RESEARCH ARTICLE

A Mutation in PMP2 Causes Dominant Demyelinating Charcot-Marie-Tooth Neuropathy

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Abstract

Charcot-Marie-Tooth disease (CMT) is a heterogeneous group of peripheral neuropathies with diverse genetic causes. In this study, we identified p.I43N mutation in PMP2 from a family exhibiting autosomal dominant demyelinating CMT neuropathy by whole exome sequencing and characterized the clinical features. The age at onset was the first to second decades and muscle atrophy started in the distal portion of the leg. Predominant fatty replacement in the anterior and lateral compartment was similar to that in CMT1A caused by PMP22 duplication. Sural nerve biopsy showed onion bulbs and degenerating fibers with various myelin abnormalities. The relevance of PMP2 mutation as a genetic cause of dominant CMT1 was assessed using transgenic mouse models. Transgenic mice expressing wild type or mutant (p.I43N) PMP2 exhibited abnormal motor function. Electrophysiological data revealed that both mice had reduced motor nerve conduction velocities (MNCV). Electron microscopy revealed that demyelinating fibers and internodal lengths were shortened in both transgenic mice. These data imply that overexpression of wild type as well as mutant PMP2 also causes the CMT1 phenotype, which has been documented in the PMP22. This report might expand the genetic and clinical features of CMT and a further mechanism study will enhance our understanding of PMP2-associated peripheral neuropathy.

Author Summary

Isolation of causative mutation is still challenging in genetic diseases with a variety of genetic causes. We discovered a mutation in a novel gene from a family exhibiting a
peripheral neuropathy by virtue of next-generation sequencing. Although the family shows characteristic clinical features of hereditary motor and sensory neuropathy, we could not find a mutation from well-known genes. To demonstrate the clinical relevance of the novel gene, we generated transgenic mice, which carry the patients’ mutation within their chromosome. The transgenic mice exhibited the same phenotype as the patients including peripheral neuropathic symptoms and reduced locomotor function. We also observed the affected peripheral nervous system through electron microscopy. It seems that the expression of the mutant protein impairs the myelin of peripheral nervous system. These data might expand the genetic, clinical, and pathophysiological features of the peripheral neuropathy and a further investigation will enhance our understanding of disease in the peripheral nervous system.

Introduction
Charcot-Marie-Tooth disease (CMT) represents a group of inherited peripheral neuropathies with a heterogeneous genetic and clinical spectrum. The typical CMT phenotype is characterized by distal weakness, sensory loss, foot deformities, and absence of reflexes [1]. CMT is broadly classified into the demyelinating type (CMT1) with slowed motor nerve conduction velocity (MNCV) of <38m/s, and the axonal defective type (CMT2) with reduced conduction velocity, >38m/s [2,3].

Although the axonal type of CMT is caused by more than 60 genes with diverse mechanisms, the demyelinating type of CMT is caused mainly by peripheral myelin proteins, the salient proteins in the myelin, or their transcription factors [4]. The most frequent genetic cause of CMT1 is alterations in PMP22, resulting in CMT1A or CMT1E, followed by MPZ mutations, which lead to CMT1B. PMP22 and MPZ account for about 5% and 50% of the total peripheral myelin proteins, respectively. PMP22 mutations account for up to 70–80% of CMT1 cases, while MPZ mutations occur in approximately 10% of CMT1 cases. In addition, a transcription factor for myelin proteins, EGR2, is the genetic cause of CMT1D [5].

Intriguingly, mutations in Myelin basic protein (MBP) and Peripheral myelin protein 2 (PMP2), which account for 18% and 5% of peripheral myelin proteins, have not been reported as a genetic cause of CMT. MBP is also a constituent of central nervous system (CNS) myelin proteins. Although mutation in MBP has not been reported in humans, deletion of the MBP gene causes the ‘shiverer’ phenotype in mice, in which mice show decreased CNS myelination, tremors, and increased severity leading to early death [6].

The PMP2 (8q21.13) was once suspected to be the causative gene because it is located in the close vicinity of the CMT4A locus (GDAP1) [7], but no mutation was found in the family studied [8]. To link the gene with CMT, a knockout mouse model for PMP2 was generated; however, there was no typical phenotype of peripheral neuropathy except for a slight reduction in the nerve conduction velocity [9]. Recently, a point mutation (p.I43N) in PMP2 was strongly suggested as a potential pathogenic mutation in a family with autosomal dominant CMT1 [10]. To demonstrate the pathogenesis of the PMP2 mutation, the researchers showed structural abnormality caused by mutant PMP2 expression in a zebrafish model. Several years ago, we also found a Korean CMT1 family harboring the same PMP2 mutation and have investigated the pathogenicity of the mutation using mouse models.

In this report, we present the detailed clinical features of a PMP2 mutation-associated autosomal dominant CMT1. In addition, the relevance of PMP2 mutation as a genetic cause of dominant CMT1 was assessed using transgenic mouse models.
**Results**

**Identification of a PMP2 mutation**

To determine the genetic cause of the FC183 family, whole exome sequencing was performed on five family members (S1 Table). The mean total sequencing yields was about 8.05 Gbp/sample, and the coverage rate of the target region (≥10x) was 91.24%. The average number of observed variants per sample was 90,653 SNPs and 6,299 indels, respectively. Of these, the number of functionally significant variants was 10,400/sample.

Exome data of the three affected members revealed ~60 functionally significant variants in CMT-related genes (S2 Table). However, none of the variants cosegregated with the affected members in the family. Most variants were polymorphic with high frequency, except for three variants with allele frequencies of less than 0.01 in Korean controls. Although three variants (p. M1I in FAM134B, p.A1315T in SBF2, and p.A952V in CTDP1) were not observed in in Korean controls, they were not considered as the underlying cause, because of noncosegregation with affected individuals in the FC83 family.

Since no variants in the CMT-related genes were considered to be an underlying cause of the CMT phenotype, we tried to identify the genetic cause from the entire exome data. A total of 5 cosegregating missense variants were isolated in RYR2, NME5, ANK1, PMP2, and AGAP11 genes. Given that our family phenotype has a late onset, the age of the proband’s daughter (IV-1, 2 years old) would provide questionable insights into the consideration or exclusion of affection; hence she was excluded from the cosegregation analysis. All five variants were not found in the 500 Korean controls and they were not reported in global human variant databases, such as the dbSNP144, 1000G, ESP, and ExAC (Table 1). A thorough look at the variants, their gene functions, conservation, and *in silico* analysis drew attention to PMP2 (c. T128A, p.I43N) mutation. First of all, PMP2 encodes a myelin protein that is an important component of the peripheral nervous system. In addition, the PMP2 mutation cosegregated within extended family members (Fig 1). The p.I43N mutation in the PMP2 protein is located in the highly conserved lipocalin/cytosolic fatty-acid binding domain, and it was predicted to be pathogenic through several *in silico* analyses using SIFT, PolyPhen2, and MUpro programs. Most of all, a recent report on the same mutation (p.I43N) also strongly suggested that the mutation is the genetic cause of this family [10].

**Clinical and electrophysiological manifestations**

The clinical manifestations are summarized in Table 2. The age at onset ranged from 6 to 18 years among the three affected individuals. In all patients, distal leg muscle weakness,
atrophy, and frequent falling were first noticed. They also exhibited bilateral hand muscle weakness that caused difficulty in writing, cooking, and fine finger control. Their hand atrophy was first noticed in the dorsal interosseous and thenar muscles, but their hypothenar muscle bulk and strength was relatively preserved.

Table 2. Clinical manifestations of the three patients with PMP2 p.I43N mutation.

| Patients | II-4 | III-1 | III-3 |
|----------|------|-------|-------|
| Sex      | Female | Male | Male |
| Age at exam (years) | 58 | 38 | 36 |
| First symptom at onset | Distal leg muscle weakness | Frequent falling | Distal leg muscle weakness |
| Age at onset of leg (years) | 18 | 6 | 8 |
| Age at onset of hand (years) | 41 | 20 | 28 |
| Muscle weakness<sup>a</sup> | | | |
| Upper limb | + | ++ | + |
| Lower limb | + | +++ | ++ |
| Muscle atrophy | Mild (U< L) | Moderate (U< L) | Mild (U< L) |
| Sensory loss | Vibration>Pain | Vibration>Pain | Vibration>Pain |
| Knee/ankle jerks<sup>b</sup> | A/A | A/A | D/D |
| Plantar response | No | No | No |
| Hand tremor | Yes | Yes | No |
| Pes cavus | Yes | Yes | Yes |
| Scoliosis | No | No | No |
| FDS | 3 | 4 | 3 |
| CMTNS | 9 | 20 | 13 |
| 9-HPT (sec) | 22.7 | 31.4 | 26.3 |
| Electromyography | Neurogenic MUAPs | Neurogenic MUAPs | Neurogenic MUAPs |
| Nerve biopsy | Demyelinating neuropathy | NE | NE |

Abbreviations: A, absent; CMTNS, CMT neuropathy score; FDS, functional disability scale; NE, not examined

<sup>a</sup>Muscle weakness in lower limbs: + = ankle dorsiflexion 4/5 on the Medical Research Council (MRC) scale; ++ = ankle dorsiflexion <4/5 on the MRC scale; +++ = proximal weakness and wheelchair dependent. Muscle weakness in upper limbs: + = intrinsic hand weakness 4/5 on the MRC scale; ++ = intrinsic hand weakness <4/5 on the MRC scale; - = no symptoms

<sup>b</sup>Deep tendon reflexes: N = normal; D = diminished; A = absent
Neurological examination indicated that muscle weakness and atrophy started and predominated in the distal portions of the legs, and were noted to a lesser extent distally in the upper limbs. All of patients had pes cavus without scoliosis. Foot dorsiflexion (MRC, G0/5 to G2/5) was markedly weaker than foot plantarflexion (G4+/5 to G5/5) and finger abduction (G4/5 to G4+/5). Among the three patients, patient III-1 exhibited the most severity in muscle disability in the upper and lower limbs. All of them were able to walk independently with or without ankle-foot orthosis. Vibration sense was more severely affected than pain sense. Areflexia was noticed in the early stages of the disease, but pathologic reflexes were not found. On neurological and electrophysiological examination, her mother (I-2) showed normal findings. Based on history taking, her father (I-3) experienced no difficulty with walking.

SNAPs of the sural nerve were not detected at the early stage of the disease. Although CMAPs of the tibial motor nerves were evoked in all patients, SNAPs of sural nerves were not elicited at all (Table 3). All three patients had reduced amplitudes for the peroneal innervated foot muscles, and those responses were lesser than the responses from the thenar muscles. Needle EMG showed scattered fibrillation potentials and neurogenic motor unit action potentials.

### Fatty replacement in the lower leg

MRIs of the three patients revealed predominant signal changes in the anterior and lateral compartments of the lower leg muscles (Fig 2). T1-weighted coronal images showed more

| Patients  | II-4 | III-1 | III-3 | Normal values |
|-----------|------|-------|-------|---------------|
| **Motor nerve conduction studies (right / left)** | | | | |
| Median motor nerve | CMAPs (mV) | 12.8 / 10.7 | 10.0 / 9.5 | 7.7 / 9.9 | ≥ 6.0 |
| | MNCVs (m/s) | 21.9 / 21.6 | 19.4 / 16.9 | 15.7 / 17.3 | ≥ 50.5 |
| Ulnar motor nerve | CMAPs (mV) | 14.5 / 13.2 | 7.9 / 9.4 | 13.0 / 13.0 | ≥ 8.0 |
| | MNCVs (m/s) | 21.2 / 22.9 | 17.7 / 16.3 | 16.8 / 17.1 | ≥ 51.1 |
| Peroneal motor nerve | CMAPs (mV) | NP / NP | NP / NP | NP / 0.1 | ≥ 1.6 |
| | MNCVs (m/s) | NP / NP | NP / NP | NP / 17.9 | ≥ 41.3 |
| Tibial motor nerve | CMAPs (mV) | 3.4 / 0.9 | 0.2 / 0.1 | 1.0 / 1.6 | ≥ 6.0 |
| | MNCVs (m/s) | 17.2 / 15.9 | 15.3 / 17.8 | 16.3 / 14.0 | ≥ 41.1 |
| **Sensory nerve conduction studies (right / left)** | | | | |
| Median sensory nerve | SNAPs (µV) | 5.4 / 4.2 | NP / NP | 3.1 / 6.4 | ≥ 8.8 |
| | SNCVs (m/s) | 19.8 / 19.5 | NP / NP | 17.3 / 16.8 | ≥ 39.3 |
| Ulnar sensory nerve | SNAPs (µV) | 5.1 / 2.4 | NP / NP | 6.0 / 2.3 | ≥ 7.9 |
| | SNCVs (m/s) | 18.0 / 17.2 | NP / NP | 12.6 / 16.8 | ≥ 37.5 |
| Sural sensory nerve | SNAPs (µV) | NP / NP | NP / NP | NP / NP | ≥ 6.0 |
| | SNCVs (m/s) | NP / NP | NP / NP | NP / NP | ≥ 32.1 |

Abbreviations: CMAP, compound muscle action potential; MNCV, motor nerve conduction velocity; NP, no potential; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity.

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muscle atrophy and fatty replacement in the calf muscles than in the hip and thigh muscles (S1 Fig). We could observe a sequential pattern of muscle involvement associated with disease duration (Fig 2). It is noteworthy that the lateral compartment muscles including the peroneus longus and brevis were initially involved and they showed the most severe fatty hyperintense signal changes. In the early stage of the disease, diffuse fatty infiltration was observed in the lateral compartment muscles, whereas it was less severe in the anterior compartment muscles including the tibialis anterior, extensor hallucis longus, and extensor digitorum longus. In later stages, the posterior compartment muscles including the soleus, gastrocnemius, and tibialis posterior in the calf were still relatively unaffected (Fig 2). When we performed follow-up lower leg MRI studies at a 5-year interval, we could observe disease progression (S1 Fig). Muscle atrophy and fatty involvement in the calf were more rapid and severe than those in the thigh and hip muscles. At the hip and thigh level, the axial MR images were almost normal except for the bilateral semitendinosus and vastus lateralis muscles. At the calf level, the anterior and lateral compartment muscles showed more prominent fatty replacement than the posterior compartment muscles. In addition, brain MRI showed normal findings in the three patients (S2 Fig).

Histopathological findings

Light microscopic examination of longitudinal sections and cross sections of nerve fibers showed a slight decrease in the size of nerve fascicles as well as a decrease in the number of large myelinated fibers (MFs), and endoneurial fibrosis. Semi-thin transverse sections showed a normal range of MFs (8,601/mm²) with loss of large MFs, myelin abnormalities, onion bulbs, and regenerating axonal clusters (Fig 3). The histogram showed a unimodal distribution pattern of MFs. The range and average diameter of MFs were 0.86–12.61 μm and 2.59 μm, respectively. After excluding the largest MF (marked with an asterisk at the right upper corner of Fig 3A, diameter: 12.61 μm), the range and average diameter of the remaining MFs were 0.86–7.04 μm and 2.57 μm, respectively. The range and average diameter of MFs in the distal sural nerve of a normal 45-year-old female are 1.8–14.8 μm and 5.2 μm, respectively. The MF% area in this case was 5.5% (distal sural nerve of a normal 45-year-old female: 26.9%). The range and average of the g-ratio were 0.33–0.86 and 0.62±0.09, respectively (mean g-ratio in the age group of 21–50 years: 0.66). g-ratios above 0.7 comprised 18.7% of MFs and g-ratios less than 0.4 comprised 0.6% of the MFs. Electron microscopic examination showed frequent onion
bulb formation, large or medium sized MFs with myelin abnormalities, regenerating axonal clusters, and thin or thick MFs (Fig 3).

Transgenic mice exhibit peripheral neuropathy

To investigate the PMP2 mutation-associated pathogenesis, we generated transgenic mouse models for both wild type and mutant PMP2. Except for the peripheral neuropathy, both mice exhibited normal phenotype including normal development and breeding. To determine whether mice overexpressing human PMP2 exhibit motor deficits, a tail suspension test was performed. Both PMP2-WT and PMP2-I43N transgenic mice showed hind limb folding into the abdomen with infrequent clasping of their hind limbs (Fig 4). Both transgenic mice also showed reduced motor performance on the rotarod test. Therefore, transgenic mice overexpressing either wild type or mutant PMP2 definitely exhibited motor deficits compared to control mice.

To determine the nerve integrity in the hind limbs, nerve conduction was examined on both sides of the sciatic nerve at 5 months of age. Both transgenic mice showed significantly reduced MNCV compared to control mice (Fig 4). However, there was no difference in CMAP between control and PMP2 transgenic mice. These data suggest that the phenotypes of both PMP2 transgenic mice were similar to CMT1 rather than CMT2, which is caused by axonal defect and shows reduced CMAP.

Demyelination and shorter internodal length in transgenic mice

Semi-thin sections of the sciatic nerve displayed a reduced number of large myelinated fibers in PMP2-I43N mice compared to control mice, while PMP2-WT mice showed a pattern similar to the control mice. The average g-ratios were $0.75 \pm 0.07$ (control), $0.71 \pm 0.09$ (PMP2-WT),
and 0.69 ± 0.09 (PMP2-I43N). Electron microscopic analysis revealed mixed forms of demyelinated or dysmyelinated fibers in both PMP2-WT and PMP2-I43N mice compared to control mice (Fig 5). When we analyzed the teased nerve, the PMP2 transgenic mice revealed more frequent occurrence of Schmidt-Lanterman incisures (SLIs) (Fig 6). In addition, the mean internodal length in PMP2-WT and PMP2-I43N mice was 259.0 ± 14.6 μm and 229.3 ± 10.9 μm, which was significantly shorter than that in healthy controls (442.4 ± 15.8 μm; \( P < 0.001 \)) (Fig 6). However, the difference in internodal length between PMP2-WT and PMP2-I43N mice was not statistically significant. Considering the shortened internodal length in the transgenic mice, the number of SLIs per myelinating Schwann cell was within a similar range of 9.6 (PMP2-I43N) to 13.1 (PMP2-WT) per cell. Collectively, these data suggest that the overexpression of either wild type or mutant PMP2 affects Schwann cell integrity, resulting in reduced internodal length as well as demyelination, which might cause a demyelinating phenotype in transgenic mice.

![Fig 4. Phenotype of PMP2 transgenic mice.](image)

![Fig 5. Aberration in myelination was observed in PMP2 transgenic mice.](image)

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Discussion

This study strongly suggests that PMP2 mutation is the underlying cause of an autosomal dominant CMT1 phenotype. The same mutation in PMP2 was also suggested as a strong candidate for the cause of another CMT1 family [10]. Moreover, the pathogenicity of PMP2 mutation had been demonstrated in a zebrafish model and the relevance of the CMT1 phenotype was also observed in our transgenic mouse models. Exome data revealed many functionally significant variants in the CMT-relevant genes from the affected individuals. Although they were not considered as the underlying cause of the CMT1 phenotype, some of them may affect clinical severity as the modifying factor(s).

The clinical features of the present family were similar to PMP22 duplication. The age at onset among the patients was the first and second decades. The nerve conduction velocities in all three patients were below 38 m/s. Muscle weakness and atrophy started and predominated in the distal portions of the legs, and were noted to a lesser extent distally in the upper limbs. Moreover, the histopathological findings including onion bulb formation were consistent with the demyelinating type. In all patients, SNAPs of the sural nerve were not evoked at the early stage of the disease.

Additionally, there were clinical heterogeneities within the present family. The ages at onset of gait disturbance were in the first decade among the affected sons (III-1 and III-3), and in the second decade for their mother (II-4). Hand muscle weakness appeared in the third decade among the affected sons (III-1 and III-3), whereas it appeared in the fifth decade in their mother (II-4). Furthermore, the results of nerve conduction studies were mild in the mother, but were more severe in her sons.

MRI analysis revealed a distinct pattern of muscular involvement in patients with PMP2 mutation. Marked fatty replacement was observed in the calf muscle compared to the thigh muscle, which was consistent with the hypothesis of length-dependent degeneration of motor axons. These results are useful for discerning motor neuron disease from peripheral neuropathies like CMT1 with PMP2 mutation. In addition, we could observe a sequential pattern of muscle involvement associated with disease duration. In the early disease stage, the peronei muscles were involved, and they showed the most severe fatty replacement. The tibialis anterior muscles were also involved to a lesser degree. In the later stage, the soleus muscle in the calf was found to be relatively spared. These findings were similar to the demyelinating MR images.

![Fig 6. Abnormal SLI and internodal length in PMP2 transgenic mice.](image-url)
of CMT1A patients with PMP22 duplication, and they differed from the axonal MR images of CMT2A patients with MFN2 mutations. Compared to CMT2A, in which severe and predominant involvement of the soleus muscles has been reported, the present patients showed relative sparing of the soleus muscles, which suggests that the etiologies and pathophysologies of CMT2A with MFN2 mutation are different. Therefore, MRIs of patients with PMP2 mutation were well related to the demyelinating CMT neuropathy.

PMP2 is a peripheral myelin protein gene that encodes a small basic P2 protein. P2 is also called M-FABP/FABP8 and is a member of a family of fatty acid binding proteins with maximal specificity of expression in the peripheral myelin [11–13]. P2 enhances myelin membrane stability and lipid dynamics [14]. P2 forms a β-barrel, which is well conserved through the species as well as throughout the FABP family subtypes with 10 anti-parallel β-strands and an N-terminal helix-loop-helix cap, where fatty acids are bound inside the barrel [15–17]. Molecular docking simulations show that the best candidate for the P2 pocket is cholesterol, one of the most abundant lipids in myelin, with a polar head that suits the electrostatic characteristics of the binding site [16]. Hydrophobic contacts surround the cavity entrance while hydrophilic contacts are located deep in the binding pocket. The mutation site (p.I43N) lies inside the pocket and substitutes a non-polar Ile with a polar Asn. Therefore, the substitution may possibly alter the dynamics of the fatty acid capacitation. In silico analyses also predict that PMP2 mutation might affect protein function and stability.

The demyelinating features were also observed in PMP2 transgenic mouse models. Both PMP2-WT and PMP2-I43N transgenic mice exhibited reduced MNCV, which is compatible with the CMT1 phenotype. We found that PMP2 transgenic mice have shorter internodal length. Since internodal length is considered to affect the velocity of nerve impulse conduction, lower MNCV in transgenic mice might be due to the shorter internodal length [18]. Previously, shortened internodal length was reported in CMT1A patients and a Periaxin null mouse model [19,20]. In addition, a cell culture model using Trembler J mouse also showed reduced internodal length [21]. Therefore, shortened internodal length might be a characteristic feature of CMT1.

Since both transgenic mice either overexpressing wild type or mutant PMP2 exhibited a similar neuropathic phenotype, we could not determine whether p.I43N mutation was pathogenic or not until the zebrafish model for PMP2 was reported [10]. Overexpression of either wild type or mutant (p.I43N) PMP2 in zebrafish also led to defects in axonal branching. Therefore, these data imply that PMP2 might result in peripheral neuropathy in the same manner as the well-characterized PMP22 gene, which affects myelination by either mutation in one allele or overdose of the wild type gene. In addition, in vitro assay showed that overexpression of both wild type and mutant PMP2 in rat Schwann cell induced the markers of endoplasmic reticulum stress, which might result in cell death (S3 Fig).

Compared to the zebrafish model, our mouse models exhibited a clear CMT1 phenotype. The zebrafish model showed that aberration in PMP2 expression exhibited motor neuron phenotypes such as axonal branching, which is somewhat different from the primary pathogenesis of CMT1. On the other hand, the zebrafish model clearly demonstrated the pathogenicity of p.I43N mutation. Overexpression of PMP2-I43N in zebrafish showed limited benefits compared to wild type PMP2. In this study, we could not directly observe the PMP2 mutation-associated neuropathy. Phenotypic severity in the PMP2-143N transgenic mouse compared to the PMP2-WT mouse was limited. Although PMP2-I43N transgenic mice exhibited worse parameters including rotarod performance, MNCV, g-ratio and internodal length than PMP2-WT mice, the differences were not statistically significant. For clear demonstration of PMP2 mutation-associated neuropathy, further studies using knock-in mouse models are needed.

In this study, we first report the clinical features of patients with CMT1 caused by PMP2 mutation as well as the characterization of transgenic mice. This report might expand the
genetic and clinical features of CMT and a further mechanism study will enhance our understanding of the PMP2 associated peripheral neuropathy.

Materials and Methods

Ethics statement
The study involving human patients was approved by the Institutional Review Board of Sungkyunkwan University, Samsung Medical Center (approval number: 2013-10-066). Written informed consent was obtained from all participants. All animal studies were conducted according to protocols approved by the Institutional Animal Care and Use Committees of Samsung Medical Center (approval number: 2013-080-2002).

Patients
This study enrolled a Korean autosomal dominant CMT1 family (family ID: FC183, Fig 1) with 9 members (3 affected and 6 unaffected members) who showed no 17p12 duplication/deletion. In addition, 500 healthy controls, who had no family history of neuromuscular disorders, were enrolled after careful clinical and electrophysiological examination.

Clinical assessment
Patients were evaluated through a detailed history including the assessment of motor impairments, sensory loss, deep tendon reflexes, and muscle atrophy. Age at onset was determined by asking the patients their ages, when symptoms such as distal muscle weakness, foot deformity, or sensory change first appeared. Muscle strengths of flexor and extensor muscles were assessed manually using the standard Medical Research Council (MRC) scale [22]. In order to determine physical disability, we used two scales, a functional disability scale (FDS) and a CMT neuropathy score (CMTNS) [23,24]. Sensory impairments were assessed in terms of the level and severity of pain, temperature, vibration and position, then pain and vibration senses were compared. The Nine-Hole Peg Test (9-HPT) was performed using both dominant and non-dominant hands; five consecutive trials for the dominant hand, followed immediately by another five consecutive trials for the non-dominant hand.

Electrophysiological examination
Electrophysiological studies were carried out in the 3 affected individuals. Nerve conduction studies were performed by placing surface electrodes on median, ulnar, peroneal, tibial, and sural nerves. MNCVs for the median and ulnar nerves were determined by stimulating at the elbow and wrist while recording compound muscle action potentials (CMAPs) over the abductor pollicis brevis and adductor digiti quinti, respectively. In the same manner, the MNCVs of the peroneal and tibial nerves were determined by stimulating at the knee and ankle, while recording CMAPs over the extensor digitorum brevis and adductor hallucis, respectively. Sensory nerve conduction velocities (SNCVs) were obtained over a finger-wrist segment from the median and ulnar nerves by orthodromic scoring, and were also recorded for the sural nerves. Electromyography (EMG) was performed for the first dorsal interosseous, biceps brachii, tibialis anterior, medial gastrocnemius, and vastus lateralis muscles.

Lower limb and brain MRI studies
The three patients (Fig 1: II-2, III-2, and III-4) underwent examination by lower limb MRIs of the hip, thigh, and calf muscles. MRI was performed using a 1.5-T system (Siemens Vision, Siemens, Germany). The imaging was conducted in the axial [field of view (FOV) 24–32 cm,
slice thickness 10 mm, and slice gap 0.5–1.0 mm] and coronal planes (FOV, 38–40 cm; slice thickness 4–5 mm; and slice gap 0.5–1.0 mm). The following protocol was used: T1-weighted spin-echo (SE) (TR/TE 570–650/14–20 ms), T2-weighted SE (TR/TE 2800–4000/96–99 ms), and fat-suppressed T2-weighted SE (TR/TE 3090–4900/85–99 ms). In addition, brain MRI was performed in the three patients (II-4, III-1, and III-3) by using a 1.5-T system (Siemens Vision, Siemens, Germany). Whole brains were scanned using a slice thickness of 7 mm and a 2-mm interslice gap to produce 16 axial images. The imaging protocol consisted of T2-weighted spin echo (TR/TE = 4,700/120 ms), T1-weighted spin echo (TR/TE = 550/12 ms), and fluid-attenuated inversion recovery (FLAIR) (TR/TE = 9,000/119 ms, inversion time 2,609 ms) images.

Histopathologic studies

Patient II-4 underwent distal sural nerve biopsy at the age of 56 years. Pathological examinations included light and electron microscopic analysis. One sural nerve fragment was fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin, modified Masson’s trichrome, Luxol fast blue, and Bodian stain. For the electron microscopic observation, another fragment was immediately fixed with 2% glutaraldehyde in 0.025 M cacodylate buffer at pH 7.4 and post-fixed in 1% osmium tetroxide. Epon-embedded semi-thin and ultra-thin sections were prepared for light and ultra-structural examinations.

DNA preparation, exome sequencing and filtering

DNA was purified from peripheral blood using the QIAamp blood DNA purification kit (Qiagen, Hilden, Germany). Exome sequencing was performed on 5 members of the FC183 family (3 affecteds: II-4, III-1, III-3; and 2 unaffecteds: II-1, II-3). Exomes were captured using the SeqCap EZ ver 3.0 (Roche-NimbleGen, Madison, WI), and sequencing was performed using the HiSeq 2000 Genome analyzer (Illumina, San Diego, CA). Sequences were mapped to the human reference genome UCSC assembly hg19, and variants were called by the SAMtools program (http://samtools.sourceforge.net/). We first selected functionally significant variants (missense, nonsense, exonic indel and splicing site variants) and then filtered out polymorphic variants registered in the dbSNP (http://www.ncbi.nlm.nih.gov), 1000 Genomes project database (1000G, http://www.1000genomes.org/), Exome Sequencing Project (ESP, http://evs.gs.washington.edu/EVS/), and Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/). Next, we further selected variants that cosegregated with the three affected individuals within the family and were not found in the 500 healthy controls. Conservation analysis of protein sequences was conducted with MEGA ver 5.05 program [25]. Genomic evolutionary rate profiling (GERP) scores were determined with the GERP++ program (http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html). In silico analyses were performed to predict the deleterious nature of the protein function due to amino acid substitution using SIFT (http://sift.jcvi.org/) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/); while protein stability distortion due to amino acid substitution was predicted with the MUpro program (http://mupro.proteomics.ics.uci.edu/).

Generation of transgenic mouse model

To obtain the human PMP2 gene, total mRNA from HEK 293 cells was used as a template for cDNA synthesis and PCR amplification. Mutation (p.I43N) of PMP2 was generated by site-directed mutagenesis using the QuiKChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and all sequences were confirmed by capillary sequencing. To establish transgenic mouse models for wild type and mutant PMP2, cloned vectors were injected into fertilized
eggs. The eggs were implanted into surrogate female mice, then transgenic mice were generated.

**Behavior and electrophysiological examination of transgenic mice**

To evaluate the motor coordination of PMP2 transgenic mice, rotarod tests were performed on a 3 cm horizontal rotating rod at a speed of 2 m/min. To adapt to the test, mice were pre-trained for one week. For the electrophysiological test, 10 of each control and transgenic mice, aged 5 months and weighing 25–30 g, were used. The mice were anesthetized with Zoletil (50 mg/kg) intraperitoneally (Virbac, Seoul, Korea) and the fur from the distal back and the hind limbs was completely removed. The CMAP and MNCV were determined by using the Nicolet VikingQuest (Natus Medical, San Carlos, CA) as previously described [26].

**Histological examination of mice nerves**

Histological analysis of mouse sciatic nerves was performed in the same manner with the patient samples. To visualize the Schmidt-Lanterman incisures (SLI) and nucleus, teased nerves were stained with rhodamine conjugated Phalloidin (Thermo Fisher Scientific, Waltham, MA) and mounted with 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI) containing Vectashield (Vector Laboratories, Burlingame, CA) as previously reported [27]. Internodal length was determined by measuring the distance of each nuclei stained with DAPI according to a previous report [28].

**Statistical analysis**

All animals were studied with a blind test. Comparison between normal and transgenic mice were made by Student’s t-test. P<0.05 was considered statistically significant.

**Supporting Information**

S1 Table. Whole exome sequencing summary for five members of the family FC183. (DOCX)

S2 Table. Functionally significant variants observed in CMT causative genes in the patients of the FC183 CMT1 family. (DOCX)

S1 Fig. Follow-up of lower limb MR images of III-1 patient aged 32 years (A-D) and 37 years (E-H). T1-weighted coronal (A, B, E and F), and axial (C, D, G and H) MRIs of the middle thigh (C and G), and calf (D and H) are shown. In these MRI studies at a 5-year interval, we can observe disease progression. Distal leg muscle atrophy and fatty replacement were more severe than those in proximal leg muscles. (A, C, E, and G) At the hip and thigh level, the axial MR images were almost normal in both patients except for the semitendinosus and vastus lateralis muscles. (B, D, F, and H) At the calf level, the anterior and lateral compartment muscles showed more prominent fatty replacement than the posterior compartment muscles. (TIF)

S2 Fig. Brain MRI of the III-1 patient at the age of 38 years with PMP2 mutation showed normal findings. (TIF)

S3 Fig. Cell death and endoplasmic reticulum (ER) stress induced by PMP2 proteins. (A) Cell death by overexpression of wild-type and mutant PMP2 or PMP22 genes were determined.
Rat Schwann cell line, RT4, was transfected with indicated vectors for 72 h, then cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The viability was displayed as % of control vector (pCMV-Myc). Data are presented as mean± SEM. *p < 0.05. (B) Standard Western blotting exhibits induction ER stress markers, BiP and CHOP, by overexpression of wild-type and mutant PMP2, which were fused with Myc-epitope. RT4 cells were transfected with indicated vectors for 48 h, then expression levels of each protein were analyzed.

Author Contributions
Conceived and designed the experiments: YBH KWC BOC. Performed the experiments: JJ GK YRC HKY DHJ EJK WMM JHY YSH SHN SMK HK. Analyzed the data: YBH JHY HK HTP. Wrote the paper: YBH HTP KWC BOC.

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