SIMPLE Mutation in Demyelinating Neuropathy and Distribution in Sciatic Nerve

Craig L. Bennett, PhD,1 Andrew J. Shirk, BSc,1 Huy M. Huynh, BSc,1 Valerie A. Street, PhD,2 Eva Nelis, PhD,3 Lionel Van Maldergem, MD, PhD,4 Peter De Jonghe, MD, PhD,1,3,5 Albena Jordanova, PhD,3,5 Velina Guergueltcheva, MD,5,6 Ivailo Tournev, MD, DSc,6 Peter Van den Bergh, MD,7 Pavel Seeman, MD,8 Radim Mazanec, MD,7 Tomas Prochazka, MD,8 Ivo Kremensky, MD, PhD,7 Jana Haberlova, MD,9 Michael D. Weiss, MD,10 Vincent Timmerman, PhD,3 Thomas D. Bird, MD,10,11 and Philip F. Chance, MD,10

Charcot–Marie–Tooth neuropathy type 1C (CMT1C) is an autosomal dominant demyelinating peripheral neuropathy caused by missense mutations in the small integral membrane protein of lysosome/late endosome (SIMPLE) gene. To investigate the prevalence of SIMPLE mutations, we screened a cohort of 152 probands with various types of demyelinating or axonal and pure motor or sensory inherited neuropathies. SIMPLE mutations were found only in CMT1 patients, including one G112S and one W116G missense mutations. A novel T74I polymorphism was identified, yet no splicing defect of SIMPLE is likely. Haplotype analysis of STR markers and intragenic SNPs linked to the gene demonstrated that families with the same mutation are unlikely to be related. The clustering of the G112S, T115N, and W116G mutations within five amino acids suggests this domain may be critical to peripheral nerve myelination. Electrophysiological studies showed that CMT1C patients from six pedigrees (n = 38) had reduced nerve conduction velocities ranging from 7.5 to 27.0m/sec (peroneal). Two patients had temporal dispersion of nerve conduction and irregularity of conduction slowing, which is unusual for CMT1 patients. We report the expression of SIMPLE in various cell types of the sciatic nerve, including Schwann cells, the affected cell type in CMT1C.


Charcot–Marie–Tooth neuropathy type 1 (CMT1) includes a large group of inherited disorders characterized by peripheral nerve demyelination affecting both motor and sensory nerves.1 The natural history of CMT1 is a slowly progressive distal muscle weakness and atrophy in the upper and lower limbs with loss of sensation.2 Hallmarks of CMT1 include reduced nerve conduction velocities (NCVs) and nerve biopsies that display “onion bulb” formation reflecting repeated cycles of demyelination followed by remyelination.

To date, five genes have been identified that, through mutation, cause subtypes of CMT1 (see http://molgen-www.uia.ac.be/CMTMutations/). Recently, linkage for CMT type 1C to chromosome 16p13.13 was established, and missense mutations (G112S, T115N, and W116G) in the small integral membrane protein of lysosome/late endosome gene (SIMPLE) were implicated as being causal for this disorder.

The SIMPLE gene (GenBank AB034747) consists of four exons and encodes a protein with a calculated molecular weight of 17.1kDa. The protein possesses a putative membrane association domain flanked by two putative CXXC motifs (high-affinity zinc binding motifs).4 The N terminus of SIMPLE possesses two PPXY motifs (WW domain binding motif) that have been shown to interact with Nedd4, an E3 ubiquitin ligase that plays a role in ubiquitinating membrane proteins.6 Ubiquitination, among other functions, has been identified as a signal for endocytosis and sorting to the lysosome for degradation.7,8 We identified an additional

From the 1Department of Pediatrics, Division of Genetics and Developmental Medicine, 2Department of Otolaryngology, University of Washington, Seattle, WA; 3Molecular Genetics Department, Flanders Interuniversity Institute for Biotechnology, University of Antwerp, Antwerp; 4Centre de Génétique Humaine, Institut de Pathologie et de Génétique, Louvain, Belgium; 5Laboratory of Molecular Pathology and 6Department of Neurology, Sofia Medical University, Sofia, Bulgaria; 7Service de Neurologie, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain, Brussels, Belgium; 8Department of Child Neurology and 9Department of Neurology, 2nd School of Medicine, Charles University, Prague, Czech Republic; 10Department of Neurology, University of Washington; and 11VA Puget Sound Health Care System, Seattle, WA.

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Address correspondence to Dr. Bennett, Department of Pediatrics, Box 356320, University of Washington, Seattle, WA.

E-mail: cbennet@u.washington.edu

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motif in the N terminus of SIMPLE known as a P(S/T)AP domain. This domain functions to bind TSG101, a class E vacuolar sorting protein that facilitates protein sorting to the lysosome via multivesicular bodies (MVBs). The subcellular localization and protein binding domains of SIMPLE suggest a role in ubiquitin-mediated lysosomal sorting.

In this study, we sought to discover new mutations in SIMPLE in patients with CMT1 as well as other demyelinating neuropathies and to correlate these mutations with clinical data. We also sought to characterize the expression of SIMPLE in various cell types of the peripheral nerve and particularly Schwann cells, which are affected in CMT1C.

**Patients and Methods**

**Patients**

Informed consent was obtained from all participants according to the ethical committee of the participating Universities and the Declaration of Helsinki. A total of 152 persons having various forms of inherited peripheral neuropathy were studied from three subject-tier for prior gene analysis and geographic location. The first tier of 63 individuals of general European descent consisted of 17 with CMT type 1, 19 with CMT type 2, 5 with intermediate CMT, 8 with CMT type unclassified, 8 with hereditary motor neuropathy, and 6 with hereditary sensory neuropathy. These individuals were taken from unmapped pedigrees and were known to lack the CMT1A duplication and mutations in PMP22, MPZ, GJB1, PERIAxin (PRX), EGR2, the neurofilament light polypeptide chain (NEFL), myotubularin-related protein 2 gene (MTMR2), and ganglioside-induced differentiation-associated protein 1 gene (GAD1). The second tier of 38 Bulgarian patients consisted of 12 with CMT type 1, 12 with CMT type 2, 4 with intermediate CMT, and 10 with CMT type unclassified but excluded for the CMT1A duplication on chromosome 17p11.2. The third tier was 50 CMT patients from the United States who had been excluded for the CMT1A duplication on 17p11.2. In addition, we examined a three-generation CMT1 pedigree of Ukrainian origin (K1552) in which a proband had tested negative for the CMT1A duplication and for mutations in the GJB1 gene. All patients were examined by a neurologist to document their features at the clinical and electrophysiological levels.

**Charcot–Marie–Tooth Neuropathy Type 1C Subjects**

CMT1C pedigrees PN282, K1552, and K1910, which were of Belgian, Ukrainian, and English descent, respectively, are shown in Figure 1. Affected individuals met widely accepted criteria for CMT including distal muscle weakness and atrophy, depressed deep tendon reflexes, and sensory impairment. Pedigrees K1550, K1551, and K2900 have been described previously.

**Mutational Analysis**

Total blood samples were obtained by venipuncture for extraction of high molecular weight DNA as described previously and used as a template for polymerase chain reaction (PCR). PCR primers used to amplify exon 2 through 4 of the SIMPLE gene have been described previously. Direct sequence analysis was performed on amplified fragments using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and chromatograms were generated on Applied Biosystems High Through-Put Capillary Electrophoresis sequencers available at the participating institutions in Seattle and Antwerp.

**Microsatellite Analysis**

Polymorphic markers used in this study (see Fig 1) were from the Genethon human genetic linkage map. PCR amplicons were detected using 6-FAM (6-carboxy-fluorescein) fluorescence sense primers obtained from Applied Biosystems. After capillary electrophoresis of the PCR products on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems), the results were analyzed using GeneScan software (Applied Biosystems). The possible haplotypes were constructed manually and exact allele lengths are given.

**Electrophysiological Examination**

Standard and universally accepted methodologies were used for all electrophysiological examinations that were undertaken across the participating institutions. This includes the detailed reexamination of four affected individuals from pedigree K2900, I.2, I.3, I.5, and I.1 (see Fig 1). Amplitude, duration, and area of the negative phase of the compound muscle action potential (CMAP) were measured.

**Peripheral Nerve Immunohistochemistry of SIMPLE**

Sciatic nerve immunohistochemistry was conducted in duplicate on postmortem tissue samples obtained from unaffected individuals by LifeSpan Biosciences (http://www.lspbio.com/). Subject 1 was a 54-year-old woman who died of a drug overdose. Subject 2 was a 74-year-old man who died of respiratory failure. The analysis was performed with a commercial murine monoclonal antibody (Ab) to SIMPLE (Transduction Labs, San Diego, CA) and a murine monoclonal Ab to PMP22 (NeoMarkers, Fremont, CA). To specifically identify Schwann cells, we stained with the anti–PMP22 Ab. A concentration of 2.5 μg/ml was found to provide the highest signal-to-noise ratio on paraffin-embedded, formalin-fixed tissues for both antibodies. To detect SIMPLE antibody, we used a DAKO LSAB2 kit utilizing secondary goat anti-mouse Ab and a DAKO DAB+ Chromogen-substrate (DakoCytomation, Glostrup, Denmark) to produce a brown precipitate. For PMP22 antibody detection, we used a Vector ABC-AP kit utilizing a Vector horse anti–mouse secondary Ab and a Vector Red substrate kit (Vector Laboratories, Burlingame, CA) producing a fuchsia precipitate.

In addition to staining for PMP22 or SIMPLE antibody alone, double-staining experiments were performed sequentially. Tissues were stained with CD31 antibody as a positive control to ensure that tissue antigens were preserved and accessible for immunohistochemical analysis. Negative controls consisted of performing the entire immunohistochemistry procedure on adjacent sections in the absence of primary ant-
Fig 1. Six Charcot–Marie–Tooth neuropathy type 1C (CMT1C) pedigrees examined in this study. Electrophysiological examination of all affected members of pedigree K2900 was undertaken. Genotypes are shown for each pedigree necessary to determine potential founder effects were present to account for the common mutations G112S (A) and W116G (B). Asterisks indicate truncated pedigrees because they have been published previously in full.15
tibody. Slides were imaged with a DVC 1310C digital camera coupled to a Nikon microscope.

Results
SIMPLE Mutation Analysis
We screened the three SIMPLE coding exons for mutations in a total of 152 patients with various peripheral neuropathies. No mutations were detected in patients with CMT2 (n = 31), hereditary motor neuropathy (n = 8), hereditary sensory neuropathy (n = 6), intermediate CMT (n = 9), or unclassified forms of CMT (n = 18). The three probands who were found to harbor SIMPLE missense mutations were from CMT type 1 pedigrees (n = 80). One mutation was found in probands from the tier one group of 17 type 1 CMT pedigrees (PN282) that were known to lack mutations in NEFL, PMP22, MPZ, GJB1, EGR2, and PDX. A second mutation was identified from the Ukrainian pedigree (K1552) that was in fact identical to that in pedigree PN282. The third proband K1910 (II.1) with a SIMPLE mutation was drawn from the tier three group of 50 CMT1 probands that had been screened only for the CMT1A duplication (see Fig 1 and Table). In the PN282 and the K1552 pedigrees, the c.334G→A transition leads to a p.G112S substitution in the SIMPLE protein. In K1910, a c.346T→G transition predicted to result in a p.W116G substitution was present. In a pedigree BUL102 (Bulgarian cohort) a c.222T→C transition (I74I) was identified (see Table) that was not present in 100 normal chromosomes. Reverse transcription PCR analysis did not show any alternately spliced products (data not shown). The two missense mutations p.G112S and p.W116G have been described previously, and the clinical features of patients with SIMPLE mutations are summarized in the Table.

Haplotype Segregation Analysis
Pedigrees segregating the c.334G→A mutation (p.G112S) were of English (K1551), Ukrainian (K1552), and Belgian (PN282) descent. To test for a possible founder effect, we determined haplotypes with a series of markers spanning a distance of approximately 5cM including the SIMPLE locus on chromosome 16p13.1. For each pedigree (K1551, K1552, and PN282), the disease-linked haplotype differed, suggesting that no founder haplotype was present (see Fig 1). Two pedigrees (K2900 and K1910) with a c.346T→G mutation (p.W116G) were both of English origin. The disease-linked haplotypes in these two pedigrees were different, indicating that no founder was present (see Fig 1).

Immunochemistry of SIMPLE in Sciatic Nerve
Antibody staining was performed in duplicate using autopsy samples from two unrelated individuals. In the sciatic nerve, staining for SIMPLE antibody was positive in Schwann cells (Fig 2A). PMP22 antibody was used as a marker for myelinating Schwann cells (see Fig 2B). Double labeling with antibodies to SIMPLE and PMP22 showed distinct PMP22 staining of myelin (red) and SIMPLE staining of associated peripheral

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>N</th>
<th>Mutation</th>
<th>Age at Onset (range)</th>
<th>Initial Symptoms</th>
<th>Muscle Weakness*</th>
<th>Muscle Atrophy*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Limbs</td>
<td>Upper Limbs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Limbs</td>
<td>Upper Limbs</td>
</tr>
<tr>
<td>PN282</td>
<td>5</td>
<td>112S</td>
<td>3 yr to adult</td>
<td>Pes cavus</td>
<td>0/+ +</td>
<td>0/+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Childadolescent</td>
<td>Weak feet</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12–15 yr</td>
<td>Unable heal</td>
<td>0/+</td>
<td>0/+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>walk/pes cavus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1551</td>
<td>15</td>
<td>112S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1552</td>
<td>3</td>
<td>112S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K2900</td>
<td>4</td>
<td>116G</td>
<td>41 (10–58)</td>
<td>Pes cavus</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal gain</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>K1910</td>
<td>1</td>
<td>116G</td>
<td>6 yr</td>
<td></td>
<td>+ +</td>
<td>+ +</td>
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<tr>
<td>K1550</td>
<td>10</td>
<td>115N</td>
<td>Childhood</td>
<td>Weak feet</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>BUL102</td>
<td>1</td>
<td>Polymorphism 174I</td>
<td>7 yr</td>
<td>Pes cavus</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Muscle weakness/Atrophy (0 = none; + = mild; ++ = a moderate; +++ = significant)
nerve Schwann cell cytoplasm (brown) (see Fig 2C, D). The SIMPLE antibody staining was positive in additional cell types, such as adipocytes (see Fig 2E), mast cells, endothelium, and vascular smooth muscle (data not shown). SIMPLE antibody staining was minimal or completely absent from adipocytes or fibroblasts, whereas PMP22 staining was, as expected, absent from all the above cell types except Schwann cells (eg, see Fig 2F). The same staining pattern was observed in both autopsy samples.

**Electrophysiological Findings**

A total of 12 motor and eight sensory nerves were retested in all four affected family members from pedigree K2900 (see Fig 1). Sensory nerve action potentials could not be elicited in the lower limbs of three patients. A moderately to severely reduced sensory nerve conduction velocity (SNCV) of 31.0 m/sec was noted for the sural nerve in the remaining patient. Moderately to severely reduced SNCVs of 35.5 ± 2.6 m/sec (standard error mean) were obtained for the median nerves. No values were obtained for motor nerve conduction velocity (MNCV) in the lower limbs of two patients. Moderate to severely reduced MNCVs of 27.0 ± 0.0 and 25.0 ± 1.0 m/sec were noted in the distal and proximal segments, respectively, for peroneal nerves and of 27.5 ± 0.5 m/sec for tibial nerves. Mild to moderately reduced MNCVs were seen in the median nerves (39.8 ± 3.0 m/sec). Based on published criteria, abnormal temporal dispersion of the CMAP was noted in the proximal segments of the peroneal and tibial nerves of one patient and the tibial nerve of another (Fig 3).

**Discussion**

In this report, we found that SIMPLE is present in Schwann cell cytoplasm. We also show SIMPLE protein is present in several cell types (eg, endothelial, mast cells, and vascular smooth muscle cells) seen in the postmortem tissues, a fact that is in agreement with the previous demonstration of ubiquitous SIMPLE gene expression from nearly all tissues examined.

A total of seven CMT1 pedigrees with SIMPLE mutations have now been identified, including six missense mutations represented by only three particular substitutions (p.G112S, p.T115N, and p.W116G). Our haplotype analysis (see Fig 1) suggests that no founder effect contributed to these high-frequency missense mutations. Furthermore, the clustering of the mutations suggests this domain plays a critical role in CMT1C.

A total of 38 patients with diagnosed CMT1C were examined. For the subset of six CMT1C pedigrees representing three SIMPLE missense mutations (see Table), a uniform clinical pattern typical of CMT1 is present. Although few patients with CMT1C have been examined to make a definitive comparison, CMT1C patients appear indistinguishable from CMT1A and meet widely accepted criteria for CMT1 including distal muscle weakness and atrophy, depressed deep tendon reflexes, and sensory impairment. The clustering of mutations seen in CMT1C.

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Sensory Loss (i)</th>
<th>Reflexes 8</th>
<th>Other symptoms</th>
<th>Motor (SD)</th>
<th>NCV (m/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper Lower</td>
<td>Pes Cavus</td>
<td></td>
<td>Median</td>
<td>Ulnar</td>
</tr>
<tr>
<td>PN282</td>
<td>+/+ 1+/0 1+/0</td>
<td>Yes</td>
<td></td>
<td>NA</td>
<td>25.3 (1.6)</td>
</tr>
<tr>
<td>K1551</td>
<td>+ 1+ 0/1+</td>
<td>Yes</td>
<td>Nerve hypertrophy, hand tremor</td>
<td>25.8 (9.0)</td>
<td>25.3 (6.0)</td>
</tr>
<tr>
<td>K1552</td>
<td>+/+ 1+/2+ 0/1+</td>
<td>Yes</td>
<td>lumbago, spondylolisthesis</td>
<td>23.4</td>
<td>NA</td>
</tr>
<tr>
<td>K2900</td>
<td>+ + 1+ 1+</td>
<td>Yes</td>
<td></td>
<td>NA</td>
<td>39.8 (6.0)</td>
</tr>
<tr>
<td>K1910</td>
<td>+ + 1+ 0</td>
<td>Yes</td>
<td></td>
<td>NA</td>
<td>15</td>
</tr>
<tr>
<td>K1550</td>
<td>+ + 0 0</td>
<td>Yes</td>
<td>Nerve hypertrophy</td>
<td>17.3 (2.0)</td>
<td>16.7 (1.0)</td>
</tr>
<tr>
<td>BUL102</td>
<td>+ + 1+ 0</td>
<td>Yes</td>
<td></td>
<td>38.8</td>
<td>43.7</td>
</tr>
</tbody>
</table>

1Sensory loss (0 = none; + = mild, +/+ = a moderate)
2Reflexes (0 = absent; 1+ = reduced, 2+ = normal, 3+ = hyperactive)
NA = not available.

Table 1(B).
Fig 2. Immunohistochemistry was performed in duplicate on tissue obtained from a 54-year-old woman who died of a drug overdose. Panels A to F depict immunohistochemistry staining for PMP22 antibody (red) and SIMPLE antibody (brown) as labeled. Scale bars (bottom left corners) = 10μM in A, B, E, and F and 2μM for C and D. The staining pattern for the two proteins is quite different, with a more diffuse pattern evident for SIMPLE (A), and the punctate myelin ring structures seen (marked by arrowheads) for both axon cross-sections or longitudinal sections seen for PMP22 (B). Panels C and D are nerve cross-sections. Arrowheads highlight the distinct PMP22 staining demarcating myelinated axons. Open arrows indicate the polarized cytoplasm of a Schwann cell in cross-section. Panels E and F show that SIMPLE is present in the cytoplasm of an adipocyte cell from nerve section, whereas PMP22 staining is clearly absent.

may account for the apparent tight phenotypic spectrum that we have thus far observed for CMT1C. Only pedigree K2900 with the p.W116G mutation had a broad age of onset that often is seen in CMT1A pedigrees (see Table). In the CMT1C pedigrees that we have examined there is 100% penetrance as determined by slowed nerve conduction velocity, another feature shared with CMT1A.

Only one mutation (p.G112S; PN282) was found in our most stringent category of 17 CMT1 probands previously excluded for mutations in the NEFL, MTMR2, GDAP1, PMP22, MPZ, CX32, PRX, and EGR2 genes. This suggests that SIMPLE mutations may be at a relatively low frequency in CMT1 patients. No SIMPLE mutations were observed in other neuropathies, yet too few patients have been observed to draw any firm conclusions currently.

Despite the fact that SIMPLE is ubiquitously ex-
pressed, the clinical presentations observed so far are restricted to the peripheral nervous system. One possible explanation stems from the acute sensitivity of Schwann cells to overexpression and/or misfolding of proteins. It is well established that CMT1 can result simply from an overexpression of PMP22, a protein that is highly expressed in Schwann cells. PMP22 is difficult to correctly fold, which is demonstrable by the fact that 80% of newly synthesized PMP22 is rapidly degraded. Schwann cells may be under particularly high protein turnover burden, such that, when challenged by a defect in the role SIMPLE putatively plays in the lysosomal degradation pathway, pathological features result. Given that there is expression of SIMPLE in brain and spinal cord, it is possible that mutations in SIMPLE could lead to central nervous system demyelination. Autosomal dominant syndromes of central nervous system leukodystrophies have been described and represent targets for possibly having mutations in SIMPLE.

The results of the NCV studies showed temporal dispersion in two of four affected individuals of pedigree K2900, representing 25% (3/12) of all motor nerves tested, a phenomenon not typically seen in patients with CMT1, with the exception of recently identified missense mutations of the MPZ gene. In addition, upper limb MNCSs were significantly higher than those in the lower limbs, some even greater than 40m/sec. Other electrophysiological studies of patients with CMT1 have emphasized uniformity of conduction slowing and the absence of segmental amplitude reductions or temporal dispersion.

Although the function of SIMPLE is unknown, the subcellular localization and putative domains present in SIMPLE suggest that it may have a role in ubiquitin-mediated lysosomal degradation. Interestingly, both Nedd4 and TSG101 are recruited by retroviral gag protein L domains for use in viral budding from the plasma membrane, a process topologically equivalent to budding into the lumen of the endosome during MVB formation. It may be that SIMPLE plays a similar role of recruiting these factors to sites of MVB formation, taking advantage of the putative membrane association domain to anchor these functions to specific subcellular locations and thereby facilitate the sorting of proteins along this pathway to the lysosome.

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