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ORIGINAL COMMUNICATION

Whole-exome sequencing in patients with inherited neuropathies: outcome and challenges

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Abstract Inherited peripheral neuropathies (IPN) are one of the most frequent inherited causes of neurological disability characterized by considerable phenotypic and genetic heterogeneity. Based on clinical and electrophysiological properties, they can be subdivided into three main groups: HMSN, dHMN, and HSN. At present, more than 50 IPN genes have been identified. Still, many patients and families with IPN have not yet received a molecular genetic diagnosis because clinical genetic testing usually only covers a subset of IPN genes. Moreover, a considerable proportion of IPN genes has to be identified. Here we present results of WES in 27 IPN patients excluded for mutations in many known IPN genes. Eight of the patients received a definite diagnosis. While six of these patients carried bona fide pathogenic mutations in known IPN genes, two patients had mutations in genes known to be involved in other types of neuromuscular disorders. A further group of eight patients carried sequence variations in IPN genes that could not unequivocally be classified as pathogenic. In addition, combining data of WES and linkage analysis identified *SH3BP4, ITPR3*, and *KLHL13* as novel IPN candidate genes. Moreover, there was evidence that particular mutations in *PEX12*, a gene known to cause Zellweger syndrome, could

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also lead to an IPN phenotype. We show that WES is a useful tool for diagnosing IPN and we suggest an expanded phenotypic spectrum of some genes involved in other neuromuscular and neurodegenerative disorders. Nevertheless, interpretation of variants in known and potential novel disease genes has remained challenging.

Keywords IPN · CMT · dHMN · HMSN · WES

Introduction

Inherited peripheral neuropathies (IPN) represent one of the most frequent inherited causes of neurological disability with an estimated prevalence of 1 in 2,500 [1]. Age at disease onset is usually within the first two decades of life but may be later as well. Clinical hallmarks comprise foot deformities, slowly progressive weakness and wasting of the distal parts of the lower limbs leading to gait disturbances, and usually distally pronounced sensory deficits. Involvement of the small hand muscles may also be present to a variable degree [2, 3]. All modes of inheritance have been described. Traditionally, IPN are subdivided into three main groups based on clinical and electrophysiological properties: the hereditary motor and sensory neuropathies (HMSN), i.e., the classical form, which is also known as Charcot–Marie–Tooth (CMT) syndrome. HMSN is further subdivided into the demyelinating form (HMSN1, CMT1) characterized by a considerable reduction of nerve conduction velocities (NCV; motor median nerve <38 m/s), an axonal variant (HMSN2, CMT2) with normal or slightly slowed NCV but low compound motor action potential amplitudes (CMAP), and an intermediate form (intermediate HMSN, ICMT) with NCV in the intermediate range (motor median nerve 25–45 m/s) [2, 3]. Patients lacking sensory disturbances both clinically and electrophysiologically have been classified as distal hereditary motor neuropathies (dHMN) [4, 5]. However, an overlap between dHMN and HMSN (mainly HMSN2) has been observed even within families [6, 7]. If patients present with predominant sensory (and autonomic) abnormalities and no or milder motor disturbances, the disease is called hereditary sensory (and autonomic) neuropathy (HSN, HSAN) [8, 9]. Furthermore, the IPN phenotype may be complicated by various additional neurological and/or non-neurological features [10–12].

Molecular genetic studies have demonstrated marked genetic heterogeneity of the IPN with more than 50 genes identified so far ([3] and are in part listed by http://www.molgen.ua.ac.be/CMTMutations/). However, many patients and families with IPN have not yet received a molecular genetic diagnosis. One explanation is that clinical genetic testing usually does not include all known IPN genes (mostly due to restricted funds and limited availability of tests). Moreover, further genetic heterogeneity has been suggested and additional causative genes have to be elucidated [13].

Next-generation sequencing techniques including whole-exome sequencing (WES) and whole-genome sequencing (WGS) have now opened promising possibilities to find the disease-causing mutation in patients harboring any Mendelian disease. Recent studies have also demonstrated the diagnostic and scientific impact of WES and WGS in IPN patients [14–18].

In the present study, WES was carried out in 27 patients with presumed rare or novel forms of IPN excluded for mutations in numerous known IPN genes (Supplementary Table 1). Thereby, eight patients (29.6 %) received an accurate diagnosis, while potentially disease-causing mutations were identified in another eight cases (29.6 %). In three families (11.1 %), we identified interesting new candidate genes for IPN. Another three probands (11.1 %) carried variants of unknown significance in genes known to cause other neuromuscular disease, but still in five patients (18.5 %) neither mutations in known IPN genes nor any strong candidate variants could be sorted out.

Patients and methods

Clinical and electrophysiological studies

Twenty-seven index probands who had received a diagnosis of IPN were included in this study. Clinical and electrophysiological studies were performed using standard methods as described previously [19]. Patients were subdivided according to the mode of inheritance and their phenotype, which either resembled HMSN or dHMN. Cases exhibiting IPN, but also additional neurological and/or non-neurological signs or symptoms, were classified as "complicated" IPN.

Ten patients showed dominant or autosomal dominant inheritance (HMSN-D1–D6; dHMN-D1–D4). Autosomal recessive inheritance was suspected in seven probands, all exhibiting an HMSN phenotype (HMSN-R1–R7). In ten patients, the disease occurred apparently sporadic. Four of these showed a "classical" HMSN-phenotype (HMSN-S1–S4), four had a complicated form of HMSN (HMSN-SC1-4) and two were classified as dHMN (dHMN-S1-S2) (Tables 1, 2, 3).

The study was approved by the local ethical committees of the Medical Universities of Vienna and Graz.

Genetic studies

Sanger sequencing

Prior to WES, up to 20 known IPN genes were excluded for mutations in the probands using Sanger sequencing

Table 1 Clinical and electrophysiological features from ten IPN index patients with dominant/autosomal dominant inheritance

Phenotype family/ patient ID	Sex	Age at onset/exam (in years)	LL: muscle involvement	UL: muscle involvement	Sensory loss/V	PTR/ ATR	MNCS-CMAP Med/peron	SNCS Med/sur	Additional symptoms	Linkage analysis	WES diagnosis (SNV)
HMSN-D1/II-1	M	40/45	+	+	++/6/8	1/0	54.0–14.0/36.0–4.5	56.9/40.7	No	No	DCTN1 [p.Y670F] het
HMSN-D2/III-1	M	12/40	+++	+++	+/3/8	0/0	nr/nr	34.8/35.9	No	No	**GJB1 [p.M1L] hem**
HMSN-D3/II-1	F	Inf/22	+	–	No/7/8	1/1	45.6–8.3/39.3–6.6	52.6/nr	No	No	ARHGEF10 [p.N202H] het
HMSN-D4/II-1	M	8/53	++	+	No	0/0	54.3–15.0/nr	51.8/27.1	Scoliosis	No	–
HMSN-D5/II-2	M	7/40	+++	+++	+++/0	0/0	16.8–0.9/nr	nr/nr	Scoliosis, foot ulcers	Yes	SH3BP4 [p.R16H] het
HMSN-D6/II-3	F	40/43	++	–	++/0	0/0	34.7–4.8/21.7–0.8	41.1/nr	Multiple cysts, proximal weakness	Yes	ITPR3 [p.T1424M] het
dHMN-D1/III-3	M	11/39	+++	+++	No/2	3/0	40.3–0.1/nr	50.0/31.3	No	No	**GARS [p.H472R] het**
dHMN-D2/III-1	M	6/43	+++	++	No/6/8	1/0	56.4–12.4/nr	50.6/44.8	Hand tremor	Yes	GDAP1 [p.K39N] het
dHMN-D3/IV-6	M	36/25	+++	+	No/6/8	1/0	55.6–13.7/44.1–0.6	49.1/37.6	CK↑, WHE	No	RYR1 [p.R552Q] het
dHMN-D4/ [16]	– [16]	– [16]	– [16]	– [16]	– [16]	– [16]	– [16]	– [16]	– [16]	– [16]	**REEP1 [c.304-2 A>G][16] het**

Interesting candidate genes are underlined, confirmed pathogenic mutations are shown in bold

IPN inherited peripheral neuropathies, *Inf* infancy, *M* male, *F* female, *LL* lower limbs, *UL* upper limbs, *nr* no response, *nd* not done, *WHE* weakness of finger extensors, *V* vibration sense, *PTR* patellar tendon reflexes, *ATR* Achilles tendon reflexes, *MNCS* motor nerve conduction studies (m/s), *CMAP* compound motor action potential (mV), *SNCS* sensory nerve conduction studies (m/s), *Med* medianus, *Peron* peronaeus, *Sur* suralis, *WES* whole-exome sequencing, *SNV* single-nucleotide variant, *CK* creatine kinase, – normal, *hem* hemizygous, *hom* homozygous, *het* heterozygous

* Gene involved in piebaldism [45]

Table 2 Clinical and electrophysiological features from seven IPN index patients with autosomal recessive inheritance

Phenotype Family/ patient ID	Sex	Age at onset/ exam (in years)	LL: Muscle involvement	UL: Muscle involvement	Sensory loss/V	PTR/ ATR	MNCS-CMAP Med/peron	SNCS Med/ sur	Additional symptoms	Linkage analysis	WES diagnosis (SNV)
HMSN-R1/III-1	F	30/38	+++	–	++/–	0/0	50.9–4.1/ 45.8–0.2	nr/nr	CK↑, distal and proximal weakness LL	Yes	HSJ1 [p.Y5C] hom
HMSN-R2/IV-2	M	7/59	+++	+	+/0	0/0	48.1–3.8/ 38.8–0.3	39.9/ nr	Scoliosis	Yes	**GAN [p.V438I] hom**
HMSN-R3/III-1	M	Inf/46	+++	+++	No	0/0	28.0–?/nd	41.0/ nd	Wheelchair dependent, distal and proximal weakness LL	Yes	<u>KLHL13 [p.L376S] hem</u>
HMSN-R4/II-3	M	Inf/49	+++	+++	+/–	0/0	42.5–5.3/nr	49.1/ 26.1	Hand tremor, mild proximal weakness LL	Yes	PEX12 [p.S190L]+ [p.R180X] het
HMSN-R5/II-2	F	Inf/37	+++	++	+/–	1/1	43.0–0.9/nr	44.0/ 31.0	No	Yes	–
HMSN-R6/IV-4	F	22/31	++	–	++/6/8	1/2	49.0–7.0/ 36.6–2.1	51.8/ 39.7	CK↑	Yes	MFN2 [p.R259H] het
HMSN-R7	M	4/5	+++	+	+/0	0/0	nd/47–0.1	nd/nd	No	No	**GAN [p.I102T] hom**

Interesting candidate genes are underlined, confirmed pathogenic mutations are shown in bold

IPN inherited peripheral neuropathies, *Inf* infancy, *M* male, *F* female, *LL* lower limbs, *UL* upper limbs, *nr* no response, *nd* not done, *WHE* weakness of finger extensors, *V* vibration sense, *PTR* patellar tendon reflexes, *ATR* Achilles tendon reflexes, *MNCS* motor nerve conduction studies (m/s), *CMAP* compound motor action potential (mV), *SNCS* sensory nerve conduction studies (m/s), *Med* medianus, *Peron* peronaeus, *Sur* suralis, *WES* whole-exome sequencing, *SNV* single-nucleotide variant, *CK* creatine kinase, – normal, *hem* hemizygous, *hom* homozygous, *het* heterozygous

* Gene involved in piebaldism [45]

Table 3 Clinical and electrophysiological features from ten IPN patients without family history

Phenotype Family ID	Sex	Age at onset/exam (in years)	LL: Muscle involvement	UL: Muscle involvement	Sensory loss/V	PTR/ATR	MNCS-CMAP Med/peron	SNCS Med/sur	Additional symptoms	Linkage analysis	WES diagnosis (SNV)
HMSN-S1	M	5/35	+++	+++	No/–	0/0	43.6–1.3/nr	nr/nr	Wheelchair dependent, proximal weakness LL, scoliosis, CK↑	No	–
HMSN-S2	F	8/32	+++	++	+/0	0/0	32.0–?/33.0–?	nr/nr	Hand tremor	No	GAA [p.G638W]+ [p.L917F] het
HMSN-S3	F	Inf/10	+++	++	No/7/8	1/0	50.5–3.6/ 36.0–1.0	46.8/46.2	–	No	**–**
HMSN-S4	M	15/75	+++	–	++/0	0/0	64.4–5.3/nr	47.0/nr	Scoliosis	No	YARS [p.E274K] het
HMSN-SC1 [24]	– [24]	– [24]	– [24]	– [24]	– [24]	– [24]	– [24]	– [24]	– [24]	– [24]	**SPTLC1 [p.S331Y]** [24] **het**
HMSN-SC2	F	6/19	+++	+++	±	0/0	nr/nr	nr/nd	Unilateral vocal cord paralysis, scoliosis	No	**SBF2 [p.R356X] hom**
HMSN-SC3	M	31/34	++	+	+/2	3/1	nd/26.8–0.2	nd/35.0	Cerebellar signs, hyperlipidemia, dementia, bilateral cataracts at 13y, Babinski pos	No	**CYP27A1 [p.T339M] hom**
HMSN-SC4	F	18/57	+++	+++	++/0	0/0	24.8–1.6/nr	nr/nr	Dysarthria, cerebellar signs, hypermobility of joints	No	PRX [p.V525A]+ [p.R1335Q] het
dHMN-S1	M	20/30	–, mild pes cavus	++	No/4/8	1/1	nr/42.0–3.7	49.0/40.0	Isolated atrophy (thenar/Int I), familial piebaldism	No	AARS [p.T608M] het, **KIT* [p.E583Q] het**
dHMN-S2	F	35/38	+++	–	No	1/1	46.5–7.7/ 46.6–8.3	48.1/54.1	No	No	–

Confirmed pathogenic mutations are shown in bold

IPN inherited peripheral neuropathies, *Inf* infancy, *M* male, *F* female, *LL* lower limbs, *UL* upper limbs, *nr* no response, *nd* not done, *WHE* weakness of finger extensors, *V* vibration sense, *PTR* patellar tendon reflexes, *ATR* Achilles tendon reflexes, *MNCS* motor nerve conduction studies (m/s), *CMAP* compound motor action potential (mV), *SNCS* sensory nerve conduction studies (m/s), *Med* medianus, *Peron* peronaeus, *Sur* suralis, *WES* whole-exome sequencing, *SNV* single-nucleotide variant, *CK* creatine kinase, – normal, *hem* hemizygous, *hom* homozygous, *het* heterozygous

* Gene involved in piebaldism [45]

techniques (Supplementary Table 1). Sequence variations detected by WES that were considered to be associated with the disease were confirmed by Sanger sequencing.

Whole-exome sequencing

Sequencing was performed on a HiSeq 2000 system (Illumina, San Diego, CA, USA) after in-solution enrichment of exonic and adjacent intronic sequences [SureSelect Human all Exon 50 Mb v3 and v4 kits (Agilent, Santa Clara, CA, USA)] and indexing of samples for multiplex-sequencing (Multiplexing Sample Preparation Oligonucleotide Kit, Illumina). We performed 100-bp paired-end runs yielding on average 8.8 Gb of sequence for 35 samples (the 27 index probands and for eight families an additional affected individual was included). The average read depth was 110 with 90 % of the targeted regions covered at least 20-fold. Read alignment was performed with BWA (version 0.6.1) to the human genome assembly hg19. Single-nucleotide variants and small insertions and deletions were called with SAMtools (version 0.1.18). We filtered variants to exclude HapMap-SNPs present in dbSNP-135 with an average heterozygosity greater than 0.02 and those present in more than four of 2,246 in-house exomes from individuals with unrelated diseases. Variant annotation was performed with custom scripts.

Linkage analysis

To perform linkage analysis, genomic DNA samples from all available affected and unaffected individuals and spouses of families HMSN-D5, HMSN-D6, HMSN-R4, and dHMN-D2 were hybridized to GeneChip Human Mapping 10 K 2.0 and 250 K Nsp SNP arrays (Affymetrix, Santa Clara, CA, USA) using the protocols recommended by the manufacturer. Parametric multipoint LOD scores and haplotypes were obtained with the ALOHOMORA program [20] based on linkage analysis from Merlin [21] under the assumption of an autosomal dominant or autosomal recessive, fully penetrant model. Pedigrees of the families, possible disease intervals, and maximum LOD scores detected for each family are shown in Supplementary Fig. 1a–1d.

Biochemical studies

Very long chain fatty acids (VLCFA) and phytanic acid concentrations in plasma were determined by GC/MS with deuterated internal standards as their methyl or dimethylsilyl derivatives according to established methods [22, 23]. Pipecolic acid was analyzed with a modified commercially available method designed for amino acid analysis by liquid chromatography/triple quadrupole mass spectrometry with pipecolic acid-D9 as internal standard (Phenomenex, Torrance, CA, USA).

Results

Clinical and electrophysiological findings in the index patients and results of WES are summarized in Tables 1, 2, and 3. Applying filtering as indicated above revealed up to 377 variants (108–377, mean 163) for each individual. First, we focused on significant SNVs in genes known to be involved in the pathogenesis of IPN. This approach resulted in 15 missense variants in 13 IPN genes (Table 4). Subsequently, we searched for variants in genes known to cause other neuromuscular or neurodegenerative diseases. Thereby we detected seven interesting variants in five genes (Table 5). Moreover, we identified three interesting novel candidate genes for IPN (Table 6). Variants were further evaluated in detail to define their potential impact on the disease (Tables 4, 5, 6). Whenever possible, further affected and unaffected family members were screened to confirm or exclude segregation.

SNVs in IPN genes regarded to be disease causing

In eight index cases we identified mutations which we regarded to be disease causing. These mutations affect amino acid residues that are well conserved among species. Most of them are predicted to be probably or possibly damaging by Polphen2 and SIFT (Tables 4, 5) and segregated within the families. Patient HMSN-D2 carried the c.1 A>T (p.M1L) missense variant in *GJB1* (gap junction protein, beta 1, 32 kDa) that was also present in his less severely affected mother. The same mutation had been detected previously in another unrelated Austrian HMSN patient (unpublished data) exhibiting a similar phenotype thus making causality of the disease highly likely. In patient HMSN-D2 this variant had been missed by conventional Sanger sequencing in a laboratory offering genetic testing. In two patients (HMSN-R2 and HMSN-R7) displaying a classical HMSN2 phenotype we identified two novel homozygous mutations in *GAN* (gigaxonin) (c.1312 G>A, p.V438I; c.305 T>C, p.I102T). Segregation within the family could be confirmed for the c.305 T>C (p.I102T) mutation. The mutation in *SPTLC1* (serine palmitoyltransferase, long chain base subunit 1) (c.992 C>A, p.S331Y, patient HMSN-SC1) resulted in an unexpected severe sensory motor neuropathy. The detailed phenotype has been reported recently [24].

Patient HMSN-SC2, exhibiting a severe HMSN phenotype with unrecordable NCV in the upper and lower limbs, was homozygous for the c.1066 C>T (p.R356X) mutation in *SBF2* (SET binding factor 2). In addition to prominent

Table 4 Mutations/sequence variations identified in IPN genes

Family ID	IPN gene	Base change	Mutation	Segregation in family	Ns SNV in 2,246 controls	Conservation of mutation	Polyphen 2	Sift	Impact on IPN disease
HMSN-D1	*DCTN1*	c.2009 A>T (het)	p.Y670F	Yes	33 (0)	+++	0.983	1	PDC
HMSN-D2	*GJB1*	c.1 A>T (hem)	p.M1L	Yes	4 (0)	+++	0.989	0	DC
HMSN-D3	*ARHGEF10*	c.604 A>C (het)	p.N202H	Yes	73 (0)	+++	0.953	0.22	PDC
HMSN-R1	*HSJ1*	c.14 A>G (hom)	p.Y5C	Yes	15 (2)	+++	0.976	0	PDC
HMSN-R2	*GAN*	c.1312 G>A (hom)	p.V438I	nt	15 (0)	+++	0.931	0.02	DC
HMSN-R7	*GAN*	c.305 T>C (hom)	p.I102T	Yes	15 (0)	+++	0.639	0	DC
HMSN-R6	*MFN2*	c.776 G>A (het)	p.R259H	No	20 (0)	++++	0.995	0.01	PDC
HMSN-S4	*YARS*	c.820 G>A (het)	p.E274K	nt	14 (2)	+++	0.182	–	PDC
HMSN-SC1	*SPTLC1*	c.992 C>A (het)	p.S331Y [24]	Yes	13 (0)	+++	0.595	0	DC
HMSN-SC2	*SBF2*	c.1066 C>T (hom)	p.R356X	nt	62 (2)	++++	–	1	DC
HMSN-SC4	*PRX*	c.604 T>C (het)	p.V525A	nt	56 (0)	+	0.001	–	PDC
	PRX	c.4004 G>A (het)	p.R1335Q	nt	56 (0)	++	0.479	0.18	PDC
dHMN-D1	*GARS*	c.1415 A>G (het)	p.H472R	Yes	25 (0)	+++	0.997	0	DC
dHMN-D2	*GDAP1*	c.117 G>C (het)	p.K39N	Yes	11 (1)	++++	0.970	0	PDC
dHMN-S1	*AARS*	c.1823 C>T (het)	p.T608M	nt	37 (2)	+++	1.000	0	PDC

Polyphen 2: Classification of a mutation: probably damaging (probabilistic score >0.85), possibly damaging (probabilistic score >0.15). The remaining mutations are classified as benign [60]

Sift: damaging (score $\leq$ 0.05), tolerated (score > 0.05) (http://sift.jcvi.org/www/SIFT_help.html#SIFT_OUTPUT)

hem hemizygous; *hom* homozygous; *het* heterozygous; *nt* not tested; *Ns SNVs in controls* number of non-synonymous single-nucleotide variations in 2,246 in home controls; *conservation of mutation* ++++ complete at position and within region, +++ almost complete, ++ moderate, + incomplete (not in mouse); *DC* disease causing; *PDC* potentially disease causing; *IPN* inherited peripheral neuropathies

distal muscle weakness and wasting, the patient presented with moderate scoliosis and a hoarse voice due to unilateral vocal fold paralysis. The c.1415 A>G (p.H472R) mutation in *GARS* (glycyl-tRNA synthetase), which had already been reported previously [25], was identified in family dHMN-D1 and was also present in the similarly affected father.

SNVs in non-IPN genes regarded to be disease causing

Proband HMSN-SC3 was initially diagnosed as HMSN2 because of prominent pes cavus, distal muscle wasting, and axonal neuropathy being the most striking feature at the beginning of the disease. However, with progression of the disease complications like bilateral juvenile cataracts, cerebellar and pyramidal tracts signs, and abnormalities of lipid metabolism became evident as well. WES identified a known homozygous mutation in *CYP27A1* (cytochrome P450, family 27, subfamily A, polypeptide 1) (c.1016 C>T, p.T339M), the gene responsible for cerebrotendinous xanthomatosis [26, 27]. The advanced clinical presentation fits well with the latter diagnosis although initial predominant peripheral neuropathy may be an unusual finding. The mutation in *REEP1* (receptor accessory protein 1) (c.304-2 A>G; family dHMN-D4) and the associated phenotype has been reported elsewhere [16].

SNVs of unknown significance in known IPN genes

A further eight patients carried novel mutations in known IPN genes (Table 4). The significance of these variations has remained unclear so far for several reasons. Mutations in *DCTN1* (dynactin 1) have been reported in patients with dHMN and amyotrophic lateral sclerosis [28, 29] but not in HMSN2 as is the phenotype of family HMSN-D1 carrying the c.2009 A>T (p.Y670F) mutation, which has not yet been reported. Only one family without clinical symptoms but slow NCV has been reported carrying a mutation in *ARHGEF10* (Rho guanine nucleotide exchange factor (GEF) 10) [30]. The two patients of family HMSN-D3 exhibit very mild intermediate HMSN and both carry a c.604 A>C (p.N202H) variant. We also identified mutations in *AARS* (alanyl-tRNA synthetase) and *YARS* (tyrosyl-tRNA synthetase), two further IPN genes [31, 32]: case HMSN-S4 carried a *YARS* mutation c.820 G>A (p.E274K) and proband dHMN-S1 carried an *AARS* mutation c.1823 C>T (p.T608M). Although all these variants alter well-conserved amino acid residues, we noted that a

Table 5 Mutations/sequence variations identified in genes known to cause other neuromuscular/neurodegenerative disorders

Family ID	IPN gene	Base change	Mutation	Segregation in family	Ns SNV in 2,246 controls	Conservation of mutation	Polyphen 2	Sift	Impact on IPN disease
HMSN-R4	*PEX12*	c.538 C>T (het)	p.R180X	Yes	16 (4)	+++	–	0.43	PDC
		c.569 C>T (het)	p.S190L	Yes	16 (4)	+++	0.996	0.03	
HMSN-S2	*GAA*	c.1912 G>T (het)	p.G638W	nt	49 (1)	++	1	0	PDC
		c.2749 C>T (het)	p.L917F	nt	49 (1)	++	0.01	0.68	
dHMN-D4	*REEP1*	c.304–2 A>G [16] (het)	–	Yes	6 (1)	++++	–	–	DC
HMSN-SC3	*CYP27A1*	c.1016 C>T (hom)	p.T339M	nt	26 (5)	++++	0.999	0	DC
dHMN-D3	*RYR1*	c.1655 G>A (het)	p.R552Q	Yes	154 (7)	+++	0.999	0.04	PDC

Polyphen 2: Classification of a mutation: probably damaging (probabilistic score >0.85), possibly damaging (probabilistic score >0.15). The remaining mutations are classified as benign [60]

Sift: damaging (score $\leq$ 0.05), tolerated (score > 0.05) (http://sift.jcvi.org/www/SIFT_help.html#SIFT_OUTPUT)

hem, hemizygous; *hom,* homozygous; *het,* heterozygous; *nt,* not tested; *Ns SNVs in controls,* number of non synonymous single nucleotide variations in 2,246 in-home controls; *conservation of mutation,* ++++ complete at position and within region, +++ almost complete, ++ moderate, + incomplete (not in mouse); *DC* disease causing; *PDC* potentially disease causing; *IPN* inherited peripheral neuropathies

Table 6 New interesting candidate genes for IPN identified by whole-exome sequencing

Family ID	IPN gene	Base change	Mutation	Segregation in family	Ns SNV in 2,246 controls	Conservation of mutation	Polyphen 2	Sift	Impact on IPN disease
HMSN-D5	*SH3BP4*	c.47 G>A (het)	p.R16H	Yes	31 (1)	+++	0.991	0	PDC
HMSN-D6	*ITPR3*	c.4271 C>T (het)	p.T1424M	Yes	69 (3)	++++	0.961	0	PDC
HMSN-R3	*KLHL13*	c.1127 T>C (hem)	p.L376S	Yes	10 (0)	++++	0.995	0	PDC

Polyphen 2: Classification of a mutation: probably damaging (probabilistic score >0.85), possibly damaging (probabilistic score >0.15). The remaining mutations are classified as benign [60]

Sift: damaging (score $\leq$ 0.05), tolerated (score > 0.05) (http://sift.jcvi.org/www/SIFT_help.html#SIFT_OUTPUT)

hem hemizygous; *hom* homozygous; *het* heterozygous; *nt,* not tested; *Ns SNVs in controls,* number of non synonymous single nucleotide variations in 2,246 in-home controls; *conservation of mutation,* ++++ complete at position and within region, +++ almost complete, ++ moderate, + incomplete (not in mouse); *DC* disease causing; *PDC* potentially disease causing; *IPN* inherited peripheral neuropathies

considerable number of rare missense variants affecting almost invariable residues occur in *DCTN1*, *ARHGEF10*, *AARS*, and *YARS* in our controls (Supplementary Table 2) and in other databases (http://evs.gs.washington.edu/EVS/; https://genomics.med.miami.edu/). Thus, caution seems warranted when evaluating these variants in these genes.

Mutations in *HSJ1* (DnaJ (Hsp40) homolog, subfamily B, member 2) have been described in one recessive dHMN family only [33]. However, the phenotype in family HSMN-R1 carrying a c.14 A>G (p.Y5C) mutation resembles HMSN2. To confirm the pathogenicity of this mutation, functional studies are currently ongoing.

Mutations in *MFN2* (mitofusin 2) have been reported in both autosomal dominant and autosomal recessive HMSN [34–37]. The heterozygous c.776 G>A (p.R259H) *MFN2* mutation in family HMSN-R6 presenting with a mild HMSN2 phenotype affects a highly conserved residue but does not segregate within the family. If this MFN2 variant was a true mutation, two carriers (III/4 and IV/1) would have remained asymptomatic (Supplementary Fig. 2). Individuals III/4 and IV/1 are neurologically and electrophysiologically normal except for diminished tendon reflexes in the lower limbs in III/4 and very mild pes cavus foot deformity in IV/1.

Moreover, we identified compound heterozygosity for *PRX* (periaxin) (c.604 T>C, p.V525A and c.4004 G>A, p.R1335Q) in a patient with a complex phenotype including dysarthria, cerebellar signs, and hypermobility of joints (HMSN-SC4). While the c.4004 G>A (p.R1335Q) mutation is novel and involves a residue moderately conserved among species, the c.604 T>C (p.V525A) variant is a known rare polymorphism affecting a weakly conserved amino acid [38].

Finally, the c.117 G>C (p.K39N) variant in *GDAP1* (ganglioside-induced differentiation associated protein 1) changes a well-conserved amino acid, segregates within the small family dHMN-D2, and is one of only three variants located with the possible linkage regions detected

(Supplementary Fig. 1d). However, a dHMN phenotype is unusual in context with dominant GDAP1-associated neuropathy.

Identification of IPN candidate genes

Linkage analysis was most helpful in selecting possible candidate genes in small IPN families. Results are summarized in Supplementary Fig. 1a–1d. WES in families HMSN-D5 and HMSN-D6 (Supplementary Fig. 1a and 1b) both classified as HMSN1 based on NCV studies did not reveal a variation in the known IPN genes. Also, the two families did not share variants within a single gene. Therefore, we scrutinized all variants located within the regions of possible linkage in each family. Given the structure of the pedigrees six chromosomal regions in family HMSN-D5 and more than ten loci in family HMSN-D6 reached the corresponding maximum LOD scores of 1.5 and 1.2, respectively. Combining data of linkage analysis and WES revealed only one candidate gene for family HMSN-D5 (*SH3BP4* gene (SH3-domain binding protein 4): c.47 G>A, p.R16H) and two for family HMSN-D6 (*ITPR3* gene (inositol 1,4,5-trisphosphate receptor, type 3): c.4271 C>T, p.T1424M and *TEC* gene (tec protein tyrosine kinase): c.700 G>A, p.V234I) (Supplementary Fig. 1a and 1b). All three variants affected well-conserved amino acid residues (Supplementary Fig. 1a and 1b). In line with data fom the linkage screens, segregation of these variants was confirmed by Sanger sequencing.

Family HMSN-R3 consists of two affected brothers exhibiting severe early onset intermediate HMSN leading to wheelchair dependence after age 30. The parents were unaffected by history. By filtering for X-chromosomal, homozygous, and compound heterozygous variants, only a variant on the X-chromosome affecting the *KLHL13* gene (kelch-like family member 13) (c.1127 T>C, p.L376S) was detected. This variant was also present in the affected brother.

Mutations in genes known to cause other neuromuscular diseases

In the remaining families, we did not find potentially relevant variants in any of the known IPN genes. However, family HMSN-R4 was compound heterozygous for two well-conserved variants in *PEX12* (peroxisomal biogenesis factor 12), a gene previously reported to cause a severe autosomal recessive infantile disease, called Zellweger syndrome [39–41]. While the c.538 C>T (p.R180X) mutation had already been reported in Zellweger patients [40], the c.569 C>T (p.S190L) variant is novel but affects a strongly conserved residue. Testing of further family members revealed segregation with the phenotype. *PEX12* was the only gene in the regions suggestive for linkage harboring two mutations (Supplementary Fig. 1c). As patients with Zellweger syndrome have elevated levels of VLCFA and phytanic acid, we tested serum of our patients for these acids. While VLCFA and phytanic acid were normal, we repeatedly found elevated levels of pipecolic acid in both patients but not in the healthy parents. In cultured fibroblasts of the patients, 70–75 % of catalyze activity was particle bound while in control fibroblasts 95–100 % are associated with peroxisomes.

In patient dHMN-D3, we detected a novel mutation in *RYR1* (ryanodine receptor 1 (skeletal)) (c.1655 G>A, p.R552Q), a gene which is involved in malignant hyperthermia and central core disease [42]. This mutation was also present in the affected father.

Patient HMSN-S2 was compound heterozygous for two SNVs in *GAA* (glucosidase, alpha; acid) (c.1912 G>T, p.G638W; c.2749 C>T, p.L917F), the gene responsible for Pompe's disease [43, 44].

Detection of mutations in genes causing other Mendelian diseases

In addition to dHMN, patient dHMN-S1 was affected with piebaldism, an autosomal dominant skin disorder that was present in three generations of the family. The genetic cause had not yet been identified. WES revealed a known heterozygous missense variant (c.1747 G>C, p.E583Q) in *KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog), the gene most frequently involved in the pathogenesis of piebaldism [45].

In total, 4–47 (mean 22) SNVs in different genes causing dominant and recessive Mendelian diseases could be detected in every proband but these variants were not further evaluated.

Discussion

Among 27 patients who had already been screened negative for many known IPN genes and were thus highly enriched for mutations in rarer IPN genes, we identified the disease-causing mutation in eight cases (29.6 %) by WES. This result is in line with previous studies [17] and highlights the power of WES in the diagnosis of rare forms of IPN. In six of these subjects, mutations in known IPN genes (*GAN*, *GARS*, *GJB1*, *SBF2*, *SPTLC1*) were detected, while in two cases the responsible gene was previously reported to cause another neurodegenerative disease (*CYP27A1*, *REEP1*). To interpret the value of each candidate variant, we used prediction programs (Polyphen 2, Sift), considered alignment of sequences with multiple organisms, and tested for segregation of the variants within

the families whenever possible. Variants in these eight patients were either predicted to be probably or possibly damaging and targeted strongly conserved amino acid residues (Tables 4, 5). Interestingly, we found a mutation in *GJB1*, a gene that is frequently mutated in HMSN and therefore included in routine diagnosis as was the case in patient HMSN-D2. This demonstrates that WES may detect variants that have been missed by conventional Sanger sequencing. It is noteworthy that two patients with classical HMSN2 carried mutations in *GAN*. None of the patients had cerebellar signs or characteristic hair changes as described previously [46–48]. A "pure" HMSN2 phenotype due to *GAN* mutations is unusual and unexpected. We therefore suggest that sequence analysis of *GAN* should more often be included in routine diagnosis in childhood-onset HMSN2 patients.

Notably, three of four individuals (i.e., 75 %) classified as sporadic and complicated IPN received a definite diagnosis. In patient HMSN-SC2, we found a novel homozygous nonsense mutation in *SBF2*. Mutations in *SBF2* are known to cause severe recessive HMSN1, which may be accompanied by glaucoma [49–51]. The latter feature was not observed in our patient. However, a hoarse voice was noted and could be explained by unilateral vocal fold paralysis, thus expanding the phenotypic spectrum of *SBF2*-associated IPN. Vocal fold paralysis is common in dominant HMSN2C caused by mutations in *TRPV4* (transient receptor potential cation channel, subfamily V, member 4) [52] and has also been reported in patients with recessive HMSN4A due to *GDAP1* mutations [53]. Patient HMSN-SC3 initially presented with pes cavus, gait abnormalities, and an axonal neuropathy in the lower limbs. Other features like ataxia, cataracts, elevated serum lipid levels, and dementia, which are typically seen in patients with cerebrotendinous xanthomatosis due to *CYP27A1* mutations developed later, thus postponing the distinct diagnosis on a clinical basis [26]. This case demonstrates that in particular conditions, WES may serve to establish a diagnosis on the basis of the genotype leading to re-assessment of the phenotype. Finally, the complicated, syndromic phenotype of patient HMSN-SC1, which has already been reported in detail, could surprisingly be explained by a distinct mutation in *SPTLC1* [24].

In a further eight subjects (29.6 %), we identified mutations in known IPN genes (*DCTN1, ARHGEF10, HSJ1, MFN2, YARS*, *PRX*, *GDAP1, AARS*). Most of these targeted well-conserved amino acid residues and were predicted to be probably or possibly damaging (Table 4). However, interpretation of clinical significance turned out to be challenging for several reasons. For some of these genes, well-conserved SNVs not reported in any database and absent in our large series of 2,246 controls are also frequently found in individuals not afflicted with IPN (Supplementary Table 2). This raises the question of how to sort out true disease-causing mutations. Testing of further family members to confirm segregation is an attractive option, but this requires availability and cooperation of larger families. As functional studies are usually expensive and time-consuming and not always readily available, systematic comparison of SNV in IPN databases will become a promising strategy to provide a correct diagnosis for patients. Currently, this service is already offered at https://genomics.med.miami.edu. Patients carrying sequence variants of unknown significance in IPN genes should be invited to genetic counseling and have to be informed that a final diagnosis based on results available by WES is currently challenging. Although dominant mutations in *GDAP1* have been reported in IPN families [54], we were cautious to define the c.117 G>C (p.K39N) mutation in our family dHMN-D2 as definitively disease-causing. To make any firm conclusions, additional patients with dHMN carrying heterozygous *GDAP1* mutations have to be identified.

As has been shown in previous studies [16], a combination of linkage analysis and WES was helpful in identifying novel candidate genes for IPN in this study. Combining linkage and WES data of four families dramatically reduced the number of SNVs and identified interesting candidate genes (Supplementary Fig. 1a–1d). In family HMSN-D5, a mutation in *SH3BP4* (c.47 G>A, p.R16H) was the only variant located within the regions of suggestive linkage. SH3BP4 functions in transferrin receptor internalization at the plasma membrane through a cargo-specific control of clathrin-mediated endocytosis [55]. It interacts with DNM2 (dynamin 2) a protein which is also involved in the pathogenesis of IPN [56]. In family HMSN-D6, two unknown SNVs remained within the regions of potential linkage. The most interesting candidate gene is *ITPR3* [57]. It encodes a receptor for inositol 1,4,5-trisphosphate, a second messenger that mediates the release of intracellular calcium. ITPR3 is expressed in distinct cellular domains of the Schwann cells, particularly in dense patches in the paranodal region. Notably, connexin 32 (Cx32), a gap junction protein responsible for HMSN X, is expressed in close proximity with ITPR3. It has therefore been speculated that Schwann cell Ca^{2+} signals control the function of the gap junctions, or that the gap junctional channels serve as conduits for rapid radial spread of Ca^{2+} signals initiated during action potential propagation [58]. Sequencing of *SH3BP4* and exon 32 of *ITPR3* in more than 30 HMSN1 families did not reveal further mutations, indicating that these genes might—if so—be a rare cause of IPN. In family HMSN-R3, we suspected autosomal recessive inheritance as the parents were reported to be unaffected. However, no homozygous or compound heterozygous mutations could be detected. Instead, we

identified a mutation in *KLHL13*, which is located on chromosome X and encodes a BTB (Bric-a-brac–Tram-track–Broad complex) and kelch domain-containing protein [59].

Of particular interest is family HMSN-R4 with severe intermediate HMSN. Autosomal recessive inheritance is likely as the parents were clinically and electrophysiologically normal after age 80. The only gene harboring biallelic mutations and being located in one of the potential linkage regions was *PEX12*. Segregation of the variants within the family was confirmed (Supplementary Fig. 1c). While one of the mutations (c.538 C>T, p.R180X) has already been reported in Zellweger's disease, the second (c.569 C>T, p.S190L) was novel. Testing for metabolic consequences of the two mutations yielded normal values for VLCFA and phytanic acids but considerably elevated levels of pipecolic acids in both affected probands but not in the heterozygous parents. Furthermore, in cell lines from both patients, a considerable fraction of catalase activity is cytosolic. Functional studies are ongoing to further explain these results. However, no firm conclusions can be drawn that particular recessive mutations in *PEX12* can produce an IPN phenotype. It cannot be ruled out that the *PEX12, SH3BP4*, *ITPR3*, and *KLHL13* variants represent harmless variants and that large insertions, deletions, chromosomal rearrangements, or intronic variants not detected by WES are the true disease-causing mutation in these families. Therefore, identification of mutations in *PEX12, SH3BP4*, *ITPR3*, and *KLHL13* in further IPN families as well as functional studies are needed to confirm whether these are indeed genes for IPN.

Finally, given the example of patient dHMN-S1 presenting with dHMN and a dominant skin disease due to a KIT c.1747 G>C (p.E583Q) mutation, this study demonstrates that WES is a suitable method for screening patients harboring more than one Mendelian disease.

In summary, we confirm that WES is an efficient tool in the diagnosis of IPN but interpretation of variants in known and potential novel disease genes has remained challenging. We suggest an expanded phenotypic spectrum of some genes involved in other neuromuscular and neurodegenerative disorders and introduce three novel IPN candidate genes. Based on the results obtained in this study, we conclude that WES should be preferred to IPN/CMT panels if patients present with a complicated phenotype and/or if many IPN genes have already been excluded.

Web resources

http://www.ncbi.nlm.nih.gov/
http://www.ensembl.org/index.html
http://genome.ucsc.edu/
http://evs.gs.washington.edu/EVS/
http://www.molgen.ua.ac.be/CMTMutations/
https://genomics.med.miami.edu/
http://sift.jcvi.org/www/SIFT_help.html#SIFT_OUTPUT

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Conflicts of interest The authors declare that they have no conflicts of interest.

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