and cognitive impairment without spasticity. The electrodiagnostic study showed axonal sensorimotor polyneuropathy. B4GALNT1 was critical to the proliferation of motor neuron cells. The compensation assay revealed that the pathogenic variants might affect the enzymatic activity of B4GALNT1.

Conclusions  This study is the first to identify a case of autosomal recessive axonal CMT associated with a compound heterozygous pathogenic variant in B4GALNT1. This finding expands the clinical and genetic spectra of peripheral neuropathy.

Keywords  Charcot-Marie-Tooth disease; whole-exome sequencing; B4GALNT1.

INTRODUCTION

A hereditary motor and sensory neuropathy commonly known as Charcot-Marie-Tooth disease (CMT) is a heterogeneous disorder of the peripheral nervous system.1,2 CMT is mainly divided into demyelinating and axonal types according to the location of the main pathogenesis.3,4 The former is associated with demyelination in the Schwann cells and the latter is caused by aberrations in the integrity of peripheral axons. The main symptoms of CMT are motor deficit and sensory loss due to peripheral degeneration, gait disturbance, and walking disability. More than 100 genes have been reported to be associated with the CMT phenotype, and their number continues to increase with the application of efficient analysis tools such as whole-exome sequencing (WES).6,7

The beta-1,4-N-acetyl galactosaminyltransferase 1 gene (B4GALNT1) transfers GalNAc to LacCer, GM3, GD3, or GT3 to generate GA2, GM2, GD2, and GT2, respectively.8-10 Gangliosides are glycosphingolipids that are highly expressed in the nervous system and are involved in various critical roles such as synaptic plasticity and signal transduction.11-13 Alterations in ganglioside metabolism affect neuronal function and are associated with neurodegenerative diseases.14 Changes in the concentrations of gangliosides are involved in the pathogenesis.

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esis of Alzheimer’s disease, Huntington’s disease, and gangliosidosis.15-17

Pathogenic variants in B4GALNT1 are involved in hereditary spastic paraplegia subtype 26 (SPG26) in an autosomal recessive manner. The clinical symptoms of SPG26 caused by pathogenic variants in B4GALNT1 include lower extremity spasticity, muscle weakness, and gait abnormality, while extrapyramidal and cerebellar signs, intellectual disability, and dysarthria have also been reported.18-20

Here we report the clinical features of an autosomal recessive CMT patient with a novel compound heterozygous pathogenic variant in B4GALNT1.

**METHODS**

**Clinical and electrophysiological assessments**

The clinical information used in the phenotype assessment included age at symptom onset, age at examination, family history, muscle impairments, joint contracture, sensory deficit, and deep tendon reflexes. Physical disability was quantified by scoring the patient on two scales. Disease severity was assessed according to the 9-point Functional Disability Scale (FDS) from 0 to 8 as follows: 0=normal; 1=normal except for cramps and fatigueability; 2=inability to run; 3=difficulty walking unaided, but still possible; 4=can walk with a cane; 5=can walk with crutches; 6=can walk with a walker; 7=wheelchair-bound; and 8=bedridden.21 The CMT neuropathy score was determined based on the symptoms as well as the results of a neurological examination and nerve conduction study (NCS).22 NCS and needle electromyography were performed at both 41 and 53 years of age. The Mini-Mental State Examination (MMSE) and neuropsychological tests were performed at 53 years of age. This research protocol was approved by the Institutional Review Board of Gangnam Severance Hospital, Korea (IRB No: 3-2021-0014). Written informed consent was exempted by the board because this was a retrospective study.

**Isolation of genetic cause**

The genetic cause of peripheral neuropathy was determined by applying WES to the patient (III-2). The total sequencing yield was 18.87 Gbp/sample, and the coverage rate of the targeted exon regions (≥10×) was 99.0%. The average read depth of the target regions was 253.0 reads. The total number of single-nucleotide variants (SNVs) and indels was 111,802 per sample, of which 43,662 SNVs were coding variants. We identified seven functionally significant variants of neuromyopathy-relevant genes (Table 1).

**Generation of B4GALNT1 mutant**

The plasmid containing human B4GALNT1, pCMV6-myc-B4GALNT1, was obtained from OriGene (Rockville, MD, USA). Site-directed mutagenesis was performed to generate c.128dupC and c.451G>A using the following primers:

- **B4GALNT1 forward**, 5’-GGA GAT CTG CCG CCG CGA TCG CCA TGT GGC TGG GCC GCC GGG CCC-3’;
- **B4GALNT1 reverse**, 5’-CTG CTC GAG CGG CCG CGT ACG CGT CTG GGA GGT CAT GCA CTG-3’;
- **B4GALNT1-128dupC forward**, 5’-CTT GCG CCG TGG GCG CCC GCA AAG CCC CCG CAG-3’;
- **B4GALNT1-128dupC reverse**, 5’-CTG CGG GGG CTT TGC GGG GGG CGC CCG CGC AAG-3’;
- **B4GALNT1-451G>A forward**, 5’-CTC CAG TAC CCC CTA CAG AGT GTG GAA GTT CAG CCC C-3’;
- **B4GALNT1-451G>A reverse**, 5’-GGG GCT GAA CTT CCA CAC TCT GTA GGG GGT ACT GGA G-3’.

All pathogenic variants were confirmed using capillary sequencing.

**Cell viability assay**

The NSC34 mouse motor neuron cell line was used to monitor the effect of B4galnt1 knockdown as described previously.23,24 To determine cell proliferation, cells (4×10^4) were transfected using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

| Table 1. Functionally significant variants of neuromyopathy-relevant genes |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gene     | Accession | Variant | Nucleotide | Amino acid | Zygosity | dbSNP138 | gnomAD exomes (version 2.11) | SIFT | PolyPhen2 | ACMG classification |
| B4GALNT1* | NM_001478.5* | c.128dupC* | p.Gln44AlafsTer14* | - | Hetero* | - | 0.00000399* | 0.000* | 1.000* | Pathogenic* |
| B4GALNT1* | NM_001478.5* | c.451G>A | p.Gly151Ser | - | Hetero* | rs750664123* | 0.00000399* | 0.000* | 1.000* | Likely pathogenic* |
| AFG3L2 | NM_006796.3 | c.242A>C | p.Lys81Thr | Hetero | - | - | 0.016 | 0.594 | VOUS |
| WASHC5 | NM_014846.4 | c.1708G>A | p.Glu570Lys | Hetero | - | - | 0.242 | 0.099 | VOUS |
| SACS | NM_014336.3 | c.6751C>A | p.Glu2251Thr | Hetero | rs747293426 | - | 0.163 | 0.965 | VOUS |
| NDUFV1 | NM_007103.4 | c.218C>T | p.Pro73Leu | Hetero | - | 0.00000398 | 0.181 | 0.005 | VOUS |
| ABHD12 | NM_015600.5 | c.718G>A | p.Val240Met | Hetero | rs572997548 | 0.00211 | 0.003 | 0.992 | VOUS |

*Pathogenic or likely pathogenic variants. SIFT: <0.05 indicates prediction of deleterious. PolyPhen2: ~1 indicates prediction of pathogenicity. ACMG classification refers to the classification of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.46 VOUS, variant of uncertain significance.
with the following B4galnt1-specific siRNAs (Bioneer, Daejeon, Korea): B4galnt1-siRNA#1, 5'-CAG UUC UGG AUA AAC UCA A-3'; B4galnt1-siRNA#2, 5'-CUU CUG UCC AGG AGA UAU A-3'; and B4galnt1-siRNA#3, 5'-CUG AUA GCU CCC GCC AAC U-3'. After 3 days of knockdown, cell proliferation was quantified by direct counting under a microscope.

The knockdown of B4galnt1 in NSC34 cells was confirmed using the reverse-transcription polymerase chain reaction (RT-PCR). Total mRNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA obtained by applying reverse transcription using SuperScript™ II reverse transcriptase (Invitrogen) was used as a template for PCR amplification. Transfections of human wild-type and c.128dupC and c.451G>A mutant B4GALNT1 plasmids were performed in combination with B4galnt1-siRNA#3. After overexpression and knockdown for 3 days, the total number of NSC34 cells was counted.

RESULTS

Clinical manifestations

A 53-year-old male (Fig. 1A, III-2) presented to our neurological clinic with gait disturbance. He did not have diabetes mellitus or alcohol abuse, and was only a carrier of hepatitis B. He first noticed a steppage gait at an age of 20 years, after which his muscle weakness progressed very slowly. When he was first examined at the age of 41 years, he displayed motor weakness and hypesthesia of the distal leg muscles. At the last examination at 53 years of age, he was able to ambulate independently. A neurological examination revealed motor weakness and atrophy of the bilateral distal leg muscles. Ankle contractures were also observed. Pain sensation was preserved, but the vibration and position senses were reduced. Knee and ankle jerks were absent, as were Babinski’s sign and ankle clonus. The patient did not exhibit facial weakness or a high-arched palate. He had an FDS score of 4 and a CMT neuropathy score of 6, and was categorized as having mild disability. He had received 12 years of education and scored 25 of 30 on the Korean version of the MMSE. Neuropsychological tests revealed cognitive impairments in multiple domains (language, ideomotor praxis, calculation, visuospatial, and memory) without any limitation in performing the activities of daily living.

Electrodiagnostic studies were performed at ages of 41 and 53 years (Table 2). NCSs showed reduced sensory nerve action potentials in the median, ulnar, superficial peroneal, and sural nerves. Needle electromyography showed mild denervation potentials in the bilateral tibialis anterior and gastrocnemius muscles at 53 years of age. These findings were con-
sistent with axonal sensorimotor polyneuropathy. MRI of the brain and spinal cord did not reveal any parenchymal abnormalities.

Identification of a compound heterozygous pathogenic variant in B4GALNT1

From the unreported functionally significant SNVs in db-SNP138 and the Genome Aggregation database (gnomAD, https://gnomad.broadinstitute.org), we identified a pair of compound heterozygous pathogenic variants transmitted from each of the parents: c.128dupC and c.451G>A in B4GALNT1 (NM_001478.5) (Fig. 1A and B). Although the parents carried a copy of each mutant allele, they did not exhibit an axonal CMT phenotype. The siblings carried wild-type alleles. The c.128dupC variant was classified as a pathogenic variant based on the following evidence: 1) it is a null variant of a gene where loss of function is a known disease mechanism, 2) the variant is not found in gnomAD exomes and genomes, 3) there are multiple lines of computational evidence for a deleterious effect on the gene or protein, and 4) an in vitro functional study supports a damaging effect on the gene. The c.451G>A change causes the p.Gly151Ser variant. Gly151 is located in a highly conserved region among different species (Fig. 1C), and in silico analyses (using SIFT and PolyPhen2) predict that it affects functional integrity. This missense variant was classified as a likely pathogenic variant based on the following evidence: 1) the variant is not found in gnomAD exomes and genomes, 2) the variant is detected in trans with a pathogenic variant, 3) there are multiple lines of computational evidence for a deleterious effect on the gene or protein, and 4) an in vitro functional study supports a damaging effect on the gene.

Mutant protein inhibits cell proliferation and viability

To investigate the role of B4GALNT1 in motor neurons, we measured its effect on cell proliferation after abrogation. Transfection of mouse B4galnt1-specific siRNAs for 72 h affected the number of NSC34 cells (Fig. 2A). RT-PCR showed that all of the B4galnt1-specific siRNAs were effective in reducing the mRNA levels in NSC34 cells (Fig. 2B). Direct cell counting showed that abrogation of B4GALNT1 significantly reduced the proliferation of NSC34 cells (Fig. 2C). The cell numbers were reduced to 50.6% in B4galnt1-siRNAs#3 treated cells compared with negative-control siRNA (NC-siRNA) treatment. This implies that B4GALNT1 is crucial for the proliferation of motor neurons.

We next investigated the function of mutant B4GALNT1 after the generation of two mutants of B4GALNT1 (c.128dupC and c.451G>A) from the wild-type gene. After the knockdown of endogenous B4galnt1 in NSC34, human B4GALNT1 plasmids were introduced. Overexpression of wild-type and mutant (c.128dupC and c.451G>A) B4GALNT1 did not affect cell proliferation in combination with NC-siRNA transfection, indicating that the mutant proteins did not show a dominant-negative effect. In the B4galnt1 cells knocked down by B4galnt1-siRNAs#3, overexpression of wild-type B4GALNT1 significantly increased cell proliferation to the control level.
Fig. 2. Knockdown of *B4GALNT1* and cell proliferation. A: Representative images of cells from the NSC34 mouse motor neuron cell line, after mouse *B4galnt1*-specific siRNAs. B: Confirmation of *B4galnt1* knockdown in NSC34 by reverse-transcription polymerase chain reaction. C: Proliferation changes after *B4galnt1* knockdown. D: Compensation of *B4galnt1* knockdown with overexpression with human *B4GALNT1* (wild type and mutants). Data are mean and standard-error-of-the-mean values. Student’s *t*-test: **p<0.01. Con, control; NC, negative control; WT, wild type.

whereas overexpression of the mutant genes had no effect (Fig. 2D). These results are consistent with the reduced expression of *B4GALNT1* in affected patients.

**DISCUSSION**

This study has identified a compound heterozygous pathogenic variant in *B4GALNT1* that is associated with axonal sensorimotor polyneuropathy and mild cognitive impairment without spasticity. Pathogenic variants in *B4GALNT1* has usually been reported to cause SPG26, which is associated with early-onset spastic paraplegia, intellectual disability, cerebellar ataxia, and peripheral neuropathy.\(^{18-20,25}\) It has recently been reported that patients with a novel homozygous pathogenic variant (c.263dupG) in *B4GALNT1* exhibit glutaric acidemia type II, which results in a sudden metabolic crisis that includes acidosis and hypoglycemia.\(^{26}\) The clinical findings of our patient have not been reported previously in other patients with pathogenic variants in *B4GALNT1*. However, many SPG-related genes, including *ATL1*, *KIF1A*, *KIF5A*, *SACS*, *SPG11*, and *TFG*, are also associated with spastic paraplegia, hereditary sensory neuropathy, or CMT diseases.\(^{27-32}\)

Sphingolipids or gangliosides play a series of important functions in neurons, such as proliferation, differentiation, and synaptic transmission.\(^{33-36}\) To date, the most well-described diseases with sphingolipid metabolism are lysosomal storage disorders such as Tay-Sachs disease and Niemann-Pick C disease.\(^{37,38}\) Recent advances in causative gene isolation have revealed that sphingolipid metabolism is associated with various types of neurodegenerative diseases, including peripheral neuropathy. Pathogenic variants in *SPTLC1* (Serine palmitoyltransferase) and longchain base subunit and *SPTLC2* are associated with hereditary sensory and autonomic neuropathy.\(^{39,40}\) Mutant *HSPB1*, a causative gene of CMT2F and distal hereditary motor neuropathy type IIB, was recently reported to decrease mitochondrial ceramide levels and modify the structural and functional changes in mitochondria via interactions with ceramide synthase 1.\(^{41}\) Therefore, dysregulation of sphingolipid metabolism also affects neuronal activity in the peripheral nerves.

Previous studies have found that *B4galnt1* disruption in mice does not severely affect the nervous system, except for a slight reduction in neural conduction velocity from the tibial
nerve to the somatosensory cortex, which suggests that complex gangliosides are predominantly required in synaptic transmission. These features are very similar to those observed in humans, such as reduced sensory nerve function, abnormal gait, tremor, and ataxia in age-related neurodegeneration. Eleven pathogenic variants in B4GALNT1 have been reported, including two nonsense and three frameshift variants. Predictions of the tertiary structure of B4GALNT1 protein suggest that most missense variants will affect protein stability, which is also supported by immunostaining data. Enzymatic assays have revealed that all of the pathogenic variants completely affect the activity, while two pathogenic variants (c.898C>T (p.Arg300Cys) and c.682C>T (p.Arg228)) show lower levels of enzymatic activity.

To determine the effect of the newly identified pathogenic variants (c.128dupC and c.451G>A) on peripheral neurons, we evaluated the proliferation of motor neurons after abrogation of mouse B4galnt1 and overexpression of human mutant B4GALNT1. Knockdown of B4galnt1 significantly reduced the proliferation of mouse motor neurons. In the absence of mouse B4galnt1, overexpression of human wild-type B4GALNT1 completely compensated for the cell proliferation. However, transfection of both mutant genes (c.128dupC and c.451G>A) did not affect cell proliferation, implying that these pathogenic variants might significantly affect the enzymatic activity of B4GALNT1. Collectively these data suggest that the loss-of-function variants in B4GALNT1 can play a role in peripheral neuropathy by disturbing ganglioside metabolism in neurons.

In conclusion, here we report for the first time that a compound heterozygous pathogenic variant in B4GALNT1 is associated with axonal CMT. The present findings suggest that alterations in sphingolipid metabolism are widely associated with peripheral neuropathy, and they expand the clinical spectrum of both B4GALNT1-associated diseases and hereditary motor and sensory neuropathy.

Availability of Data and Material

All data generated or analyzed during the study are included in this published article (and its supplementary information files).

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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