**RESEARCH ARTICLE**

**PMP22 exon 4 deletion causes ER retention of PMP22 and a gain-of-function allele in CMT1E**

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**Abstract**

**Objective:** To determine whether predicted fork stalling and template switching (FoSTeS) during mitosis deletes exon 4 in peripheral myelin protein 22 KD (PMP22) and causes gain-of-function mutation associated with peripheral neuropathy in a family with Charcot–Marie–Tooth disease type 1E. **Methods:** Two siblings previously reported to have genomic rearrangements predicted to involve exon 4 of PMP22 were evaluated clinically and by electrophysiology. Skin biopsies from the proband were studied by RT-PCR to determine the effects of the exon 4 rearrangements on exon 4 mRNA expression in myelinating Schwann cells. Transient transfection studies with wild-type and mutant PMP22 were performed in Cos7 and RT4 cells to determine the fate of the resultant mutant protein. **Results:** Both affected siblings had a sensorimotor dysmyelinating neuropathy with severely slow nerve conduction velocities (<10 m/sec). RT-PCR studies of Schwann cell RNA from one of the siblings demonstrated a complete in-frame deletion of PMP22 exon 4 (PMP22Δ4). Transfection studies demonstrated that PMP22Δ4 protein is retained within the endoplasmic reticulum and not transported to the plasma membrane. **Conclusions:** Our results confirm that FoSTeS-mediated genomic rearrangement produced a deletion of exon 4 of PMP22, resulting in expression of both PMP22 mRNA and protein lacking this sequence. In addition, we provide experimental evidence for endoplasmic reticulum retention of the mutant protein suggesting a gain-of-function mutational mechanism consistent with the observed CMT1E in this family. PMP22Δ4 is another example of a mutated myelin protein that is misfolded and contributes to the pathogenesis of the neuropathy.

**Introduction**

Charcot–Marie–Tooth (CMT) disease is the most common inherited peripheral neuropathy affecting approximately 1:2500 individuals.¹ Roughly half of these patients have CMT1A,² a demyelinating neuropathy caused by a 1.4-Mb duplication in chromosome 17p12 that contains the peripheral myelin protein 22 gene, PMP22, an integral membrane protein expressed in PNS compact myelin.³,⁴ CMT1A is hypothesized to occur as a result of nonallelic homologous recombination (NAHR)⁵-⁷ in a region of 17p12 surrounding and including the PMP22 gene, causing increased expression of the normal gene product.

Point mutations in PMP22 gene can cause a relatively severe demyelinating or dysmyelinating peripheral neuropathy, in which myelin never forms normally during development. Mutations within the PMP22 gene are classified as CMT1E.² Most CMT1E mutations are missense mutations probably resulting in a gain-of-new protein function.⁸ In contrast, some PMP22 point mutations...
produce a loss-of-protein function by a shift in the protein reading frame, leading to premature termination of translation. A similar loss of function can also be caused by the reciprocal deletion of PMP22 within 17p12 that caused the CMT1A duplication. Loss of PMP22 function leads to an electrophysiologically distinct inherited neuropathy known as hereditary neuropathy with pressure palsies. Recently, it was proposed that abnormalities of fork stalling and template switching (FoSTeS) or microhomology-mediated break-induced replication (MMBIR) caused a complex rearrangement within an individual PMP22 allele that resulted in CMT1 in a family in which two siblings, but neither parent, developed CMT1. The rearrangements were identified in DNA from white blood cells and were hypothesized to result from three separate FoSTeS/MMBIR-mediated template switching events that were predicted to specifically disrupt the sequence of PMP22 exon 4 in the patients. The mother was found to be mosaic for the same rearrangement with lower levels in the mutated allele in her WBC. Both siblings had markedly reduced nerve conduction velocities, whereas the conduction velocities of the mother were normal.

We had the opportunity to examine both affected siblings and further test this hypothesis by evaluating PMP22 expression from myelinated nerves obtained from skin biopsies. RT-PCR analysis from the skin biopsies revealed the absence of exon 4 sequences in PMP22 mRNA, consistent with the analysis of genomic DNA as described by Zhang and coworkers. Transfection studies demonstrated that the mutant protein is retained in the endoplasmic reticulum, which is the most parsimonious explanation for its pathogenesis via a gain-of-function mutational mechanism.

Methods

Clinical evaluation

The two siblings were evaluated clinically at the University of Iowa CMT clinic at ages 9 and 13 years. Neurological examination and neurophysiological and skin biopsy studies were performed on the patients during their clinic visit. A skin biopsy was obtained following informed consent as part of an orthopedic procedure at Washington University, St. Louis. Informed consent according to the Declaration of Helsinki and the Institutional Review Boards at the participating centers approving the study was obtained from both siblings and their parents.

Skin biopsy and RT-PCR

The skin sample was evaluated as described previously. The sample was treated in freshly prepared RNALater (Qiagen, CA, Cat. No.: 1017980). RNA was isolated with RNeasy Micro Kit (Qiagen, CA, Cat. No.: 74004) followed by the RT reaction in SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, CA, Cat. No.: 12371-019) and then the PCR in Platinum PfX DNA Polymerase (Invitrogen, CA, Cat. No.: 11708-015). The upstream and downstream primers were 5′-GGGCAGAAAACCTCGGCTGACGAGGAGA-3′ and 5′-GTACGCTAGGCCCTCAGACGAC-3′, respectively. PCR conditions were 95°C 2 min/95°C 15 sec, 60°C 30 sec, 68°C 1 min for 35 cycles/68°C 10 min. Sanger sequencing was performed to analyze the products after cloning with Zero Blunt TOPO PCR Cloning Kit with One Shot TOP10 Electrocamp Chemically Competent Escherichia coli (Invitrogen, CA, Cat. No.: K2860-20).

Preparation of wild-type and mutant PMP22 constructions

The cDNAs encoding both wild-type and mutant human PMP22 mRNA sequences were synthesized from the patient’s skin biopsy RNA by RT-PCR and cloned into an expression vector with an HA tag at its 3′ end (Cat. No.: A921149-1, Lucigen Corp. Middleton, WI). The HA tag was obtained from the pME-HA vector (Cat. No.: A921149-1). Both of these constructs were used in the transfection studies described below.

Cell culture, transfection, and immunocytochemistry

Cos 7 cells (American Type Culture Collection) and RT4 rat Schwann cells (Gift from Dr. John Svaren) were separately grown on four-well chamber slides in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin and transfected with 2-µg plasmid DNA using Lipofectamine™ 3000 (Invitrogen). Cells were transfected with the following plasmids: (1) WT-PMP22, (2) PMP22-exon 4 deletion – each plasmid with the hemagglutinin epitope tag fused to the N-terminus. Forty-eight hours after transfection, immunocytochemistry was carried out as described previously. In brief, cells were rinsed twice in PBS and then fixed in 4% paraformaldehyde for 5 min. After being washed three times in PBS, cells were incubated with primary antibodies at 4°C overnight: Antihemagglutinin (anti-HA), 1:1000 (Cell Signaling Technology® Cat#2367 [mouse]; #3724 [rabbit]); anticalnexin (marker for endoplasmic reticulum), 1:300 (Abcam, Cat# ab22595); antigiantin (marker for Golgi apparatus), 1:300 (Abcam, Cat# ab37266). Following three 10-min washes in PBS and 1 h incubation with secondary antibodies (Cy3-conjugated donkey anti-rabbit IgG, Jackson Immuno Research, Cat#711-165-152 or anti-mouse,
Cat#711-165-1521; fluorescein [FITC]-conjugated Affini-Pure Donkey anti-mouse IgG, cat#712-095-151; or anti-rabbit IgG cat#711-095-152), the coverslips were mounted applying Anti-Fade 4-6-diamino-2-phenylindole (DAPI) mounting media that labeled nuclei (Invitrogen Cat# P36931).

We determined the transfection efficiency by taking 10 images under 40 × objective lens per group; WT-PMP22 and PMP22-exon 4 deletion. HA-tag-labeled cells were counted as a positively transfected cell. We then divided this number by total nuclei number stained with DAPI. Transfected RT4 cells were imaged (× 63 magnification, repeated 3 times) magnification with a Zeiss 710 confocal microscope in order to demonstrate cell surface expression, endoplasmic reticulum or Golgi retention of PMP22-exon 4 deletion mutation or PMP22wt. The number of colocalized cells that showed overlap of HA-PMP22 and ER or Golgi was divided by the total number of transfected cells to obtain percentage of colocalization.

Results

Clinical summary

We evaluated two sisters who had been previously demonstrated to have complex rearrangement of PMP22 exon 4 that they had inherited from their asymptomatic mother who was mosaic for this unusual mutation.17

Both sisters had evidence of a significant sensorimotor dysmyelinating peripheral neuropathy that began in
infancy. Neither sister walked independently until 16–17 months of age. The older sister was diagnosed based on diffuse weakness and absent deep tendon reflexes prior to the age of 2. She was initially thought to have chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), received treatment with periodic intravenous gamma globulin (IVIG) between ages 2 and 3 and she did not improve. The IVIG was discontinued when the younger sister was also found to have neuropathy and it was realized that the neuropathy was probably genetically based. The older sister presented to us with symmetrical bilateral foot drop. She had been prescribed ankle foot orthotics (AFOs) that she initially rarely wore. At age 13, she did not consider balance to be a major problem and was falling rarely. She denied difficulties with fine hand movements such as manipulating buttons, zippers, writing, or manipulating small objects. Two years later she was falling more frequently and was using AFOs regularly, at least for longer distances. The younger sister was thought by her parents to be more severely affected in part because of asymmetrical proximal weakness of the left leg, which began to develop at between the ages of 7 and 8 years. She was diagnosed with mild hip dysplasia on the right, asymptomatic side for which she underwent surgery. However, the asymmetrical weakness on the left progressed until the left hip extensor strength was only 2/5 MRC scale. Ultrasound at age 10 showed reduced thickness of the left leg muscles including the quadriceps (Fig. 1A). However, ultrasound studies also showed enlargement of both right and left femoral nerves (Fig. 1B) as was the case with other nerves evaluated (data not shown). Additionally, ultrasound of both sisters, ages 2 and 6 years, showed diffuse nerve enlargement, with median and ulnar nerves three to five times larger than normal (data not shown). The possibility of a superimposed inflammatory component was considered at age 10 and she responded to twice weekly corticosteroid treatment with return of measurable strength in left quadriceps and iliopsoas (4/5 MRC). At age 11 years, the younger sister does use the elevator at school to go upstairs. The clinical examination of both siblings is provided in Table 1. Motor nerve conduction velocities

### Table 1. Clinical features of siblings with PMP22 exon 4 deletion.

| Patient age (years) | CMTNSv2 | Distal weakness LL | Proximal weakness LL | Distal weakness UL | Proximal weakness UL | Vibration LL | Vibration UL | Cutaneous LL | Cutaneous UL |
|---------------------|---------|---------------------|----------------------|-------------------|---------------------|--------------|--------------|--------------|--------------|
| 86687-0001          | 13      | (moderate)          | + (4,5)              | – (5,5)           | +(4,5,4)            | – (5,5,5)    | Red toes, ankles, and knees | Red toes, wrist, and elbows | Red toes, Normal |
| 86687-0100          | 9       | (moderate)          | + (4,5)              | – (2,4,4)         | + (4,5,4)           | – (5,5,5)    | Red toes, ankles, and knees | Red toes, wrist, and elbows | Red toes, Normal |

Motor weakness based on MRC scale (0–5): + = weakness present, – = no weakness detected. LL distal weakness assessed by anterior tibialis and gastrocnemius, LL proximal weakness assessed by ilio psoas and quadriceps; UL distal weakness assessed by first dorsal interosseous, abductor pollicis brevis, and adductor digitii minimi; UL proximal weakness assessed by deltoids, biceps brachii, and triceps. Vibration based on Rydell tuning fork with “5” on scale of “8” being considered normal and cutaneous based on pinprick sensation: Normal is no decrease compared to the examiner, Red is reduced, and abs is absent up to level indicated. Both motor and sensory evaluations were based on worst score observed of the two limbs. CMTNSv2 scores are separable into <10 (mild), 11–20 (moderate), or >20 (severe) impairment. CMTPeds scores range from 0 to 44 with higher numbers suggesting greater disability.

### Table 2. Motor NCVs.

| Individual | Age | Side | Ulnar nerve | Median nerve | Peroneal nerve |
|------------|-----|------|-------------|--------------|---------------|
|            |     |      | DML (ms)    | NCV1 (m/sec) | 0.13          |
| 86687-0001 | 13  | L    | <3.4 49     | >50 >2.8     | 10 1.4        |
| 86687-0100 | 9   | L    | 21.6 6      | 5 0.13       | 31.6 8        |
| 86687-0100 | 9   | R    | 9.7 8       | 5.3 2.5      | –             |

Normal values for each nerve in top row under DML, NCV, and CMAP. DML, distal motor latency; NCV, nerve conduction velocity; NCV1, NCV between wrist/elbow; NCV2, NCV around elbow; NCV3, NCV between ankle/knee; NCV4, NCV around knee; CMAP, compound muscle action potential. Bold letters signify abnormal values.
(MNCV) were severely reduced to between 5 and 10 m/sec in the upper extremities for both sisters (Table 2). Sensory nerve conduction velocities were unobtainable (data not shown).

**Genotyping and sequencing mutant PMP22**

We extracted RNA from dermal nerve tissue in skin biopsies of both the older sister and an unaffected control, and then amplified the PMP22 transcript by RT-PCR. In the proband’s lane, we identified two bands: a 552-bp band that migrated to the size predicted for the normal PMP22 transcript length, as seen in the positive control lane, and a 411-bp band that suggests a truncated PMP22 transcript (Fig. 2A). The 141 bp truncation was the predicted size of PMP22 mRNA for this RT-PCR product for which exon 4 is deleted (Fig. 2B). We compared transcript sequences between the two bands; the sequencing confirmed an exon 4 in-frame deletion resulted in the smaller band, with no changes to the sequence at the PMP22 C-terminus postdeletion (Fig. 2C). Sequencing of the upper band revealed the full PMP22 coding sequence from the normal allele including exon 4. The deletion of exon 4 in the mutant transcript of our proband resulted in the loss of 47 amino acids (AA#60-106), most of which comprise the hydrophobic second transmembrane region of PMP22 protein. Translation of subsequent amino acids (AA#107-160) was not affected (Figs. 2D and 3).

**Figure 2.** RT-PCR and sequencing analysis of PMP-22 transcript. RT-PCR products amplified from RNA extracted from the proband and control skin biopsies and a control sural nerve biopsy. The band of approximately 552 bp corresponds to the normal PMP22 transcript and the smaller band of approximately 411 bp corresponds to the misspliced PMP22 transcript of the proband. (B) Exon-intron structure of PMP22 shows the skipping of exon 4 caused by aa417 + 2 T>G. The nucleotide numbers in parenthesis indicate the predicted size of coding sequences for each transcript; the wild type and the mutant. (C) The PCR products were sequenced. Comparisons between wild-type and mutant transcripts demonstrate the loss of exon 4.
Cos7 localized to the plasma membrane. PMP22 transfected cells. The majority of wtPMP22 in transfected RT4 rat Schwann cells, and investigated their fate in separate cultured cell lines: non-neuronal Cos7 cells and introduced the exon 4 deletion performed a series of transient transfection studies. We To determine the consequences of the exon 4 deletion we performed studies with a cDNA construct encoding the mutant protein demonstrated that PMP22Δ4 is retained within the ER and is not expressed on the cell surface. Taken together, these data suggest that PMP22Δ4 causes CMT1E by a toxic gain-of-function mechanism.

Three FoSTes/MBIR events with microhomologies at breakpoint junctions were previously identified in our patients by high-density oligonucleotide array comparative genomic hybridization (aCGH). These events were predicted to result in at least a partial deletion of exon 4.17 Our RT-PCR, which is based on mRNA expression, confirmed this prediction by demonstrating the complete deletion of exon 4 in myelinating Schwann cells in the proband. Primers used for the RT-PCR spanned the PMP22 gene from exon 1 to the 3’ UTR and were therefore predicted to detect any splicing abnormalities in the resultant mRNA. Since only the wild-type and exon 4 deleted bands were observed (Fig. 1), this suggested to us that the only splicing abnormality that occurred was the deletion of exon 4. Thus, in the proband, there was no evidence of variability in spliced products from the exon 4 rearrangement, and we think it unlikely that variability of splicing caused the transient asymmetry in the younger sister. We speculated on why there were significant asymmetries in the younger sister; in particular, the quadriceps weakness of the left leg. Because the results from her sister’s biopsy revealed expression of only wild-type PMP22 and PMP22Δ4, we considered it unlikely that myelinating Schwann cells ensheathing the femoral nerve from the

**Figure 3.** Peripheral myelin protein 22 (PMP22) secondary protein structure. Amino acids circled in red are encoded by exon 4 which is deleted in the patients.
Figure 4. ER retention of PMP22Δ4. Transient transfection studies were performed in Cos7 (A) and RT4 (B) with WT PMP22 or PMP22Δ4 DNA constructs. Arrows point to PMP22Δ4 (HA) colocalization with the ER (calnexin). Arrowheads demonstrate PMP22 transported to cellular membrane. In PMP22Δ4 transfected cells, significantly more cells were retained in the ER compared to wild-type cells (C).
right leg expressed a different PMP22 mRNA than the left leg. Furthermore, ultrasound showed no asymmetry of the femoral nerves. We recognized that HNPP, which is caused by the deletion of an entire PMP22 allele, is characterized by episodes of asymmetric weakness or sensory loss, particularly at sites of compression.18 However, there were no other features that suggested HNPP in either sibling. For example, neither sister had previously noted transient focal symptoms and their overall presentations were quite different from those of HNPP. HNPP, a clinical phenotype resulting from PMP22 loss of function, typically has focal areas of slowing on nerve conduction studies, but outside of these regions the NCV are often nearly normal.19 Moreover, outside of the focal episodes, most patients with HNPP have at least relatively normal neurological examinations. These two girls were not only more affected clinically than most patients with HNPP, but they were also more affected than many patients with CMT1A which is caused by PMP22 gain of function – most often resulting from a duplication of PMP22. Accordingly, we hypothesized that our patients’ phenotype is caused by a toxic gain of function caused by the mutant PMP22 lacking exon 4. Given the recovery of significant quadriceps function, this seems to be a superimposed problem, perhaps related to an inflammatory process. However, we were not able to explain the younger sisters asymmetries based on the specific deletion of exon 4.

Most PMP22 missense mutations and other mutations within the PMP22 gene cause neuropathies that are clinically more severe than CMT1A and are therefore also likely to induce toxic gain of function abnormalities in myelinating Schwann cells.8 Mutations that cause severe neuropathies beginning in infancy are said to cause a Dejerine–Sottas syndrome (DSS) and those that prevent normal myelination are said to induce congenital hypomyelination (CHM).8 Met69Lys and Ser72Leu PMP22 have been reported to cause DSS,20 Ser72Leu PMP22 has also been reported to cause CHO21 as has Cys109Arg PMP2222 to provide several examples. Alternatively, other PMP22 mutations have been reported to cause a typical HNPP phenotype. These include Leu4Ter,9 Leu7 fs,10,11 Gly94 fs,12,23 Tyr97 fs,24 and Thr99 fs25 PMP22. Mutations that cause an HNPP phenotype eliminate the mutant allele by processes such as nonsense-mediated decay.11,26 Thus, these are complete loss-of-function alleles similar to traditional HNPP in which the PMP22 allele is deleted.13 How other mutations cause a gain of function is not known, but clues about mechanisms can be inferred from studies performed in the naturally occurring Trembler (Tr) and Trembler J (TrJ) mouse models of CMT1E. Tr and TrJ result from Gly150Asp27 and Leu16Pro PMP22,28 respectively. Both Tr29 and Tr30,31 mutations cause DSS in humans, and the mice can be used to model the human neuropathies. Leu16Pro PMP22 has been shown to be retained in the ER of Schwann cells in TrJ mice.32 Moreover, the mice have demonstrated improvement following treatment with curcumin to reduce ER stress.32 Similar results have been found in two missense mutations in myelin protein zero (MPZ) that cause CMT1B. The Ser63del and Arg98Cys MPZ mutations causing CMT1B are retained within the ER and are thought to cause neuropathy in part by activating the unfolded protein response (UPR) secondary to ER stress.33,34 The three arms of the UPR work together in part to relieve ER stress by reducing the overall translation of proteins by the cell.35 Therapies directed at relieving ER stress and attenuating the UPR have improved the neuropathy of both Ser63del33,36 and Arg98Cys16 mice. Our data suggest that this process may also occur with PMP22A4, further expanding the role of ER stress and UPR activation in the pathogenesis of what are often more severe forms of CMT1.

Acknowledgments

The study was supported in part by the INC (Shy PI) (U54NS065712), which is a part of the NCATS Rare Diseases Clinical Research Network (RDCRN). RDCRN is an initiative of the Office of Rare Diseases Research (ORDR), NCATS, funded through a collaboration between NCATS and the NINDS. The work was also supported by the funding from the Muscular Dystrophy Association (MDA) and Charcot–Marie–Tooth Association (CMTA). Dr. Lupski acknowledges support from the NIH (R01 NS058529).

Authors’ Contributions

Mr. Wang, Dr. Wu, and Dr. Bai performed the experiments involved in cloning, tissue culture, transfections, and RT-PCR studies. Dr. Zaidman performed the ultrasound studies and was involved in the interpretation of the data. Dr. Kamholz and Lupski were involved in the interpretation of the data and in the writing of the manuscript. Dr. Connolly was involved in evaluating the patients and analyzing their clinical phenotype. Ms. Grider was involved in caring for and evaluating the family as well as contributing to the writing of the paper. Dr. Shy designed the experiments, evaluated the patients and designed, and was the primary writer of the manuscript.

Conflicts of Interest

Dr. Lupski: Baylor College of Medicine (BCM) and Miraca Holdings, Inc., have formed a joint venture with
shared ownership and governance of Baylor Genetics (BG), formerly the Baylor Miraca Genetics Laboratories (BMGL), which performs clinical exome sequencing. J. R. L. is an employee of BCM, serves on the Scientific Advisory Board of the BG, and derives support through a professional services agreement with the BG. JRL has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals, has stock options in Lasergen, Inc., and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting.

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