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Pathogenic mutations and sequence variants within mitofusin 2 gene in Polish patients with different hereditary motor-sensory neuropathies

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At the time of its first description in 2004, *MFN2* was considered the most frequently mutated gene in hereditary motor and sensory neuropathy type 2 (HMSN 2). However recent studies have shown that the frequency of *MFN2* gene mutations in HMSN II patients is surprisingly low. To date, no systematic studies devoted to HMSN IIa in Poland have been carried out. In this study, we searched for *MFN2* gene mutations in Polish patients representing the population of nearly 40 million. We decided to include a wide spectrum of clinical phenotypes in the study, proving able to detect, in a group of 67 affected patients:

1) 3 pathogenic mutations
2) 3 sequence variants of unknown pathogenic status
3) 9 rare *MFN2* gene sequence variants
4) 6 common polymorphisms

The frequency of *MFN2* gene mutations in the whole group of patients is 4.5%. Due to the high frequency of *MFN2* gene sequence variants within single patients we could not definitely exclude the cumulative effect of these contributing to the HMSN II phenotype. The *MFN2* gene should therefore be considered in Polish HMSN II patients, though it is still not possible to determine its position in HMSN II molecular diagnostics.

Key words: mitofusin 2, polyneuropathy, mutation, sequence variant

This study we dedicate to Professor Irena Hausmanowa-Petrusewicz who passed away on 7 July 2015.

INTRODUCTION

The gene encoding the MFN2 protein has been mapped at locus 1p36.22. The *MFN2* gene was shown to be mutated in axonal polyneuropathy (HMSN II; Charcot-Marie-Tooth disease type 2) in 2004, by Züchner and colleagues, on the basis of linkage studies in 7 families (Züchner et al. 2004). Through detailed study they precluded mutations in the 14 genes known in those days in this region, including the *KIF1B* gene at the same locus, which was considered an excellent candidate for the mutation which could promote the development of CMT2A. The mutation in this gene was found by a Japanese group only in a single family (Zhao et al. 2001). Finally Züchner and colleagues have found mutations in the gene encoding the mitofusin 2 protein. They also included in the study additional 36 families too small for linkage analysis. This first publication presented 10 different mutations in patients of Italian, Russian, Turkish, Japanese, Iranian/Iraqi, North American and European origin in general. In later years, it proved possible to identify mutations in the *MFN2* gene in other European populations (e.g. German, Norwegian, Italian and Spanish) (Bergamin et al. 2014, Braathen et al. 2010, Gess et al. 2013, Sivera et al. 2013), recently also in the Czech Republic and Russia (Brozkova et al. 2013, Khidiyatova et al. 2013).

The mutations in the gene encoding the mitofusin 2 protein are causative for the HMSN II disease mani-

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festing with muscle weakness and atrophy, first in the distal parts of lower limbs and, in some patients, in the upper limbs; symmetrical foot drop; paresis in distal parts of the lower limbs and sometimes in the upper limbs; weakened or absent deep tendon reflexes in lower limbs and *pes cavus* deformity. The symptoms of the disease can be mild, or lead to wheelchair dependency in some patients (Verhoeven et al. 2006). In some cases the mutations in the *MFN2* gene also segregate with optic nerve atrophy (HMSN VI) and mild hearing loss (Züchner et al. 2006). Certain mutations in the *MFN2* gene are also associated with the HMSN II disease with pyramidal signs, known as HMSN V (Zhu et al. 2005). Patients carrying the mutations in the *MFN2* gene were also seen to manifest changes in the central nervous system (Brockmann et al. 2008, Del Bo et al. 2008). Generally there are over 100 mutations within the *MFN2* gene identified in worldwide populations (Bergamin et al. 2014). Most often these mutations occur in patients with early-onset motor-sensory neuropathy with a moderate to severe course. Only very rarely are they present in patients with late onset (at ages 50 or over) (Feely et al. 2011). There are different modes of inheritance associated with *MFN2* gene mutations in inherited motor-sensory neuropathy – an autosomal dominant in the vast majority of patients, and very rare autosomal recessive (Polke et al. 2011).

The mechanism of the pathogenicity of *MFN2* gene mutations is not clear, but is certainly associated with impaired function of the MFN2 protein. The MFN2 protein in mammals is 757 amino acids in length. It is a large protein with GTP-ase activity. The MFN2 protein is anchored in the mitochondrial outer membrane in such a manner that both the C and N termini face the cytosol. The MFN2 protein consists of one GTP-ase domain, two coiled-coil domains (CC1, CC2) (also called heptad repeat domains HR1 and HR2) and one transmembrane domain divided into two parts, anchoring the protein in the phospholipid bilayer. The GTP-ase domain hydrolyses GTP, as is necessary for the dimerization of the mitofusins, which in turn allows for the

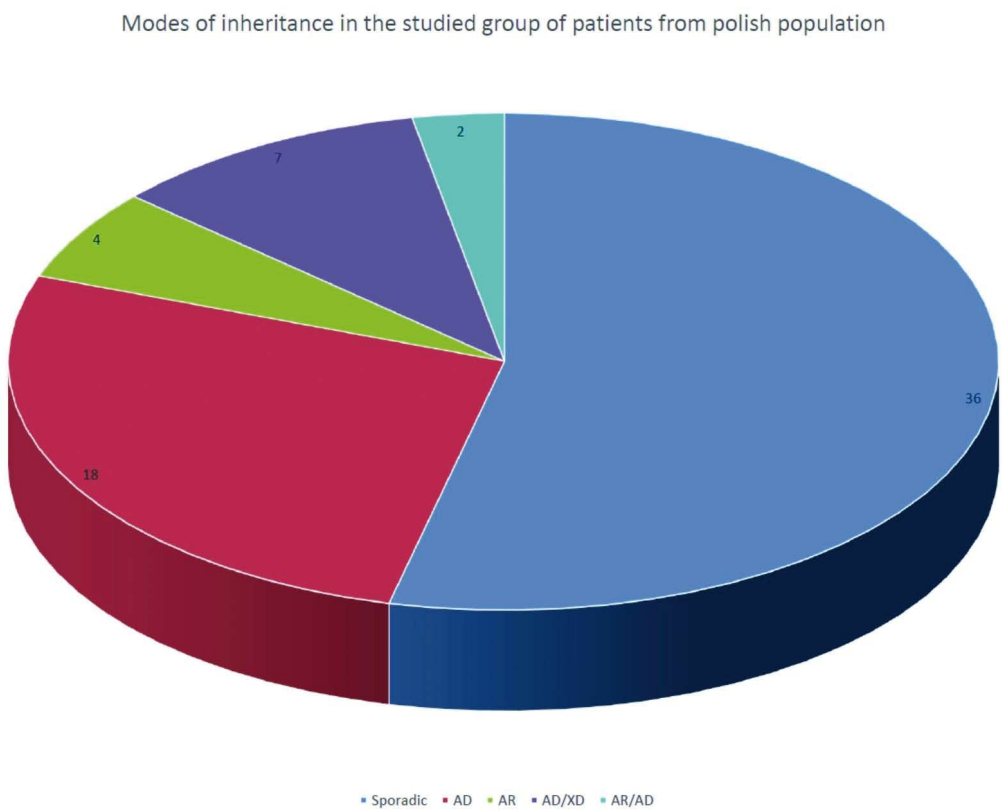

Fig. 1. Modes of inheritance in the studied group of patients diagnosed with polyneuropathy from the Polish population. AD denotes an autosomal dominant mode of inheritance, AR – autosomal recessive, AD/XD – from the pedigree we were unable to preclude either of the modes of inheritance, n/a – no data, AR/AD – unclear from the family history.

Table I

Pathogenic mutations and variants of unknown significance in the *MFN2* gene found in this study

	Nucleotide change	Protein change	De novo	NCBI id	Phenotype	Reference
Nº	Pathogenic mutations					
1.	c.311+1G>T		+	–	HMSN II	Novel
2.	c.820C>T	Arg274Trp	+	–	HMSN II with additional mild intellectual disability	Novel
3.	c.281G>A	Arg94Gln	+	rs28940291	HMSN II with additional macrocephaly	Previously reported
	Variant of unknown significance					
1.	c.1287+50G>T		+	–	EDMD/HMSN II	Novel
2.	c.2113G>A	p.Val705Ile	+	rs142271930	HMSN II	Previously reported
3.	c.1717G>A	p.Val573Ile	+	rs370335693	HMSN II	Previously reported

fusion of membranes. The linking itself (between two proteins in the outer membrane of adjacent mitochondria) occurs through the coiled-coil domains (for review see: Cartoni and Martinou 2009).

The MFN2 protein is an important component in the maintenance of the mitochondrial dynamic network, i.e. balance between fusion and fission. The mitofusin 2 protein is a molecule, whose activity ensures a broad range of effects in mediating the mitochondrial fusion. It is located in the mitochondrial outer membrane and is dependent on membranes in both adjacent mitochondria being fused (Song et al. 2009). The MFN2 protein is also observed in the endoplasmic reticulum (ER), where it tethers the ER and mitochondrion and controls the flow of the calcium ions from the ER to the mitochondrion (de Brito and Scorrano 2009). It also plays a role in axonal transport of mitochondria, connecting the mitochondrion with the microtubule along which the axonal transport of mitochondria takes place (Misko et al. 2010). Impairment of the mitochondrial fusion thus affects the metabolism of the cell indirectly, through altered mitochondrial respiration (Pich et al. 2005), Ca^{2+} signaling and axonal transport. These facts militate in favor of mutations in the gene encoding MFN2 protein altering the natural functions of the protein and contributing to the development of the inherited motor-sensory neuropathy.

In this study we aimed to present the first *MFN2* gene data from the population of Polish patients suffering from the diseases HMSN II, HMN, HSN and axonal-demyelinating HMSN (CMT1/2). The purpose of this study was thus to perform an analysis for the mitofusin 2 gene in Polish patients, and to determine the profile for the mutations and their location within the mitofusin 2 protein.

MATERIALS AND METHODS

We obtained written informed consent from all the patients included in our study. We examined 67 patients with identified axonal motor-sensory polyneuropathy, axonal motor polyneuropathy, axonal sensory polyneuropathy or intermediate axonal-demyelinating polyneuropathy (Table III). Among the patients there are 32 familial cases and 37 sporadic cases (Fig. 1). The 100 healthy anonymous individuals, whose DNA was obtained from the Forensic Medicine Department in Warsaw (200 chromosomes), were used as controls.

Genomic DNA and whole RNA were extracted from peripheral-blood lymphocytes using the salting-out method. Duplication/deletion of the *PMP22* gene was excluded by means of the quantitative real-time polymerase chain reaction (qRT-PCR) with TaqMan probes (Aarskog and Vedeler 2000). The coding

sequence and intron-exon junctions of the mitofusin 2 gene (*MFN2*) were amplified using PCR. This region was divided into 16 fragments amplified separately. The primers used for the genomic sequence of the mitofusin 2 gene were published by V.H. Lawson and colleagues (Lawson et al. 2005). The primers for exons 4, 9 and 16 and adjacent intron-exon junctions, and for the cDNA, were designed by us with Primer3 software (primers available on request).

Additionally, in 32 patients we analyzed the region of the promoter of the *MFN2* gene (from c.−10 032 to c.−8 957). The region was amplified with use of three pairs of primers designed with Primer3 software. We didn't include the whole group into the study due to the lack of mutation s in our pilot group.

The amplicons were sequenced using a Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), with analysis on an ABI Prism 373 device. The analyzed fragments were compared with reference sequence of the isoform 1 mRNA NM_014874.3, of the genomic DNA NG_007945.1 and of the protein sequence NP_055689.1. Reverse transcription was performed using a RevertAid First Strand Synthesis Kit (Fermentas), with oligo(d)T primers, according to the manufacturer's protocol.

To determine the number of copies of particular fragments of the *MFN2* gene, in 10 patients we also performed the MLPA analysis using SALSA MLPA probemix P143-B1 MFN2-MPZ (MRC-Holland. The resulting fragments were analyzed on an ABI Prism 3010 device. The MLPA method is not the standard method used in the *MFN2* gene studies in HMSN II patients, so we did only the pilot study with use of this method.

The *MFN2* gene promoter fragments were analyzed by means of the SSCP method. PCR products showing the band patterns different from remaining amplicons were sequenced, as described hereinbefore.

cDNA was amplified using primers designed on the boundary of exons two and three (forward primer) and in exon 6 (reverse primer), resulting in a 270 bp fragment and primers designed in exon 11 (forward primer) and exon 14 (reverse primer), resulting in the 391 bp fragment sequenced with the same Big-Dye Terminator v3.1 Cycle Sequencing Kit, and analyzed on the ABI Prism 373 device. The screening of the 100 healthy controls (200 chromosomes) for the mutation c.311+1G>T was performed using RFLP-PCR with the *Mse I* (Fermentas) restriction enzyme. Analysis of the restriction fragments was visualized on agarose gels, with a single band (279 bp) corresponding to the wild-type sequence, and two bands (210 bp and 69 bp) corresponding to the mutated sequence (data not shown) (Kotruchow et al. 2013). The screening of the 100 healthy controls (200 chromosomes) for the mutation c.1287+50G>T was performed using RFLP-PCR with the *Sch I* (Fermentas) restriction enzyme. The analysis of the restriction fragments was visualized on agarose gels with the three bands (325 bp, 145 bp and 26 bp) corresponding to the wild type sequence and two bands (325 bp and 171 bp) corresponding to the mutated sequence. The data were confirmed by Gene Scan analysis using the same ABI Prism 3010 device (data not shown). The probable significance of the position of the sequence variant was assessed by using ESEfinder 3.0 software (http://rulai.cshl.edu/cgibin/tools/ESE3/esefinder.cgi?process=home).

We screened 27 patients and 63 controls, for the Gln98Leu mutation in the *KIF1B* gene using the SSCP method. Samples that have displayed the different migration patterns were sequenced as described previously.

RESULTS

Duplication/deletion of the *PMP22* gene was excluded prior to investigation of the *MFN2* gene relative to values established in our laboratory (Kabzinska et al. 2009).

The Gln98Leu mutation in the *KIF1B* gene was excluded in 27 out of 69 patients and in 63 healthy controls. No mutations have been detected in MLPA approach performed in the pilot group of patients.

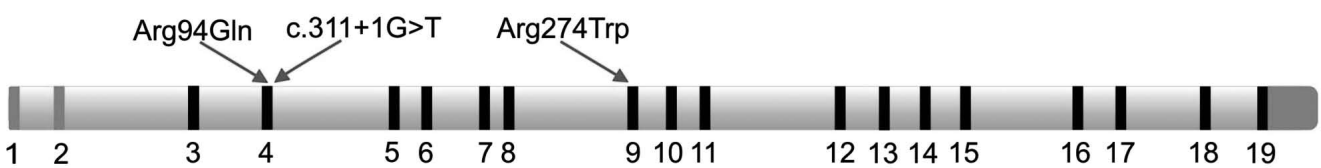

Fig. 2. Schema of the *MFN2* gene with pathogenic mutations reported herein. Black stripes depict coding exons of the gene, green stripes non-coding exons 1 and 2 and the non-coding region of the 19th exon.

Direct sequencing of the mitofusin 2 gene revealed a spectrum of numerous sequence variants among the patients studied. Most of these are common polymorphisms, with a significant average heterozygosity (Table II). Some of the polymorphisms we assumed to be rare (below the average heterozygosity value of 0.1), while four we report for the first time (Table II). We identified three pathogenic mutations (Fig. 2) and three variants of unknown status as regards pathogenicity (Table I).

One of the mutations is a missense mutation c.281C>A (Arg94Gln) commonly reported worldwide and identified in a 4 year-old patient diagnosed with HMSN II and hyperopia (Fig. 3a). The mutation was not identified in his parents, so we assume that it arose *de novo*. The second mutation is also a missense mutation c.820C>T (Arg274Trp) associated with a severe type of HMSN II (Fig. 3b). The mutation in this codon has already been reported in the literature, but arginine changes to another amino acid – glutamine (Arg274Gln) (Züchner et al. 2004). It is also a *de novo* mutation, as it does not occur in the parents of the proband. The amino acid change we identified has not been reported previously. Both mutations, as they are already reported in the literature, might be in hot spot positions. The third identified mutation is in an intron downstream of exon 4, disrupting a splice site (c.311+1G>T) (Fig. 3c). A detailed clinical description of the phenotype associated with this mutation was reported previously (Kotruchow et al. 2013). In the short words, this mutation was identified in a patient with very slowly progressive HMSN II with onset over the age of 50. The heterozygous c.311+1G>T *MFN2* gene mutation results in a presence of an additional shorter transcript, with skipped exon 4, and this leads to a premature STOP codon in the protein.

The first sequence *MFN2* gene variant of unknown status as regards pathogenicity is the missense variant p.Val705Ile. The p.Val705Ile was identified in two unrelated patients. One of the patients is a sporadic case of HMSN II with additional macrocephaly. The second patient is a sporadic case of HMN with a mild clinical course.

A second sequence variant of unknown status (rs370335693) is already reported as a nonpathogenic polymorphism, but there are no relevant frequency data in the NCBI database. We classified it to the group of sequence variants of unknown status as regards pathogenicity, due to the type of variant – it is a missense mutation causing the p.Val573Ile amino acid change, although the PolyPhen software, predicting functional effects of single nucleotide polymorphisms, suggests it should be a benign sequence variant.

A third sequence variant of unknown status as regards pathogenicity is in the intron downstream of exon 12 (c.1287+50G>T). This sequence variant coexists with a mutation in the lamin A/C gene in the patient with Emery-Dreifuss dystrophy (EDMD2) and a mild form of the axonal motor-sensory polyneuropathy. Detailed clinical characteristics of the patient harboring this mutation have been presented in a separate study

In 34 patients from the studied group of patients we analyzed the promoter region of the *MFN2* gene. We identified 1 known variant, rs2236053 present in three patients. We also identified one novel variant not reported hitherto (c.−9891 htz del T).

DISCUSSION

In this study we analyzed the gene encoding the mitofusin 2 protein, identifying three mutations causative for axonal HMSN (HMSN II) disease, six frequent sequence variants with an average heterozygosity value above 0.1, nine rare sequence variants with an average heterozygosity value below 0.1 and three sequence variants of unknown status as regards pathogenicity.

This study sought to include in its group of patients a broad spectrum of the phenotypes of axonal neuropathies. The clinical spectrum of HMSN in our study in fact ranged from hereditary motor neuropathy (HMN) through an intermediate form of HMSN and pure motor-sensory axonal neuropathy (HMSN II) to hereditary sensory neuropathy (HSN). The group of patients also included cases of congenital axonal neuropathy. Since mutations in the *MFN2* gene were identified previously in axonal polyneuropathy with optic nerve atrophy (HMSN VI) (Züchner et al. 2006) and pyramidal signs (HMSN V) (Zhu et al. 2005), we also included such patients in the study.

This is the first study of the *MFN2* gene in the Polish population, among HMSN II patients. Pilot studies were performed in 2006 on a group of 20 Polish patients diagnosed with HMSN II (Verhoeven et al. 2006). The results with 2 identified *MFN2* gene mutations in one family among 20 probands, were

Table II

Rare and frequent sequence variants of the *MFN2* gene found in this study

	Nucleotide change	NCBI ID	Phenotype	Reference
Nº	Rare sequence variants of mitofusin 2 gene			
1.	c.600-116G>A	rs41278624	HMSN II	Previously reported
2.	c.1039-22T>C	rs6680984	HMSN II, HMN	Previously reported
3.	c.1569C>T	rs1042837	HMSN II, HMN	Previously reported
4.	c.1872+63T>C	rs2273295	HMSN II, HMN	Previously reported
5.	c.311+101G>A	–	HMSN II	Novel
6.	c.1160+45A>G	–	HMSN II	Novel
7.	c.1495+184G>T	rs181445368	HMSN II	Previously reported
8.	c.1495+186A>G	–	HMSN II	Novel
9.	c.2204+15T>C	–	HMSN II	Novel
	Frequent sequence variants of the mitofusin 2 gene			
1.	c.474+65C>T	rs2236056	HMSNII, HMN, CMT1/2, HMSN VI/HMSN V, HNPP	Previously reported
2.	c.600-25T>C	rs41278626	HMSN II, HMN,	Previously reported
3.	c.1160+45A>G	rs2236057	HMSNII, HMN, CMT1/2, HMSN VI/HMSN V, HMSN VI, HMSN V, HNPP, EDMD/ HMSN II	Previously reported
4.	c.2069+82C>T	rs11586204	HMSN II, HMN	Previously reported
5.	c.2204+15T>C	rs77262016	HMSN II, HMN	Previously reported
6.	c.*58A>G	rs1042842	HMSNII, HMN, CMT1/2, HMSN VI/HMSN V, HMSN VI, HMSN V, HNPP, EDMD/ HMSN II	Previously reported

very promising, so we were prompted to carry out more extensive research. Regarding our further findings, the result of this pilot study, which gave prevalence of *MFN2* mutations of 10%, was not representative for general population.

In our group, pathogenic *MFN2* mutations were only found in pure motor-sensory axonal neuropathy (HMSN II). Given the frequency of *MFN2* mutations limited to a group of pure HMSN II the frequency of *MFN2* gene mutations reaches 7.7%. However, in a group with a broad spectrum of phenotypes, the *MFN2* gene has a mutation frequency of only 4.5%. This is in contrast to worldwide populations, in which mutations of the *MFN2* gene are present in about 20% of familial

cases of hereditary motor-sensory neuropathy (Verhoeven et al. 2006). However, recent studies have shown, that the frequency of *MFN2* gene mutations in European populations is very heterogeneous. Recent study concerning the Spanish population reports a 2.5% frequency of *MFN2* gene mutations (Sivera et al. 2013) that is in agreement with our observations. This contrasts with the earlier Spanish studies, in which the

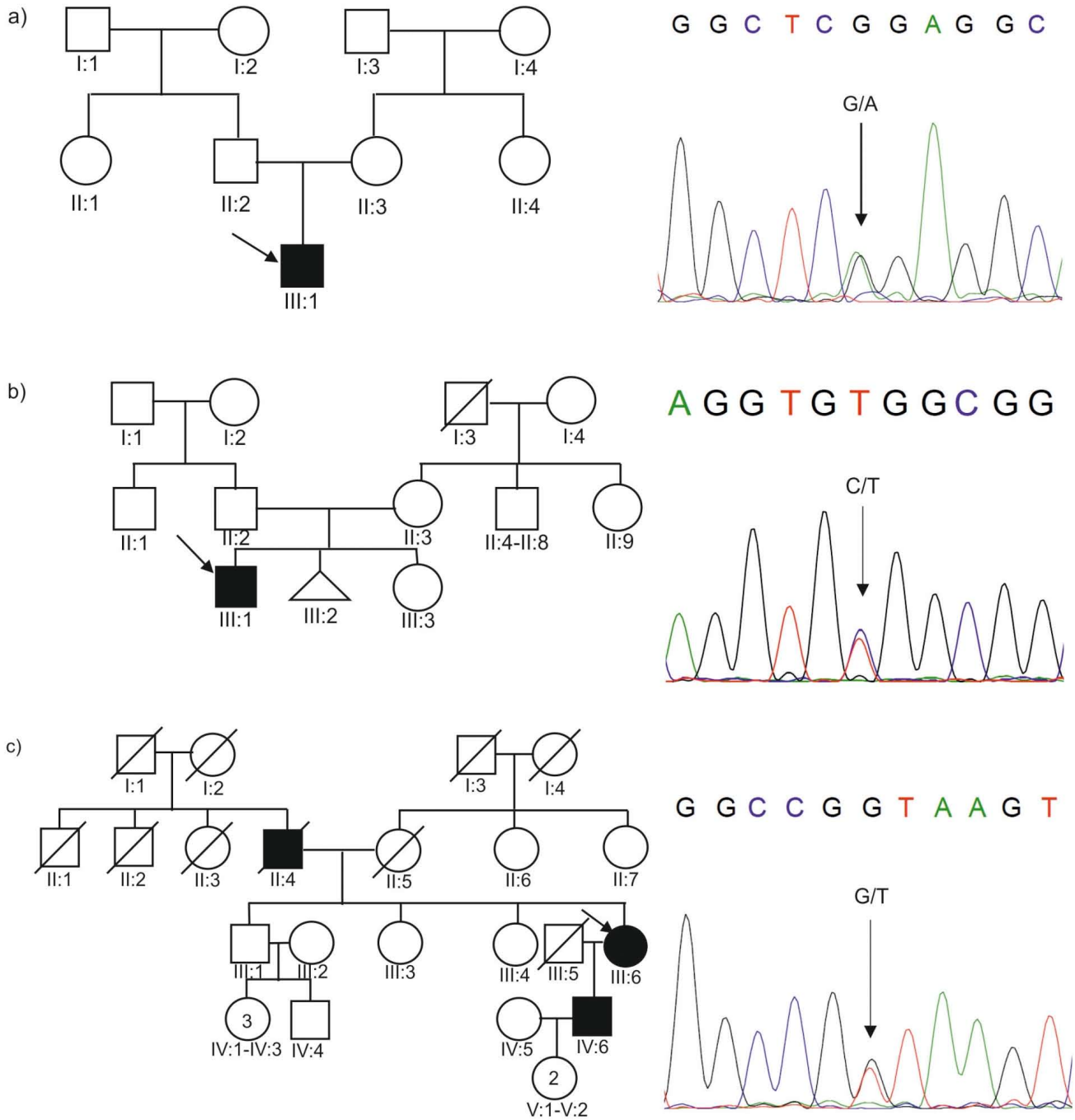

Fig. 3. Family trees and corresponding chromatograms with heterozygous mutations indicated with an arrow; (a) represents the pedigree of a proband with the c.281C>A (Arg94Gln) mutation; (b) that of a proband with the c.820C>T (Arg274Trp) mutation and (c) that of a proband with the splice site mutation (c.311+1G>T). Probands are indicated with an arrow, while circles depict females, squares males, and triangles a miscarriage. Filled symbols denote affected individuals and crossed symbols those who are deceased.

Table III

List of patients with short description of the mode of inheritance, initial diagnosis, age of onset, clinical course and additional symptoms, where present

	Patient	Mode of inheritance	Initial diagnosis	Age of onset	Clinical course	Additional symptoms
1	GT	Sporadic	HMSN II	13	Moderate	–
2	SM	AD	HMSN V	Childhood	Severe	
3	BK	Sporadic	HMSN II	6	Moderate	Scoliosis
4	CP	Sporadic	HMSN II	Congenital	Severe	Ataxia, demyelinating changes in CNS
5	CD	AD	HMSN II	10	Moderate	Facial dysmorphia
6	CO	AD	HMSN II	11	Moderate	–
7	SP	Sporadic	HMSN II	15	Mild	From the sural nerve biopsy: intermediate CMT (CMT1/2)
8	PN	AD/XD	CMT1/2	4	Mild	–
9	FA	AD	EDMD/HMSN II	2nd decade of life	Mild	EDMD caused by the c.1130G>A (p.Arg377His). mutation in *LMNA* gene
10	GJ	Sporadic	HMN	4th decade of life	Severe	Hearing deficiency; proximal muscle involvement
11	PW	Sporadic	HMN	21	Moderate, rapidly progressive	Proximal muscles involvement, in EMG: myopathy signs
12	KM	Sporadic	HMSN II	n/a	Moderate	Vivid knee reflexes
13	NM	AD/XD	HMSN II	n/a	Mild	Facial dysmorphia
14	MK	AR	HMSN II	Congenital	Severe	Scoliosis, interphalangeal joint contractures
15	WR	Sporadic	HMN	42	Too short period of the disease to determine	Proximal muscle paresis
16	RP	AD	HMSN II	n/a	Moderate	Scoliosis

17	KK	Sporadic	HMSN II	Childhood	Severe	Scoliosis, mental retardation, spastic gait
18	SM	Sporadic	HMSN II	33	Moderate	Brisk deep tendon reflexes in the upper and lower extremities
19	PT	Sporadic	HMSN II	5	Severe	Foci of demyelination in the CNS, intellectual disability
20	GM	Sporadic	HMSN II	15	Severe	Postural tremor of hands, hyperopia, mild intellectual disability
21	KN	Sporadic	HMN	6	Mild	Tremor of hands
22	OL	Sporadic	CMT1/2	50	Mild	–
23	PK	Sporadic	HNPP	23	Mild	Hashimoto disease
24	MD	AR	HMSN II	Congenital	Severe	–
25	WM	Sporadic	HMN	15	Mild	–
26	RM	Sporadic	HMSN II	8	Severe	Intellectual disability
27	KM.2	Sporadic	HMSN II	20	Mild	Psoriasis
28	CA	AR/AD	HMSN VI	27	Severe	Optic atrophy
29	SJ	AD	HMN	n/a	n/a	n/a
30	CM	AD/XD	HMN	27	n/a	n/a
31	WA	Sporadic	HMSN II	9	Hard to determine	Brisk reflexes and Babinski sign
32	MJ	Sporadic	Suspected cytopathy mitochondrial (diagnosis difficult to verify – shortages in the documentation)	15th month of life	n/a	n/a
33	MM	AD/XD	CMT1/2	5	n.d.	–
34	RJ	Sporadic	CMT1/2	Early childhood	Severe	–
35	TK	Sporadic	HMSN II	Congenital	Severe, wheelchair dependent	Numerous bone defects

36	KK.2	Sporadic	HMSN II	Congenital	Severe	Macrocephaly, delayed psychomotor development, cortical atrophy in the forehead area
37	MM.2	Sporadic	HMSN II	Childhood	Hard to determine	Right optic nerve atrophy, epilepsy
38	GK	AR/AD	HMSN VI/HMSN V	n/a	Severe	Optic atrophy, spastic paraparesis
39	ŻH	AD	HMSN II	50	Mild	–
40	PM	AR	HSN	n/a	n/a	Cerebellar syndrome
41	BS	AD	HMSN II	Childhood	Mild	Diabetes
42	CK	AD/XD	HMSN II	13	Moderate	–
43	RT	AD	HMSN II	3rd decade of life	Mild	–
44	SD	Sporadic	HSN	Early childhood	Hard to determine	Intellectual disability
45	CJ	AD	HMSN II/HMSN V with brisk reflexes	17	Severe	Dysmorphia, bone defects
46	OJ	AD/XD	HMSN II	15	Moderate	–
47	MM	AD	HMSN II	Childhood	Severe	–
48	WZ	AD	HMSN II	7	Mild	–
49	SG	Sporadic	HMSN II	33	Severe, wheelchair dependent	–
50	SK	Sporadic	HMSN II	4	Hard to determine	Macrocephaly
51	RB	AD/XD	HMSN II	50	Mild	–
52	WM	Sporadic	HMSN II	24	Moderate	–
53	PP	Sporadic	HMSN II	20	Mild	Tremor of fingers
54	WA	Sporadic	HMSN II	n/a	Moderate	n/a
55	BK	AD	HMSN II	13	Mild	–
56	BG	AD	HMSN II	39	Mild	Thrombocytopenia

57	SN	AD	HMN	40	Mild	Tremor of fingers
58	SK	Sporadic	HMSN II	4	Severe	Hyperopia
59	WM	AD	HMSN II	35	Mild	–
60	SA	Sporadic	HMSN II	35	Hard to determine	Scoliosis, depression
61	PT	AR	HMSN II	n/a	n/a	n/a
62	GK	Sporadic	HMSN II	9	Moderate	–
63	DK	Sporadic	HMSN II	4–5	n/a	n/a
64	KW	Sporadic	HMSN II	n/a	Mild	Type 2 diabetes
65	LM	AD	CMT1/2	25	Mild	–
66	LB	Sporadic	HMSN II	Childhood	Mild	Dysmorphia, obesity, mild mental retardation
67	WJ	AD	HMSN II	n/a	n/a	n/a

AD – autosomal dominant mode of inheritance, AR – autosomal recessive mode of inheritance, AD/XD – autosomal dominant mode of inheritance, but we couldn't exclude X dominant mode of inheritance, AD/AR – from the clinical history of the family was not clear, weather the father of the probands was affected; HMN – hereditary motor neuropathy, HMSN II – hereditary motor-sensory neuropathy type II, HSN – hereditary sensory neuropathy, HNPP – hereditary neuropathy with liability to pressure palsy

value exceeded 20% (Casasnovas et al. 2010). Italian work also presents a very high percentage for the occurrence of *MFN2* gene mutations among patients with HMSN II (at nearly 30%) (Bergamin et al. 2014). The study of Norwegian population reported by Braathen and others gave eight *MFN2* gene mutations out of 189 CMT familial cases, which constitutes the prevalence of 3,4% (Braathen et al. 2011). However, recent studies of the *MFN2* gene mutation profile among Russian patients of heterogeneous ethnicity have revealed a low percentage of *MFN2* gene mutations (about 3%). They have reported four mutations in the group of 170 unrelated patients (Khidiyatova et al. 2013). In our group, the additional exclusion of HMSN II with an autosomal recessive mode of inheritance gives an *MFN2* mutation frequency of 8.3%. Additionally, in our population all the found *MFN2* gene mutations were in sporadic cases, so the study of only familial cases of CMT wouldn't give us any results regarding the *MFN2* gene mutations. A question remains as to why these eastern populations are characterized by such a low prevalence of the *MFN2* mutations. In our study generally, we included patients diagnosed with axonal neuropathy. Wee included into the studied group a very wide spectrum of phenotypes and additional symptoms, such as pyramidal signs, optic atrophy, mental retardation, and patients with hereditary motor neuropathy (HMN), and with intermediate CMT, as all these phenotypes can correlate with *MFN2* gene mutations (Braathen et al. 2010). The results obtained by the Norwegian group also show a small percentage of mitofusin 2 mutations among patients with demyelinating HMSN, HMSN II, intermediate axonal-demyelinating HMSN and HMN, this suggesting that none of these phenotypes should be excluded from the genetic analysis of the *MFN2* gene.

In most studies worldwide, genetic analysis mainly included familial cases of HMSN disease. In our study, in contrast, sporadic cases predominate across the group, which was brought together over about 10 years, and represents every region of Poland (Table III).

We identified three *de novo* mutations. The c.281G>A (Arg94Gln) mutation has already been described in the literature. There are over 30 reported mutations in the 94th codon of the *MFN2* gene (Bergamin et al. 2014). Among them there are both familial and sporadic cases with the classical phenotype of the HMSN II disease. Our result, as the next case of a mutation in the given position in the *MFN2* gene, supports the idea that a hot spot exists at this location.

The mutation in the 274th codon of the *MFN2* gene was previously described with the proviso that the amino-acid change was from arginine to glutamine (Züchner et al. 2004). We report here the novel *de novo* c.820C>T (Arg274Trp) mutation in a patient with a severe form of HMSN II. We also refer here to a mutation within the splice site downstream of exon 4 (c.311+1G>T). A complete clinical description of this patient was reported by us (Kotruchow et al. 2013). The recent publication concerning the splice-site mutation in the Finnish family describes patients mildly affected by HMSN II disease resembling clinical phenotype observed in our patient. The authors also suggest that the pathogenic mechanism is due to a dominant-negative or toxic gain-of-function mechanism (Martikainen et al. 2014). This statement is consistent with our observations related to mutations in the *MFN2* gene. There is also evidence that complete loss of function in the homozygous state, in the case of mitofusin 2 gene mutations inherited in an autosomal dominant manner are lethal, in early developmental stages, as stated in studies performed on the murine models (Chen et al. 2003, Detmer et al. 2008). The other study concerning mutations inherited in an autosomal recessive manner performed on the zebrafish, showed loss-of-function mutations in the *MFN2* gene that did not cause embryonic death, but disturbed functions associated with the functionality of the mitofusin 2 protein, i.e. progressive loss of motor function and defects in axonal transport of mitochondria (Chapman et al. 2013). This may partly explain the unresolved case of the Polish family reported in 2006 (Verhoeven et al. 2006), in which the Arg400X mutation in the heterozygous state did not give symptoms of the disease, whereas a compound heterozygote of this mutation together with the Arg250Trp mutation led to the development of the HMSN II disease. Such an idea is supported by the study of Polke and colleagues, in which the authors suggest that patients with the mutated alleles in the heterozygous state might not be considered affected by the disease (Polke et al. 2011).

It is known that patients with a particular mutation in the *MFN2* gene may have variable symptoms within one family (Klein et al. 2011). The authors underline that, in a single family with one mutation in the *MFN2* gene, there can be a whole spectrum of symptoms. Their results suggest the occurrence of variable penetrance of mutations. They did not observe a worsening of symptoms with successive generations, but severely-affected patients had mildly affected children or *vice versa*. This could be the reason why parents of the probands in the previously reported family (Verhoeven et al. 2006) did not exhibit any symptoms. It is also possible that in one patient coexist more than one mutation. That could be the reason why patients with the same mutation in one gene, here *MFN2* gene, show different phenotypes. Such a case is already reported and most reasonable way to examine such a great number of genes (over 40 genes known for CMT) is a new generation sequencing (Høyer et al. 2014).

Alongside the clearly pathogenic variants we also identified others of unknown pathogenic status. One of these is the c.1287+50G>T sequence variant identified in a patient diagnosed with Emery-Dreifuss syndrome with a mild component of the polyneuropathy. This sequence variant was not observed in 100 healthy persons (200 chromosomes). In the course of research on this patient, beside the described mitofusin 2 sequence variant it also proved possible to identify an already-known mutation in the *LMNA* gene. We think that the c.1287+50G>T *MFN2* gene sequence variant might be a possible modifier of the polyneuropathy giving, together with the mutation in the *LMNA* gene, the so-called overlapping syndrome or modulating the clinical course of the disease.

A further sequence variant of the mitofusin 2 gene already known in the literature is c.2113G>A, p.Val705Ile. It was identified in 2 patients in our group, both of which were sporadic cases. To date there have been several studies reporting this *MFN2* gene sequence variant, which was considered pathogenic in the German study (Engelfried et al. 2006) and in a Norwegian one (Braathen et al. 2010). In both cases the segregation study was not performed. The recent works of Auranen and others (Auranen et al. 2013) and Albulym and colleagues (Albulym et al. 2013) show that the Val705Ile sequence variant is a non-pathogenic variant of the *MFN2* gene, as it was identified in both probands and in healthy relatives. Furthermore, the Finnish authors (Auranen et al. 2013) identified this

variant together with the mutation in the *GDAP1* gene causative for CMT2 disease. They noted that patients having both variants display a more severe phenotype than patients with the *GDAP1* gene mutation only, suggesting that Val705Ile might be a factor modifying the polyneuropathy phenotype. We suggest that more extensive studies should be carried out, in regard to the segregation of the sequence variants of the *MFN2* gene. There are known cases of an identified sequence variant causing an amino-acid change that does not segregate perfectly with the occurrence of the disease (Østern et al. 2014). This could be due to incomplete penetrance of the sequence variant, or limited pathogenicity of this variant.

We also identified the sequence variant (c.1717G>A, Val573Ile; rs370335693) that changes the amino acid, but is referred to as a polymorphism. Analysis with the PolyPhen software revealed that this amino-acid change is benign.

There is also a phenomenon, whereby the same HMSN II phenotype is associated with different genetic origins (Zhu et al. 2005), this making it more difficult to select the exact phenotype to be tested for the one particular gene, here the *MFN2* gene. The widening of the HMSN II phenotype would probably result in the identification of a larger number of *MFN2* gene mutations, but there is a question as to what exactly such a characteristic should include. Our results indicate that additional symptoms occurring in an HMSN patient should not exclude the patient from *MFN2* gene analysis, but the group of patients manifesting a pure motor-sensory axonal polyneuropathy should be screened in the first place for *MFN2* gene mutations.

In the case of the gene encoding the mitofusin 2 protein there is a problem with the interpretation of the results, because of the large number of polymorphisms in the *MFN2* gene sequence in particular patients. The majority of the polymorphisms are common, according to the NCBI database, but some of them are less frequent. There remains the open question of whether the rare polymorphic variants and novel missense variants are in fact pathogenic.

We analyzed also the promoter region of the *MFN2* gene, identifying two polymorphic sequence variants. To the best of our knowledge, such an analysis of the *MFN2* gene promoter among HMSN patients has not been performed before. Such a study is complementary to the *MFN2* coding region analysis, and may give insight into the whole gene sequence variants profile. In the literature, there are known mutations in the promoter region of the gene that are clearly pathogenic, e.g. those in the promoter region of the *GJB1* gene that cause the polyneuropathy linked to the X chromosome (Li et al. 2009).

In conclusion, we present here the first study of the mitofusin 2 gene in the Polish population, among the group of patients diagnosed with axonal polyneuropathy. The frequency of occurrence of the *MFN2* gene mutations is low (<5%), this being in agreement with the studies on Russian and Norwegian populations. The statement that a novel missense *MFN2* sequence variant is pathogenic is extremely difficult to sustain, given the large number of polymorphisms in this gene in a single patient, and thus suggesting a probable cumulative effect, as well as incomplete penetrance by the *MFN2* gene mutations, and variable patterns of segregation with the disease.

The previously reported studies on *MFN2* gene mutations indicate the familiar character of HMSN II as an indicator for *MFN2* gene analysis. In our study, we propose a next criterion for *MFN2* analysis, i.e. the phenotype of the pure axonal motor-sensory neuropathy.

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