Acta Biochimica Polonica

Vol. 51 No. 1/2004

273–280

QUARTERLY

Communication

Screening of the myelin protein zero gene in patients with Charcot-Marie-Tooth disease✪

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Received: 30 October, 2003; revised: 05 February, 2004; accepted: 08 February, 2004

Key words: Charcot-Marie-Tooth disease, *MPZ* gene, SSCP, heteroduplex analysis

The myelin protein zero gene (*MPZ*) coding for the most abundant protein of the peripheral myelin was shown to be mutated in Charcot-Marie-Tooth type 1B disease (CMT1B). Later on *MPZ* mutations have been shown in axonal type of CMT (CMT2). Recently three novel *MPZ* gene mutations were reported in congenital hypomyelinating neuropathy (CHN). In contrast to the previously reported studies, focused on CMT1B disease, we aimed to analyze the coding and promoter sequences of the *MPZ* gene in a group of patients with three CMT phenotypes i.e.: CMT1, CMT2 and CHN. Over 500 PCR products were screened by single strand conformation polymorphism analysis (SSCP) and heteroduplex analysis (HA). In one CMT2 family we founded the E56K mutation in the *MPZ* gene and in one CHN patient the T124K substitution was detected. In agreement with previously reported studies we conclude that *MPZ* gene screening should be performed for wide phenotype spectrum of CMT.

Myelin protein zero gene (*MPZ*) codes for the major structural component of peripheral nerve myelin (Lemke *et al.*, 1988). *MPZ* gene maps to chromosome 1q22-q23. Ten years ago, two missense mutations i.e. Asp90Glu and Lys96Glu have been found in the *MPZ*

✪This study was supported by the State Committee for Scientific Research (KBN, Poland) grant No. 6P05E05820.

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Abbreviations: AR, autosomal recessive; CHN, congenital hypomyelinating neuropathy; CMT, Charcot-Marie-Tooth disease; HA, heteroduplex analysis; SSCP, single strand conformation polymorphism analysis.

gene in two families, in patients suffering from demyelinating type of Charcot-Marie-Tooth disease linked to the 1q22-q23 locus (CMT1) (Hayasaka *et al.,* 1993*)*. By contrast to the CMT1A disease caused by submicroscopical duplication in the 17p11.2-p12 region, CMT1 caused by mutations in the *MPZ* gene was designed as CMT1B (Raeymaekers *et al,* 1991; Bird *et al.*, 1982). Three years after the first description of the MPZ mutations segregating with CMT1B disease, the Gln215stop mutation was reported in patient suffering from congenital hypomyelinating neuropathy (CHN) (Warner *et al.,* 1996). Recently, two novel mutations in the *MPZ* gene were found in patients suffering from congenital hypomyelination neuropathy (Lupski *et al.,* 2003)**.** Although MPZ protein has previously been shown to be expressed in the myelinating Schwann cells, the Ser44Phe mutation was detected in a large Sardinian family consisting of patients suffering from the axonal type of Charcot-Marie-Tooth disease (CMT2) (Marrosu *et al.,* 1997). Up to now, over ninety mutations have been reported in the *MPZ* gene. The majority of them segregate with CMT1 disease. Nineteen mutations have been shown to segregate with more severe form of CMT1, designed as Dejerine-Sottas-syndrome (DSS). The mutations were reported in congenital hypomyelination neuropathy (CHN) and in CMT2 disease (http://molgen-www.uia.ac.be/CMTMutations).

FAMILIES AND PATIENTS

The study included a total of 46 CMT patients (Table 1). Based on electrophysiological and morphological criteria, 15 patients were diagnosed with CMT1 (motor median nerve conduction velocity (MNCV) < 38 m/s), 23 had CMT2 (MNCV > 38 m/s), 4 had CHN (sural nerve biopsy revealing thin myelin sheet and MNCV < 6 m/s) and 4 patients had an unspecified peripheral neuropathy based on clinical records only.

From only 9 families the possible trait of inheritance could be deduced from the pedigree analysis. By contrast, CMT diagnosis in 24 families was established in the sporadic cases. The autosomal dominant trait of inheritance was observed in 8 pedigrees, whereas "possible" autosomal recessive (AR) trait was apparent in two families. Although, no paternity testing was performed, we tended to find an AR trait, if at least two individuals were CMT affected and their parents were healthy. In the family F16 the autosomal dominant CMT2 transmission was present within three generations. In family F1 two sons of the healthy parents were CMT affected, suggesting the AR trait of inheritance. The patients included in this study represent four CMT phenotypes. Firstly, CMT1 disease was diagnosed in 13 families. In 11 families CMT2 was observed. Congenital hypomyelination neuropathy was observed in 4 families, in 4 individuals.

Molecular genetic analysis

DNA was isolated from white blood cells.

In 12 patients diagnosed with CMT1 and in two CMT patients DNA was screened for duplication of the *PMP22* gene (Young *et al.,* 1998) Six exons of the *MPZ* gene were amplified in the polymerase chain reaction (PCR). Standard PCR reaction was carried out in a 25 μl volume containing: 100–500 ng of genomic DNA template, 5 mmol dNTPs and 25 pmol of each primer. Respectively, six pairs of primers were used, for exon 1: forward 5′-CCCGTTCAGTTCCTGGT-3′ (reverse 5′-GTCCCAAGACTCCCAGAGTA-3′), for exon 2: forward 5′-CTTCCTCTGTATCCCTTACTG-3′ (reverse 5′-CTCCTTAGCCCAAGTTATCT-3′), exon 3: forward 5′-TCATTAGGGTCCTCTCACATGC-3′ (reverse 5′-GCCTGAATAAAGGTCCTTAGGC-3′), exon 4: forward 5′-GGAGTCCTACATCCTCAATGCAG-3′ (reverse 5′-CCCACCCACTGGAGTAGTCTCCG-3′), exon 5: forward 5′-GAAGAGGAAGCTGTGTCCGC-3′ (reverse 5′-CACATCAG-

TCACCGAGCGACT-3′), exon 6: forward 5′-AGTCGCTCGGTGACTGAT-3′ (reverse 5′-TAGCTCCATCTCGATGACCA-3′). In addition, the PCR fragment including promoter sequence of the *MPZ* gene (from –194 upper transcription start site to +42 downstream of transcription start site) was amplified with *MPZ* promoter primers: forward 5′-CCTCTGTGTATGGGGTGGTATG-3′ (reverse 5′-CATCTGTGGGGTTGAGAAAGTG-3′). PCR reaction was performed through 30 cycles of 1 min at 94.0°C, 1 min at the respective annealing temperature (exons 1, 3, 4, 5 – 59.0°C, exon 2 – 58.0°C, exon 6 – 61.0°C and for promoter – 61.5°C), and 1 min 30 s at 72.0°C. Single strand conformation polymorphism (SSCP) analysis was performed to screen PCR-products. PCR products were mixed with SSCP-buffer, (95% formaldehyde, 20 mM EDTA, Bromophenol blue).

After denaturation for 5 min, at 95°C, samples were rapidly loaded on ice. Heteroduplex analysis (HA) of the *MPZ* gene was performed with primers used in SSCP.

Samples (3 μl) were mixed with DNA-loading buffer (saccharose, 0.5 M EDTA, 10 M Tris, Bromophenol blue) and denaturated for 5 min at 95°C. For renaturation, samples were placed at 65°C for 45 min. Samples were loaded on non-denaturing 6% acrylamide gel (acrylamid/bis-acrylamide, 99:1, w/w) at 8 W for 20 h. After electrophoresis gels were silver stained. DNA fragments disclosing different, from wild, migration pattern were direct sequenced. The Prism Sequenase Terminator Double Stranded Sequencing Kit was used. Enzyme restriction analysis was performed to confirm nucleotide changes in family F6.

RESULTS

Over 500 PCR products corresponding with six exons of the *MPZ* gene have been analysed by SSCP and HA. In 17 PCR products SSCP (17) and HA (12) revealed an altered migration pattern. DNA sequencing was performed in 7 individuals. In one family RFLP analysis was performed (Table 2).

In patient F6MJ, the SSCP and HA of the exon 3 of the *MPZ* gene showed an altered mobility of the PCR fragment. Sequence analysis of exon 3 in this patient revealed a C to A base change, resulting in an amino acid substitution of Thr to Lys at the codon 124. (Fig. 1).

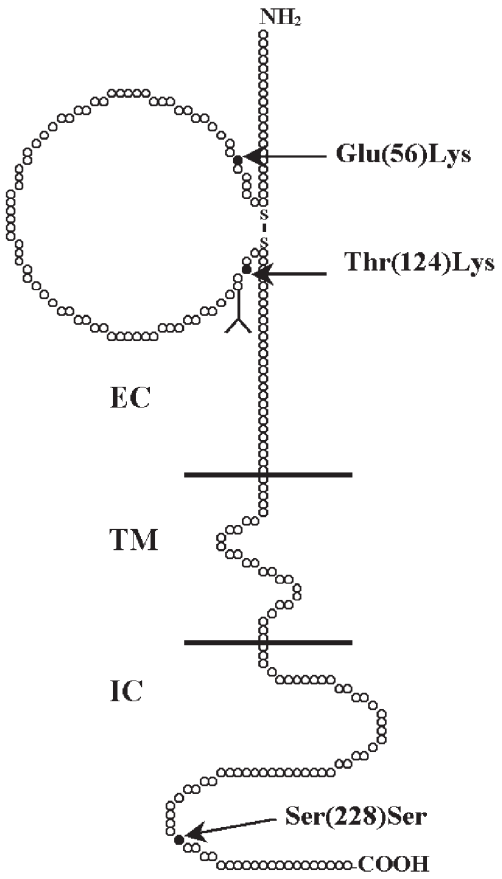

Figure 1. Schematic representation of the site of mutations and polymorphism in P_0 protein found in this study.

Arrows indicate positions of mutations and polymorphism in P_0 amino-acid sequence. (EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain).

The SSCP analysis performed in parents of F6MJ did not reveal any mobility alteration suggesting that exon 3 of the *MPZ* gene in them was most probably not mutated. Due to the the *Mae*II restriction site loss, caused by heterozygous C(371)A transversion, an addi-

tional 190 bp band was observed in patient F6MJ on RFLP analysis. RFLP analysis with *Mae*II endonuclease, performed in 10 control samples revealed only one 190 bp band (Fig. 2). The Thr124Lys mutation is a novel heterozygous substitution located in the exon 3 of the *MPZ* gene found in the 14-year-old boy diagnosed with congenital hypomyelination neuropathy (Kochanski *et al.*, in press).

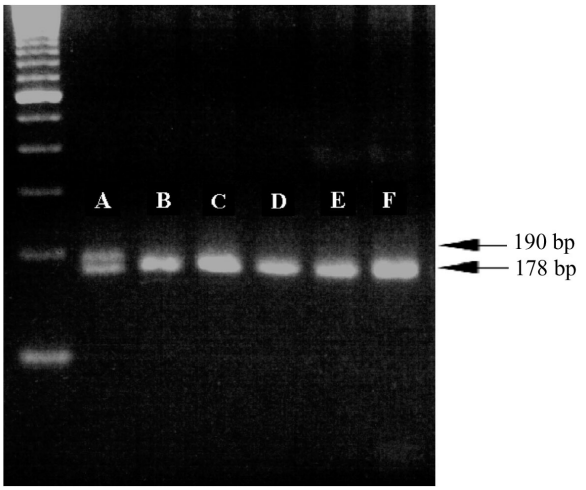

Figure 2. Restriction fragment length polymorphism analysis (RFLP) was performed in family F6.

From the left: 100 bp DNA leader; A, proband; B, proband's mother; C–F, negative controls. The size of alleles is indicated with arrows.

The SSCP analysis of the exon 2 of the *MPZ* gene in patient F16KB revealed an altered migration pattern. In the sequencing of the exon 2 of the *MPZ* gene an heterozygous G-A transition was detected leading to the Glu to Lys amino acid substitution at the codon 56 of the *MPZ* gene. The Glu56Lys mutation in the exon 2 of the *MPZ* gene was found in an 40 years-old female suffering from CMT2 disease (Kochanski *et al.*, in press).

In 11 individuals (see Table 2) both SSCP and HA analysis of exon 6 of the *MPZ* gene revealed an altered migration pattern. The automated sequencing of the exon 6 of the *MPZ* gene disclosed an heterozygous C to T base change at position 684, which did not change the Ser at codon 228. In one healthy individual from family F23 (Fig. 3) the SSCP analysis of exon 6 of the *MPZ* gene disclosed an altered migration pattern, whereas HA did not

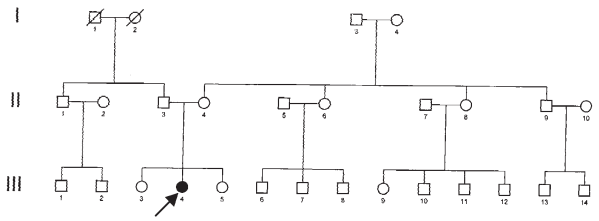

Figure 3. Pedigree of the CMT1 family (F23).

Proband was marked with an arrow. III5 individual is homozygous for S(228)S, II3 and II4 are heterozygous for S(228)S polymorphism.

reveal any migration changes. By sequencing of exon 6 of the *MPZ* gene the homozygous replacement of C to T at position 684 which did not change Ser at codon 228 was observed. In 11 individuals SSCP analysis of the promoter region of the *MPZ* gene disclosed three different migration pattern (Fig. 4). The HA of the *MPZ* promoter region did not reveal any migration changes. Sequencing analysis of *MPZ*

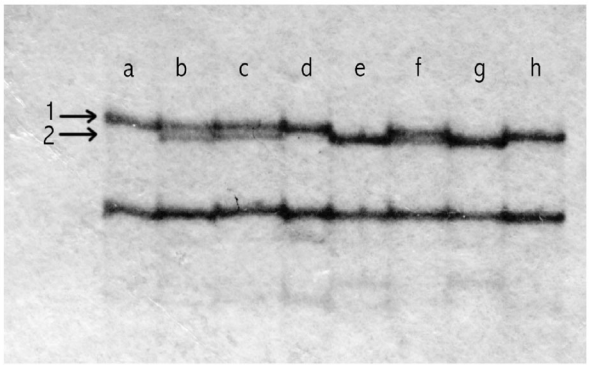

Figure 4. Result of SSCP analysis in *MPZ* promoter, three different migration pattern are shown: (1,1) (1,2) (2,2)

Lanes: a, F30KM; b, F37MM; c, F35GA; d, F27PA; e, F24CA; f, F23MW; g, F23MP; h, F29KL.

promoter region was performed in two individuals (F32GA and F26DM) showing an altered SSCP migration pattern. In patient F**32**GA an homozygous single nucleotide G insertion was found at position –140 within

Table 1. Characteristics of CMT patients and their relatives, AD — autosomal dominant trait of inheritance, AR — autosomal recessive trait of inheritance

Proband	Diagnosis	Trait of inheritance	Number of analysed DNA samples from CMT affected individuals	Number of analysed DNA samples from healthy individuals
R1LL	CMT1	AR	2	2
R2JA	CMT1	?	1	3
R3KBG	CMT2	?	1	0
R4NM	CMT1	?	1	0
R5HJ	CMT2	?	1	1
R6MJ	CHN	?	1	2
R7ZB	CMT1	?	1	2
R8KS	CMT1/2	AD	3	0
R9GT	CMT	?	1	0
R10NG	CMT2	?	1	0
R11FJ	CMT	AD	1	2
R12ZK	CMT1	?	1	1
R13WT	CHN	?	1	2
R14ML	CMT2	AD	2	0
R15CM	CMT	?	1	2
R16KB	CMT2	AD	3	3
R17SG	CMT2	AD	3	0
R18GS *	CMT	?	1	0
R19WE	CHN	?	1	2
R20KE	CMT2	?	1	0
R21KL *	CMT1	?	1	2
R22SK	CHN	?	1	1
R23MW	CMT1	?	1	5
R24CA*	CMT1	AD	2	1
R25GA	CMT2	AD	3	0
R26PA	CMT1/2	?	1	2
R27KM	CMT1	?	1	2
R28DJ	CMT2	?	1	1
R29BR	CMT2	AR	2	0
R30DK	CMT1	?	1	1
R31KR	CMT1	?	1	0
R32GA	CMT1	?	1	1
R33PJ	CMT2	?	1	0
R34MM*	CMT1	?	1	1

The patients in whom duplication of the PMP22 gene was found are marked by asterix.

the CAAT sequence in the sense strand. The antisense strand sequencing did not revealed any abnormality in the analysed region. In patient F19WE the abnormal migration pattern was visible in SSCP analysis, whereas no migration changes were detected in HA. No sequence alteration was found in the exon 3 of the *MPZ* gene in the patient F19WE.

DISCUSSION

The screening of the *MPZ* gene revealed the presence of two novel mutations i.e. T124K and E56K. From the molecular genetics point of view, the T124K and E56K substitutions represent conservative amino acid changes located in the exons 2 and 3 of the *MPZ* gene corresponding with the extracellular part of the MPZ protein (Fig. 5). As far, the vast majority of the *MPZ* gene mutations have been reported in the extracellular part of the MPZ protein which is thought to be responsible for its adhesive properties (Filbin *et al.*, 1990).

Interestingly the novel mutations were not found in the most common form of CMT1B disease associated with *MPZ* gene mutations but in the CMT2 disease and congenital hypomyelination neuropathy.

By contrast to the previously published *MPZ* gene screening's data, we decided to extend the spectrum of phenotypes for *MPZ* molecular genetic analysis. Besides the classical CMT1 phenotype associated with *MPZ* gene mutations, we analysed the *MPZ* gene in patients diagnosed with CMT2, and CHN. The reason for that was the wide spectrum of phenotypes associated with mutations in the

Table 2. Overview of the mutations and polymorphisms detected by single stranded conformation polymorphism analysis (SSCP) and/or heteroduplex analysis in the *MPZ* gene

PCR fragment	Patient	SSCP	HA	Sequence changes	Position of amino acids changes	Restriction site changes	
						achieve	loss
exon 2	F16KB F16KP F16TK	+	-	G(166)A htz.	Glu(56)Lys	_____	_____
exon 3	F6MJ	+/-	+	C(371)A htz	Thr(124)Lys	_____	Mae II
exon 3	F19WE	+	-	no revealed	_____	_____	_____
exon 6	F1LW F1LB F1LL F2JA F2JE F2JP F3KB F12ZK F12ZA F24CA F24CH	+	+	C(684)T htz	Ser(228)Ser	Rsa I	_____
exon 6	F23MP	+	-	C(684)T hmz	Ser(228)Ser	Rsa I	_____
promoter	F22SK	1,2 #	1,2 #	no changes	_____	_____	_____
	F23MW	1,2	1,2				
	F23MP	2,2	2,2				
	F24CA	2,2	2,2				
	F26DM	1,1	1,1				
	F27PA	1,1	1,1				
	F29KL	1,1	1,1				
	F27KM	1,1	1,1				
	F32GA	1,2	1,2				
	F33PJ	1,2	1,2				
	F34MM	1,2	1,2				

+,different migration pattern, –, no different migration pattern +/– SSCP results depend on conditions of electrophoresis; #SSCP showed three different migration patterns (1,1),(1,2),(2,2), details are shown in Fig. 4.

MPZ gene. In 12 individuals the heterozygous S228S polymorphism was detected. Although SSCP analysis disclosed the altered migration pattern of exon 6 of the *MPZ* gene for heterozygous (11 samples) and homozygous (one sample) S228S polymorphism, no changes were observed in the HA for homozygous S228S polymorphism.

Two types of mismatches have been detected by sequencing analysis. Firstly, in patients F16KB and F6MJ the G–T/A–C mismatch may be deduced from sequencing analysis. Interestingly, the same G–T/A–C mismatch found by HA in heterozygous individuals in the F17 family was not observed in the F16KB patient. Since the same G–T/A–C mismatch was located in exons 2 (F16KB) and 3 (F6) of the *MPZ* gene, it is possible that two different *MPZ* related sequences, "surrounding" the same mismatch, affected the HA sensitivity.

In patient F6MJ sequencing analysis revealed the C–T/A–G mismatch in the distal part of the PCR product corresponding with exon 3 of the *MPZ* gene. By contrast, the same type mismatch located in the central part of the exon 3 of the *MPZ* gene, 78 nucleotides away from that found in patient F6MJ was not detected in HA (Nelis *et al.,* 1996).

Although, the mismatch position at the end of the PCR product was shown previously to result in a decreased HA sensitivity, our results indicate, that the more distally located changes are easier to detect than those located in the central portion of the PCR product (White *et al.,* 1992). Therefore, it can be

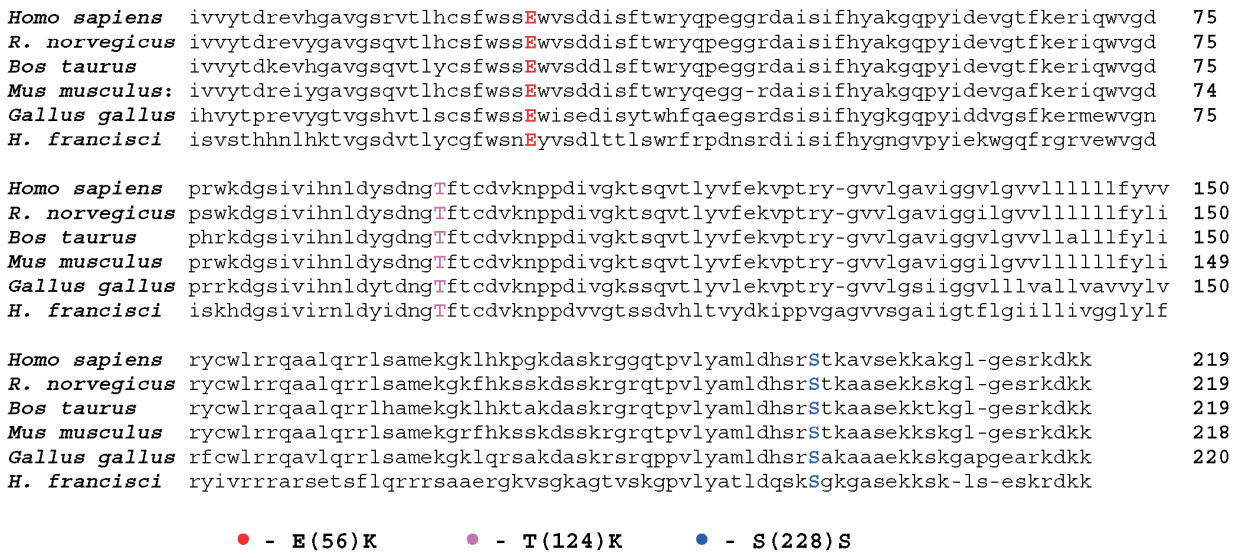

Figure 5. Amino-acid sequences comparison of myelin protein zero (P_0) of six species.

Colours represent individual missense and silent mutation found in this investigation. Both missense mutations are located in evolutionary conserved region of the P_0 protein.

said that mismatch position within analysed sequence determines the HA sensitivity.

Eight different polymorphisms were reported in the coding sequence of the *MPZ* gene up to now. In this group, only the S228S polymorphism was reported in families originating from Spain, Belgium, United States and Russia (Nelis *et al.,* 1994; Roa *et al.,* 1996; Bort *et al.,*1997; Mersiyanova *et al.,* 2000). The estimated frequency of the S228S polymorphism was 4.1% (Nelis *et al.,* 1994). It seems that S228S is the most common MPZ sequence variant occurring in various populations. It is a subject of interest if S228S mutation may represent an effect of common ancestor or, if does reflect the presence of the potential *MPZ* gene "hot- spot" at codon 228.

Interestingly, we found one healthy individual with homozygous S228S polymorphism. Given, the 4.1% estimated frequency of S228S polymorphism, the frequency of homozygous S228S polymorphism may be calculated as 0.00166. In addition to the analysis of the coding sequence of the *MPZ* gene we also decided to screen its promoter sequence. To date, no mutations or polymorphisms have been detected in the promoter sequence of the *MPZ* gene. Interestingly, we observed three different patterns of the PCR fragments corresponding with *MPZ* gene promoter in the SSCP analysis.

The presence of the homozygous insertion in patient F32GA at the position –140, detected in the sequencing of the sense strand was not confirmed by the antisense strand sequencing. For this reason we tend to classify this homozygous guanosine insertion rather as the polymerase-induced-error than a novel variant of the *MPZ* gene promoter sequence.

I am grateful to Prof. Dr. Irena Hausmanowa-Petrusewicz who enabled me to perfom molecular genetics analysis in the Neuromuscular Unit, Medical Research Centre, Polish Academy of Sciences. I would like to thank Dagmara Kabzinska MSc for support.

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Electronic-Database Information: Inherited Peripheral Neuropathies Mutation Database http://molgen-www.uia.ac.be/CMT Mutations.