

BRIEF COMMUNICATION

**Partial duplication of *DHH* causes minifascicular neuropathy
A novel mutation detection of *DHH***Naoko Saito Sato^{1,2}, Risa Maekawa², Hiroyuki Ishiura¹, Jun Mitsui¹, Hiroya Naruse^{1,2},
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Abstract

Minifascicular neuropathy (MN) is an extremely rare developmental malformation in which peripheral nerves are composed of many small fascicles. Only one patient with MN with 46XY gonadal dysgenesis (GD) was found to carry a mutation affecting the start codon in *desert hedgehog* (*DHH*). We identified an identical novel rearrangement mutation of *DHH* in two consanguineous families with MN, confirming mutations in *DHH* cause MN with 46XY GD. The patients with the 46XY karyotype developed GD, whereas a patient with the 46XX karyotype did not. These findings further support that *DHH* has important roles in perineural formation and male gonadal differentiation.

Introduction

Minifascicular neuropathy (MN) is an extremely rare developmental malformation of peripheral nerves and is characterized by the presence of many small fascicles with glove and stocking-type sensory impairment.¹ Two unrelated Japanese patients with 46XY gonadal dysgenesis (GD) have been reported.² MN was also reported in an Italian patient, who had the 46XX karyotype and whose gynecological ultrasound examinations showed normal findings.³ On the basis of the finding that *DHH* knockout mice show MN, Umehara et al. performed mutational

analysis of *DHH* and found a homozygous mutation involving the translation initiation codon in the first Japanese family found to be affected by MN.⁴ However, no *DHH* mutations were detected in the second Japanese family reported by Sugie et al.² or in the Italian family, and genes and mutations responsible for MN have remained to be elucidated. We have recently identified Japanese MN patients with or without GD born to consanguineous parents. Employing linkage analysis and detailed mutational analyses, we identified a novel rearrangement mutation in *DHH*. We further confirmed the same mutation in the second Japanese family.² Our

findings provide further evidence that *DHH* is the gene responsible for MN in humans.

Subjects and Methods

Written informed consent was obtained from all the participants. The present study was approved by the institutional review board of the University of Tokyo. Genomic DNA was prepared from peripheral blood leukocytes of the participants in accordance with the standard procedure.

Subjects

MN-1 family

Two patients with MN born to consanguineous parents were investigated in the study (Fig. 1A). The 55-year-old patient (patient 1) was born by normal delivery and raised as a female. She started to suffer from muscle cramps on exercise since childhood. Gynecological examination for amenorrhea revealed the absence of ovaries and uterus at the age of 16, when chromosomal analysis showed 46XY. At the age of 43,

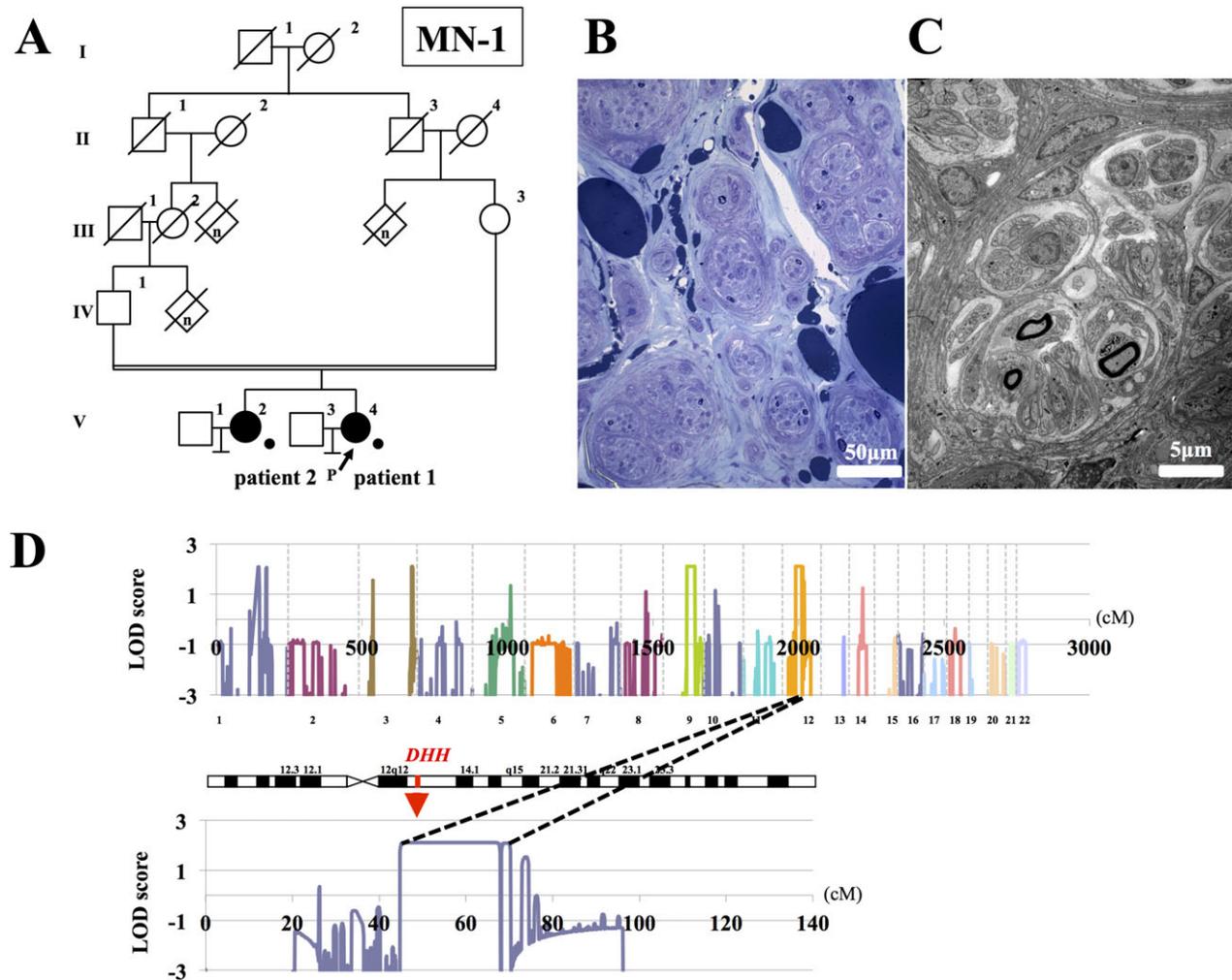


Figure 1. (A) Pedigree chart of MN-1. Squares and circles indicate males and females, respectively. Affected persons are indicated by filled symbols. A diagonal line through a symbol represents a deceased person. The person with the arrow is the index patient. Persons with available genomic DNAs are indicated by dots. (B) Light microscopy findings of sural nerve specimens stained with toluidine blue. Sural nerve contains many small fascicles. Many fascicles have smaller fascicles inside them. (C) Electron microscopy findings of sural nerve specimens. There are few myelinated fibers. Minifascicles contain several myelinated and unmyelinated fibers. Endoneurial cells carry on processes and encircle the minifascicles. (D) Multipoint parametric linkage analysis (autosomal recessive model) of MN-1 family. Multipoint parametric LOD scores spanning all the chromosomes are shown above. The horizontal axis is the cumulative genetic distance (centimorgan) starting at the short arm of chromosome 1. The vertical axis represents cumulative LOD scores. Regions on chromosome 12 give the highest multipoint parametric LOD score of 2.1. Multipoint parametric LOD scores of chromosome 12 is shown below. The horizontal axis is the genetic distance (centimorgan) starting at the arm of chromosome 12. The vertical axis shows multipoint parametric LOD scores.

pigmentation of the nails and hyperkeratosis of the sole skin appeared. She underwent amputation of some of her toes because of ulceration. On examination at the age of 55, skin pigmentations in the four extremities were observed. Pelvic magnetic resonance imaging (MRI) confirmed the absence of ovaries and uterus with a blind-ended vagina and an undescended testicle in the left inguinal canal. Neurological examinations showed sensory disturbance and the absence of tendon reflexes. The Wechsler Adult Intelligence Scale (WAIS-III) test revealed high IQ score (131; normal 90–109). Nerve conduction study revealed sensory motor dysmyelinating polyneuropathy (Table S1). The left sural nerve was biopsied. Light microscopy examination revealed numerous nerve fascicles, ranging in diameter from 20 to 200 μm subdivided into several minifascicles with connective tissue. The density of myelinated fibers was markedly reduced in each fascicle (Fig. 1B and C).

Her elder sister (patient 2) also complained of muscle cramps on exercise since childhood. She showed pigmentation of the nails and hyperkeratosis of the sole skin, and she had some of her toes amputated. Nerve conduction studies showed sensory motor dysmyelinating polyneuropathy (Table S1). Although she did not have a child, her menstrual cycle was regular until the menopause and her karyotype was 46XX (Table 1).

MN-2 family

The clinical presentations of patient 3 (Fig. S1) were described previously.² Briefly, she noticed distal numbness

and weakness in the four extremities at the age of 39, which gradually progressed. At the age of 47, a nerve conduction study revealed sensory dominant neuropathy. Sural nerve biopsy revealed minifascicle formation. She had amenorrhea and gynecological examinations revealed normal female external genitalia and a blind-ended vagina. Chromosomal analysis revealed 46XY. Direct nucleotide sequence analysis revealed no point mutations in the sex-determining region Y gene (*SRY*) and *DHH*.⁴

Whole exome sequence analysis

Exonic sequences were enriched using a SureSelect Human All Exon V4+UTRs kit (Agilent Technology, Santa Clara, CA, USA), followed by massively parallel sequence analysis (2×100 bp paired end reads) using Hiseq 2000 (Illumina, San Diego, CA, USA). The sequences were aligned against the reference genome (hg19) using Burrows-Wheeler Aligner (BWA), and SAMtools^{5,6} was used for extracting single-nucleotide variants (SNVs) and small insertions and deletions (indels) with default parameter settings.

Linkage analysis and haplotype analysis

Three affected family members of the two families (MN-1 and MN-2) were genotyped using Genome-wide Human SNP array 6.0 (Affymetrix, Santa Clara, CA)⁷ following the manufacturer's instructions. The genotype of each family member was determined using Genotyping

Table 1. Clinical and genetic findings in four neuropathologically confirmed MN families.

Patient	1	2	3	4	5
Age	55	60	47	27	28
Age at onset	Childhood	Childhood	39	26	2
Sex (phenotype)	F	F	F	F	F
Family history	+	+	–	–	+
Consanguinity	+	+	+	+	–
Neuropathy	Sensory dominant	Sensory dominant	Sensory dominant	Sensory dominant	Motor dominant
Weakness	–	–	+	–	+
Muscle cramp	+	+	–	–	–
Cutaneous ulcer	+	+	–	+	–
NCS (median nerve)					
CMAP (mV)	12	5.5	ND	6.3	2
MCV (m/sec)	39.6	38.1	37.2	48	38
Minifascicular formation	+	NE	+	+	+
Karyotype	46XY	46XX	46XY	46XY	46XX
Gonads/Internal genitalia	Bilateral streak gonads Hypoplastic uterus and ovaries	No abnormality was pointed out	Hypoplastic uterus	Right-sided streak gonads Hypoplastic uterus and ovaries	Normal
Mutation	c.304-572_492dup	c.304-572_492dup	c.304-572_492dup	c.2T>C	ND
Source	Patient 1	Patient 2	Patient 3 (2)	4	3

CMAP, compound muscle action potential; MCV, motor conduction velocity; NCS, nerve conduction studies; ND, not detected, NE, not examined.

Console 4.0 (Affymetrix). Parametric multipoint linkage analysis (autosomal recessive model with complete penetrance) was conducted using the pipeline software SNP-HITLink⁸ and Allegro version 2⁹ employing single-nucleotide polymorphisms (SNPs) satisfying a $P > 0.001$ in the Hardy–Weinberg test, a call rate of >0.98 , a genotyping confidence score of <0.02 , a minor allele frequency in the controls >0 , and intermarker distances of 80–120 kb. Haplotypes were reconstructed manually.

Long-range PCR analysis of *DHH*

We amplified the entire *DHH* region by long-range PCR using the primer set: F1 (5'-GCAGCTTCCAAGTCA-3') and R1 (5'-GCTGATATGCCCTTGTGTTAGGG-3'). To search for the breakpoint junctions, we performed genomic PCR using the primer set: F2 (5'-CTACCATCGACTCAGATTCT-3') and R2 (5'-GCTCCCCTCCTCCGCCTGA-3') (Fig. 2C), followed by direct nucleotide sequence analysis using a Big Dye Terminator version 3.1 and an ABI 3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA).

RT-PCR analysis of *DHH* mRNA expression in sural nerves

Total RNAs were extracted from the sural nerves of patient 1 and a control individual using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was performed using the ReverTra Ace- α -TM (Toyobo, Osaka, Japan) with an oligo dT primer. RT-PCR was performed using *DHH*-specific primer sets: f1 (5'-TCGTGCCGCTACTTACAAG-3'), r1 (5'-GCCGCCAAAACCCAGTCTC-3'), f2 (5'-TCGTGCCCAACTACAACCC-3'), and r2 (5'-TTCA CCCGCTCCTTACAACG-3') (Fig. 2E). As an internal control, we performed RT-PCR of *ACTB* using the primer set: (5'-AGCGAGCATCCCCCAAAGTT-3') and (5'-GGGCACGAAGGCTCATCATT-3').

Results

Mutation discovery

Multipoint parametric linkage analysis of the MN-1 family with the assumption of autosomal recessive inheritance revealed five regions in four chromosomes with the highest multipoint LOD score of 2.1. Notably, the candidate regions overlapped with the locus of *DHH* on chromosome 12 (Fig. 1D). In whole exome sequencing analyses, no single-nucleotide variants or small insertion–deletions were determined by SAMtools in the region of *DHH*. We next tried to amplify the entire *DHH* region employing a long-range PCR method using the primers F1 and R1.

The PCR products obtained from patients 1 and 2 were larger than those from a control (Fig. 2A), raising the possibility of a rearrangement such as insertion and/or multiplication. Direct nucleotide sequence analysis of the PCR products revealed that a 761-bp genomic segment was tandemly duplicated with the 5' end located in intron 1 (chr12:49,485,744 in hg19) and the 3' end in exon 2 (chr12:49,484,984) (Figs. 2B and C). We also conducted a mutational analysis of *DHH* of the patient 3 in MN-2 family employing the same strategy. The same mutation was found in the patient (Figs. 2A and B).

To investigate whether mutant *DHH* mRNA is expressed in sural nerves, we extracted total RNAs from the sural nerves of patient 1 and a control individual. RT-PCR analysis revealed that *DHH* mRNA was not expressed in the patient's sural nerve (Fig. 2D).

Haplotype analysis of the two families revealed that three individuals shared a substantially large region of 4.34 Mb in their disease-associated haplotypes with the centromeric and telomeric boundaries defined by rs10735810 and rs11170044, respectively (Fig. S2). The result suggested the existence of a common founder.

Discussion

We identified a novel rearrangement mutation as the cause of MN with 46XY GD in two Japanese families. The duplication spans 761 bp and one end of the breakpoints includes exon 2. Given the structure of the duplication and the absence of *DHH* mRNA expression in the sural nerve in patient 1 assessed by RT-PCR analysis, we suppose that the mutation would result in the loss of function of *DHH*.

DHH located on 12q12-q13.1 comprises three exons, encoding a polypeptide of 396 amino acids.¹⁰ *DHH* is a member of the hedgehog gene family which also includes sonic hedgehog and Indian hedgehog.¹¹ Paramantier et al.¹² found that the peripheral nerves of *dhh* null mutant mice showed minifascicular formation. Analysis of null mutant mice demonstrated that *dhh* signaling plays an essential role in the regulation of mammalian spermatogenesis.¹³ Because mammalian hedgehog genes share a striking homology to the *Drosophila* segment polarity hedgehog gene, these findings suggest that *DHH* plays crucial roles in both peripheral nerve sheath development and spermatogenetic regulation in humans.

Umehara et al.⁴ discovered a homozygous mutation involving the start codon, which presumably leads to the failure of the transcription of *DHH*, in an MN patient with 46XY GD. In this study, we revealed that the patients in MN-1 and MN-2 had an identical rearrangement mutation in *DHH*. This finding further confirms that mutations in *DHH* cause MN.

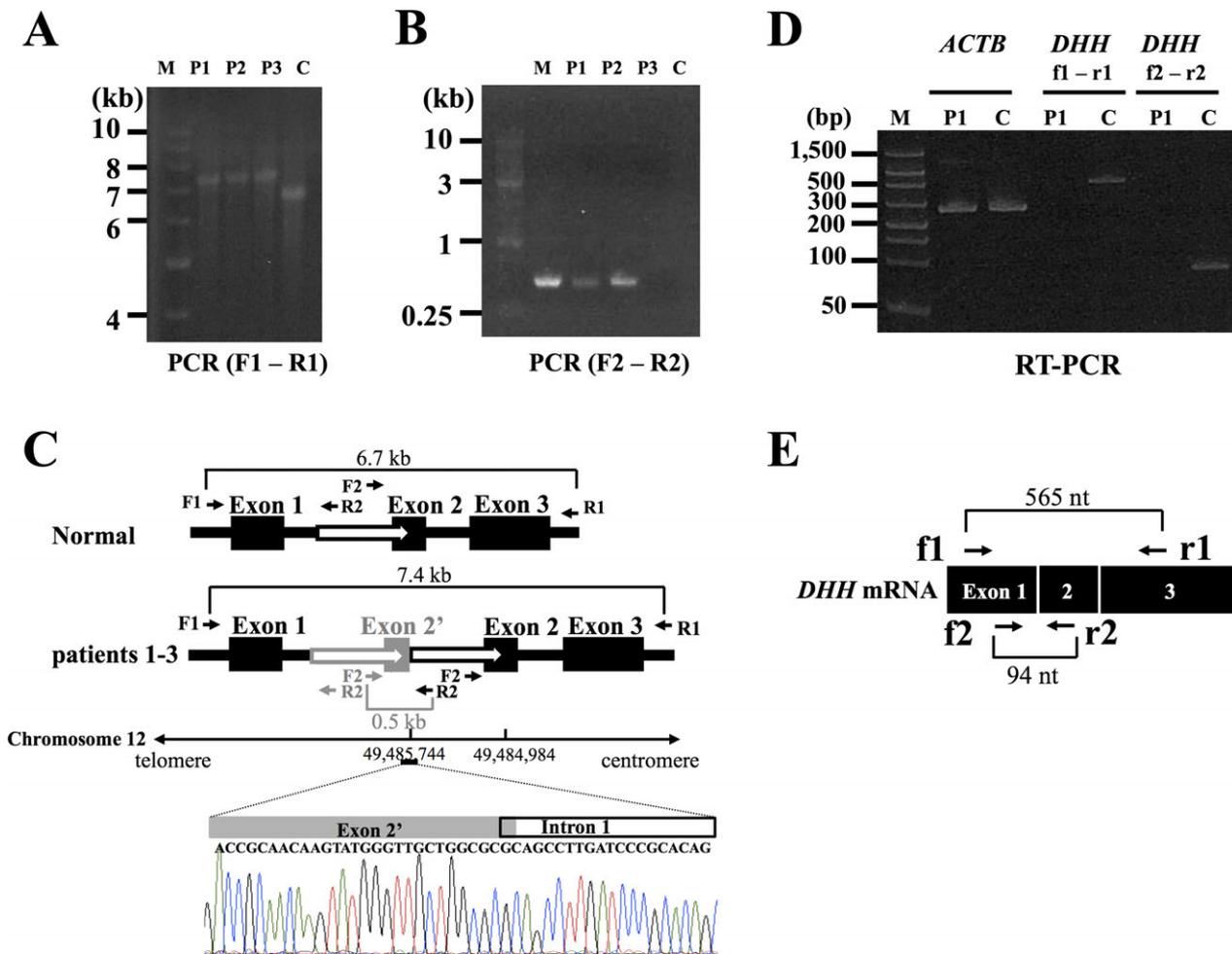


Figure 2. (A) Long-range PCR amplification of the whole region of *DHH* in the three MN patients (left). We amplified the entire *DHH* region including all three exons using the following primer set: F1 (5'-GCAGCTTCCAAGTCA-3') and R1 (5'-GCTGATATGCCTTGTAGGG-3'). *DHH* is approximately 700 bp longer in the three cases than in the normal control. M, size standard marker (1 kb DNA ladder); C, normal control; P1, patient 1; P2, patient 2; P3, patient 3. (B): Detection of breakpoint junction by PCR. We performed genomic PCR using the following primer set: F2 (5'-CTACCATCGACTCAGATTCT-3') and R2 (5'-GCTCCCCTCCCTCCGCTGA-3'). M, size standard marker (1 kb DNA ladder); C, normal control; P1, patient 1; P2, patient 2; P3, patient 3. Only the patients' genomic DNAs carrying the duplication can be PCR amplified. (C) Structure of mutation in *DHH*. Direct nucleotide sequence analysis of the PCR products amplified using a primer set (F2 and R2) showed the breakpoint junctions. One of them was found in exon 2 and the other in intron 1 (chromosome 12: 49,484,984–49,485,744). The black boxes represent exons and the white arrows duplicated regions. (D) RT-PCR analysis of *DHH* mRNA expression in the sural nerves. In the RT-PCR analyses of *DHH* using primers located on exons 1 and 3 (f1 and r1 in Fig 2E) and primers located on exons 1 and 2 (f2 and r2 in Fig 2E), the PCR products (565 bp and 94 bp bands) were revealed only in a control subject, confirming the absence of *DHH* mRNA expression in the sural nerve of the patient 1. RT-PCR of *ACTB* revealed bands corresponding to 285 bp in both the patient 1 and a control subject. M, size standard marker (FlashGel DNA marker); C, normal control; P1, patient 1; *ACTB*, actin beta. (E) A schematic presentation of normal *DHH* mRNA along with primers used in the RT-PCR analysis.

The rearrangement mutation of our subjects results in the absence of *DHH* expression. To date, seven *DHH* mutations have been reported.^{14–16} Three of the seven mutations, including the mutation found in the study, cause 46XY GD with neuropathy, whereas some mutations did not cause neuropathy. (Table S2). Although nerve conduction studies, nerve biopsy, and/or functional

study of the mutations were performed in a limited number of patients, mutations presumably leading to complete loss of function of *DHH* (c.304–572dup, c.2T>C, c.485T>C,¹⁷ and c.57–60dupAGCC) does not always cause both neuropathy and GD. It is still uncertain whether there is a phenotype correlation with complete loss versus partial loss of function of *DHH* gene.

One of our patients (patient 2) in MN-1 and the patient reported by Malandrini et al.⁴, both of whom karyotype was 46XX, showed MN and normal sex characteristics. We suggest that *DHH* may be involved in different manners in peripheral nerve development or gonadal genesis.

To detect medium-sized structural variants (100–1000 bp) as found in the study is still a challenge. Indeed, the rearrangement mutation which we detected was not revealed by standard whole exome sequencing. The finding in this study indicates that we should always give attention to structural variants, even if standard exome sequencing did not reveal mutations. The causative mutations of a patient with minifascicular neuropathy³ and a patient showing 46XY GD and neuropathy without minifascicular formation¹⁸ have not been identified. There may be clinical and genetic heterogeneity of this rare clinical entity, but our finding suggests that mutations that may have been overlooked by Sanger sequencing or standard exome sequencing should be assessed in these patients.

In conclusion, we identified a novel mutation in human *DHH*. The patients with the 46XY karyotype exhibited MN with 46XY GD, whereas the patient with the 46XX karyotype showed MN, but apparently normal sexual development. These findings further confirm that *DHH* is an important gene in both perineural formation in peripheral nerves and male gonadal differentiation.

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Authors' Contribution

N.S.S. and R.M. contributed equally to this study.

Conflicts of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Pedigree chart of MN-2.

Figure S2. Haplotypes around *DHH* locus constructed using SNPs of two families are shown.

Table S1. Nerve conduction study findings of patients 1 and 2 in MN-1.

Table S2. Clinical and genetic findings in 11 patients who have mutations in *DHH*.