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Genes for hereditary sensory and autonomic neuropathies: a genotype–phenotype correlation

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Hereditary sensory and autonomic neuropathies (HSAN) are clinically and genetically heterogeneous disorders characterized by axonal atrophy and degeneration, exclusively or predominantly affecting the sensory and autonomic neurons. So far, disease-associated mutations have been identified in seven genes: two genes for autosomal dominant (*SPTLC1* and *RAB7*) and five genes for autosomal recessive forms of HSAN (*WNK1/HSN2*, *NTRK1*, *NGFB*, *CCT5* and *IKBKAP*). We performed a systematic mutation screening of the coding sequences of six of these genes on a cohort of 100 familial and isolated patients diagnosed with HSAN. In addition, we screened the functional candidate gene *NGFR* (*p75/NTR*) encoding the nerve growth factor receptor. We identified disease-causing mutations in *SPTLC1*, *RAB7*, *WNK1/HSN2* and *NTRK1* in 19 patients, of which three mutations have not previously been reported. The phenotypes associated with mutations in *NTRK1* and *WNK1/HSN2* typically consisted of congenital insensitivity to pain and anhidrosis, and early-onset ulcero-mutilating sensory neuropathy, respectively. *RAB7* mutations were only found in patients with a Charcot-Marie-Tooth type 2B (CMT2B) phenotype, an axonal sensory-motor neuropathy with pronounced ulcero-mutilations. In *SPTLC1*, we detected a novel mutation (S331F) corresponding to a previously unknown severe and early-onset HSAN phenotype. No mutations were found in *NGFB*, *CCT5* and *NGFR*.

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Overall disease-associated mutations were found in 19% of the studied patient group, suggesting that additional genes are associated with HSAN. Our genotype–phenotype correlation study broadens the spectrum of HSAN and provides additional insights for molecular and clinical diagnosis.

Keywords: HSAN; *SPTLC1*; *RAB7*; *WNK1/HSN2*; *NTRK1*

Abbreviations: AD = autosomal dominant; AR = autosomal recessive; CIPA = congenital insensitivity to pain and anhidrosis; CMT = Charcot-Marie-Tooth disease; HMSN = hereditary motor and sensory neuropathy; HSAN = hereditary sensory and autonomic neuropathy; NCV = nerve conduction velocity; STRs = short tandem repeats; TM = transmembrane domain; SF = Rab subfamily domain; PG/M = conserved domain implicated in binding of phosphate/Mg2+ and guanine binding; F = Ras family domain; PK = protein kinase domain; AI = autoinhibitory domain; CC = coiled coil domain; SP = signal peptide; Cys = cysteine cluster; LRM = leucine rich motif; Ig = immunoglobulin-like domain

Introduction

Hereditary sensory and autonomic neuropathies (HSAN) are a clinically and genetically heterogeneous group of inherited peripheral neuropathies, primarily affecting the peripheral sensory and autonomic neurons (Dyck, 1993). Patients usually exhibit prominent distal sensory loss with manifest insensitivity to pain in some. The prominent distal sensory loss frequently leads to chronic ulcerations in feet and hands, sometimes resulting in severe complications such as extensive soft tissue infections, osteomyelitis necessitating amputations of toes and fingers or, in rare instances, even of more proximal parts of the extremities (Dyck, 1993). Autonomic dysfunction, such as anhidrosis, fever, blood pressure fluctuations and gastro-intestinal disturbances are present in some patients. Electrophysiologically, axonal nerve damage of the sensory neurons is often found, but additional demyelination may also be present (Auer-Grumbach *et al*., 2003).

HSAN can be transmitted as an autosomal dominant (AD) or autosomal recessive (AR) trait. Isolated patients have also been described (Dyck, 1993; Auer-Grumbach, 2004). The AD types of HSAN usually present in the second or third decade of life with marked sensory involvement and minimal autonomic and variable motor involvement, while AR HSAN present either as congenital syndromes with striking sensory and autonomic abnormalities or as almost pure autonomic disorders (Verpoorten *et al*., 2006*a*).

A classification of the hereditary sensory neuropathies into types HSAN I–V (Dyck, 1993) was made based on age at onset, inheritance pattern and additional features. Although the clinical classification of these HSAN types is based on a small number of individuals, it still stands after the molecular characterization of the subtypes in recent years. There is variable motor involvement in the AD form of HSAN, making the distinction with hereditary motor and sensory neuropathies (HMSN) or Charcot-Marie-Tooth disease (CMT) difficult. In CMT2B, sensory loss and the associated ulcerations are such prominent phenotypic features that inclusion within the HSAN-spectrum is justified (Vance *et al*., 1996; Verpoorten *et al*., 2006*a*). However, due to the concomitant motor involvement with distal muscle atrophy and weakness, this phenotype was originally classified as HMSN (Kwon *et al*., 1995).

So far, seven genes have been identified for the different types of HSAN (http://www.molgen.ua.ac.be/CMTMutations/). Two genes have been associated with AD HSAN: missense mutations in serine palmitoyltransferase long chain subunit 1 (*SPTLC1*) are found in families and individuals with HSAN type I, an adult-onset sensory neuropathy (Bejaoui *et al*., 2001; Dawkins *et al*., 2001). Mutations in the small GPTase late endosomal protein *RAB7*, cause CMT2B (Verhoeven *et al*., 2003; Meggouh *et al*., 2006). Mutations in the *WNK1/HSN2* gene [protein kinase with-no-lysine(K)-1/hereditary sensory neuropathy type 2] cause AR HSAN type II, an early-onset ulcero-mutilating sensory neuropathy (Lafreniere *et al*., 2004). HSAN type III, also known as Familial Dysautonomia or Riley–Day syndrome, presents with typical prominent autonomic manifestations early in life and is caused by mutations in the inhibitor of kappa-light polypeptide gene enhancer in B cells, kinase complex associated protein (*IKBKAP*) (Slaugenhaupt *et al*., 2001). Mutations in neurotrophic tyrosine kinase, receptor type 1 (*NTRK1*) are reported in families with congenital insensitivity to pain, anhidrosis and mental retardation (CIPA or HSAN type IV) (Indo *et al*., 1996). HSAN type V, a phenotype closely related to CIPA but with normal mental development and less pronounced anhidrosis, can be caused by mutations in nerve growth factor beta (*NGFB*) (Einarsdottir *et al*., 2004) but also by *NTRK1*-mutations (Houlden *et al*., 2001; Einarsdottir *et al*., 2004). Apart from these six HSAN subtypes other forms with distinct additional features exist, e.g. HSAN with gastroesophageal reflux and cough (Kok *et al*., 2003) and HSAN with spastic paraplegia (Bouhouche *et al*., 2006*b*). Recently, the gene for this last form has been identified as cytosolic chaperonin-containing *t*-complex peptide-1 *(CCT5)* (Bouhouche *et al*., 2006*a*). The identification of causative genes for the HSAN forms in recent years has provided preliminary insights in the pathogenesis of these rare neuropathies although the fundamental underlying pathomechanisms still remain to be unveiled (Verhoeven *et al*., 2006).

In this study, we investigated a cohort of 100 familial and isolated patients who had a clinical diagnosis compatible with any of the subtypes of HSAN listed above, and we determined the contribution of mutations in the known genes associated to the distinct phenotypes. *IKBKAP* was not screened since our cohort did not contain patients with familial dysautonomia. The cohort included 16 index patients of families that have previously been reported in manuscripts describing novel genes and related phenotypes. We broadened the screening of the individual genes to non-associated phenotypes in order to establish potential new genotype–phenotype correlations. Furthermore, we performed

the first large-scale mutation screening of *WNK1* and *CCT5*. Additionally, we screened the functional candidate gene *NGFR* (*p75/NTR*) because of its importance in development and function of sensory neurons (Lee *et al*., 1992).

Patients and Methods

Selection criteria

For this study, we selected a group of 100 individuals from our patient database. These were individuals presenting with a clinical phenotype compatible with any of the HSAN subforms described earlier (Dyck, 1993; Auer-Grumbach *et al*., 2006). The majority of patients presented with progressive distal sensory loss, often associated with one or several of the following additional features: skin changes (e.g. hyperkeratosis, ulcerations), spontaneous fractures, amputations and autonomic features. Because variable motor involvement under the form of distal muscle wasting and weakness can be present in some subtypes of sensory neuropathies, we also included a group of patients diagnosed with CMT2B, a variant of axonal motor and sensory neuropathy (HMSN II), with prominent ulcero-mutilations (Vance *et al*., 1996; Verpoorten *et al*., 2006*a*). To avoid inclusion of classic axonal CMT variants unrelated to the HSAN spectrum, we only included patients with motor and sensory neuropathies if their clinical presentation was complicated by the development of ulcerations. Overall, the cohort in this study could be described as a group of hereditary ulcero-mutilating and sensory neuropathies. Autonomic symptoms were only seen in patients who also presented with sensory abnormalities. Our cohort did not contain patients with predominant or pure dysautonomia, the hallmark feature of HSAN type III or Riley–Day syndrome. Diagnosis was based on clinical presentation, complemented with nerve conduction velocity (NCV) measurements and EMG. No strict electrophysiological selection criteria were applied to our cohort, given the broad range of electrophysiological features associated with the various HSAN phenotypes. Typically, a predominantly sensory axonal neuropathy was found, which was often more severe in the lower limbs. Occasional electrophysiological signs of demyelination can also be found in HSAN. Electrophysiological abnormalities in motor nerves such as reduced amplitudes of compound muscle action potentials and slightly reduced motor NCV can be found, illustrating the overlap between HSAN and HMSN. In CIPA patients, nerve conduction studies can be within normal range (Shatzky *et al*., 2000; Auer-Grumbach *et al*., 2003; Axelrod and Gold-von Simson, 2007). In several patients, nerve and skin biopsies were performed.

Patient cohort

The cohort consisted of 100 index patients who were referred to our laboratory for molecular genetic testing in the context of HSAN. Genomic DNA samples were provided through Neurologic and Paediatric Departments and Neuromuscular Centres worldwide. The majority of samples were of European origin. In 43 patients, autonomic features were noted, 44 had a pure sensory neuropathy and the remaining 13 were diagnosed as sensory-motor neuropathy with ulcero-mutilations. In two patients, the HSAN phenotype presented with an associated spastic paraplegia. For 21 out of 100 index patients, a dominant inheritance pattern, based on a parent to child transmission, could be determined. For eight index patients, a recessive inheritance pattern characterized by the presence of affected siblings in the pedigree could be determined. Twenty-four patients were referred as 'isolated' since they did not have a familial history of neuropathy in the first- and second-degree relatives. No family history was available for the remaining 47 patients. In five index patients, there was a clear indication of consanguinity of the parents. For 44 patients, detailed information about the age at onset was available; in nine patients, first symptoms occurred in the first year of life, in 11 patients the disease started in the first decade and in 12 patients in the second decade. In the remaining 12 patients, onset was after the age of 20 years. In 35 patients, ulcerations were present and 17 out of them displayed additional complications such as osteomyelitis or amputations. Detailed electrophysiological data were available for 37 patients, the remaining patients had an electrophysiological evaluation by their referring physician but detailed information was not available. In 17 patients, a nerve biopsy or a combined sural nerve/muscle biopsy was performed showing abnormalities compatible with a diagnosis of HSAN. All referring clinicians were neurologists, orthopaedic surgeons or paediatricians active in the field of rare neuromuscular diseases and well acquainted with the clinical presentation of HSAN and related phenotypes. The referring clinicians obtained informed consent from all patients or their legal representatives prior to enrolment in this study.

Molecular genetic analysis

All DNA samples were amplified using the whole genome amplification kit 'GenomiPhi V2 DNA Amplication Kit' (GE Healthcare, Waukesha, USA). The protocol was performed according to the manufacturer's instruction.

The coding regions and exon–intron boundaries up to 100 bp up- and downstream of the exons of *SPTLC1, RAB7, WNK1/HSN2, NTRK1, NGFB*, *CCT5* and *NGFR* were PCR-amplified using primer oligonucleotides designed with the Primer3 and SNPbox software tools (Rozen and Skaletsky, 2000; Weckx *et al*., 2004). PCR conditions are available upon request. PCR products were cleaned up using the Exonuclease I-Shrimp Alkaline Phosphatase enzyme (USB, Cleveland, USA). Mutation screening was performed by direct sequencing of the purified PCR fragments using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Fragments were separated on an ABI3730xl DNA Analyser (Applied Biosystems, Foster City, USA). The resulting sequences were aligned and analysed with the novoSNP (Weckx *et al*., 2005) and SeqMan™II (DNASTAR Inc., Madison, USA) programs. The nucleotide numbering of the genes is relative to the ATG translation initiation site with A as +1 of the corresponding cDNA sequences (*SPTLC1*: NM_006415.2; *RAB7*: NM_004637.5; *NTRK1*: NM_002529.3; *NGFB*: NM_002506.2; *CCT5*: NM_012073.3; *NGFR*: NM_002507). The nucleotide numbering of *WNK1/HSN2* is relative to the first nucleotide of the HSN2-specific exon of *WNK1* (NM_213665.1). Mutations are described according to the latest conventions on the nomenclature of DNA sequence variants (http://www.hgvs.org/mutnomen). Sequence variants were confirmed by repeated PCR on original DNA samples and bidirectional sequencing. Where possible, segregation of the mutation with the disease phenotype was analysed in the family.

Genotyping and paternity testing

Paternity was tested using 15 highly informative short tandem repeats (STRs) distributed throughout the genome (ATA38A05, D1S1646, D1S1653, D1S1360, D2S2256, D3S3037, D4S2382, D4S3240, D7S509, D8S1759, D9S1118, D12S1056, D12S2082, D16S2619

and GATA152H04). STRs were PCR-amplified and PCR fragments were loaded on an ABI3730xl DNA Analyser. Genotypes were analysed using Local Genotype Viewer, a software program developed in-house (http://www.vibgeneticservicefacility.be/).

Analysis of exon skipping

Analysis of exon skipping was performed by RT-PCR on mRNA isolated from lymphoblast cell lines of patients CMT-841.01 and CMT-886.01. mRNA was first purified from peripheral blood lymphoblasts using the RNeasy mini kit (Qiagen, Hilden, Germany). DNA inactivation was performed using the Turbo DNA free kit (Ambion, Austin, USA) and subsequently analysed for splicing defects by RT-PCR and sequencing using exonic primers located in exons 1 and 5 for patient CMT-886.01 (forward: 5′-CTGCTGGCTTGGCTGA TACT-3′, reverse: 5′-CACTGCAGCTTCTGTTCAGG-3′) and exonic primers in exons 12 and 16 for CMT-841.01 (forward: 5′-CCTTGTG CTCAACAAATGTGG-3′, reverse: 5′-AGCCAGCAGCTTGGCAT-3′) (Superscript III First-Strand Synthesis System for RT-PCR; Life Technologies, San Diego, USA).

Results

In 19 index patients, out of a cohort of 100, pathogenic mutations were found in four HSAN disease associated genes: *SPTLC1, RAB7, NTRK1* and *WNK1/HSN2*. These mutations were absent from 600 European control chromosomes. No pathogenic variations could be detected in *NGFB*, *CCT5* and *NGFR*. Clinical and electrophysiological data on these 19 index patients are summarized in Tables 1 and 2.

Mutations in *SPTLC1*

In Patient CMT-791.01, we detected a heterozygous missense mutation (c.992C>T; p.Ser331Phe), which was absent in both healthy parents (Fig. 1A). Paternity was confirmed in this family, pointing to a *de novo* mutation. In contrast to previously reported HSAN type I patients, the patient displayed a severe phenotype characterized by congenital onset with severe growth and mental retardation, hypotonia and vocal cord paralysis. In Patient CMT-186.05 a missense mutation (c.1055C>T; p.Ala352Val) was identified (Fig. 1B). A third heterozygous missense mutation (c.1160G>C; p.Gly387Ala) was found in Patient CMT-155.01 and her twin sister CMT-155.02 (Verhoeven *et al*., 2004). This mutation was also found in Patient CMT-820.01. However, the healthy mother of this index patient has the same variant in the homozygous state. This finding suggests that the Gly387Ala variation is not pathogenic, but a rare polymorphism. This has recently been confirmed by the analysis of serine palmitoyl transferase (SPT) activity by measuring the incorporation of [U-^{13}C]-L-serine in protein extracts from stably transfected HEK293 cells and complementation testing using an SPTLC1-deficient CHO cell line (Hornemann *et al*., 2009).

Mutations in *RAB7*

In our cohort, two different missense mutations in *RAB7* have been identified in seven anamnestically unrelated index patients. The index Patients CMT-90.01 and CMT-195.01 of two multigenerational pedigrees as well as two additional Patients with a positive familial history (CMT-186.28 and PN-626.01) carried the same heterozygous transition c.484G>A resulting in a Val162Met missense mutation. The c.385C>T (p.Leu129Phe) missense mutation was detected in three Austrian index Patients: CMT-126.01, CMT-140.01 and CMT-186.26. Additional haplotype analysis revealed that the Val162Met mutation arose independently in the reported families/patients, but the Leu129Phe mutation resides on a common disease haplotype indicating a founder effect (Verhoeven *et al*., 2003). All index patients carrying a *RAB7* mutation present with an adolescent or adult-onset HMSN II phenotype and are characterized by distal atrophy and weakness in the lower limbs with pronounced distal sensory loss complicated by ulcerations and amputations.

Mutations in *WNK1/HSN2*

A recent report showed that *HSN2* is a nervous system-specific exon of the *WNK1*-gene. A compound heterozygous mutation in *WNK1* and *HSN2* was identified as the cause for HSAN type II (Shekarabi *et al*., 2008). In view of this recent finding, the entire coding region of *WNK1* was screened in our patient cohort. No additional disease-related sequence variants were identified outside of the *HSN2* exon.

We identified a total of four different *WNK1/HSN2* mutations in three previously reported patients: one compound heterozygous mutation consisting of a 1bp-deletion resulting in a frameshift mutation with a premature stop codon (c.254delC; p.Pro85HisfsX14) and an insertion of a thymine (c.1089_1090insT) predicted to cause a frameshift mutation with premature stop codon (p.Gln364SerfsX16) in Patient CMT-451.01. One homozygous non-sense mutation (c.550C>T; p.Gln184X) and one homozygous 2-bp deletion (c.1064_1065delTC) predicted to cause a frameshift and premature stop codon (p.Ile355AsnfsX7) were detected in Patients CMT-260.01 and CMT-178.01, respectively (Coen *et al*., 2006).

Mutations in *NTRK1*

In our cohort, seven mutations in *NTRK1* were found, of which one was a novel homozygous missense mutation (c.1697G>A; p.Arg565Gln) in Patient CMT-841.01 (Tables 1 and 2). An affected sibling CMT-841.02 carried the same homozygous mutation (Fig. 1C). This mutation targets the tyrosine kinase domain of the neurotrophin tyrosine kinase receptor (Fig. 2). The mutated nucleotide is the last base pair of exon 14, resulting in aberrant splicing (Fig. 1D). Furthermore, we identified a known 9 bp-deletion (c.354_359 + 3delTCGCCTGAA) (Tuysuz *et al*., 2008) in a recently reported Turkish Patient (CMT-886.01) (Kilic *et al*., 2009). This deletion spans the splice-donor site of exon 3. cDNA analysis revealed two splice variants; one with skipping of exon 3 and the second with skipping of exons 2 and 3. These exons contain a part of the leucine-rich motif of NTRK1, important for ligand binding and signal transduction. The patient displayed a CIPA phenotype complicated by recurrent infections

Table 1 Clinical features of HSAN patients with proven mutation

Patient	Gene	AA change	Origin	Diagnosis	Inheritance	AAO	SAO	ALE	Sensory loss	Skin changes	Amputations	Bone complications	Autonomic symptoms
CMT-186.05	*SPTLC1*	Ala352Val	Austria	HSAN1	IC	16y	Sensory abnormalities	46y	Severe LL	–	–	–	–
CMT-791.01	*SPTLC1*	Ser331Phe	France (Gypsy)	HSAN/ CIPA	IC	cong	Insensitivity to pain	7y	Severe loss of superficial touch distal LL, insensitivity to pain	eschar and ulceration foot	–	–	Gastro-oesophageal reflux
PN-626.01	*RAB7*	Val162Met	Belgium	CMT2B	AD	37y	Osteomyelitis toes	54y	Diminished L>R	Ulcerations toes	+ (toes)	Osteomyelitis in the toes	–
CMT-90.01	*RAB7*	Val162Met	UK	CMT2B	AD	28y	Ulcerations toes	64y	Severe in distal LL, position sense conserved, UL less pronounced	Ulcerations toes	+ (all toes R foot)	Unk	–
CMT-126.01	*RAB7*	Leu129Phe	Austria	CMT2B	AD	15y	Weakness in LL	75y	Severe	Ulcerations feet	–	Osteomyelitis and -necrosis	–
CMT-140.01	*RAB7*	Leu129Phe	Austria	CMT2B	AD	13y	Ulcerations toes	46y	Moderate	Ulcerations and foot callus	+ (toes)	Osteomyelitis and -necrosis	–
CMT-186.26	*RAB7*	Leu129Phe	Austria	CMT2B	AD	20y	Ulcerations toes	49y	Severe in LL (toes and feet)	Ulcerations toes	–	Osteomyelitis and -necrosis	–
CMT-186.28	*RAB7*	Val162Met	Austria	CMT2B	AD	15y	Steppage gait	34y	No clinical sensory loss	Multiple ulceration toes	+ (lower leg L, several toes R)	Osteomyelitis	–
CMT-195.01	*RAB7*	Val162Met	USA	CMT2B	AD	ad	Gait disturbances	Unk	Distal LL	Ulcerations feet	Unk	Unk	–
CMT-178.01	*HSN2*	Ile355AsnfsX7	Belgium	HSAN2	IC	<2y	Ulcerations hand and feet, poor healing	5y	Distally severely reduced for all modalities, LL>UL	Ulcerations toes and fingers	–	Spontaneous fractures LL + Charcot atropathy	–
CMT-260.01	*HSN2*	Gln184X	Austria	HSAN2	IC	inf	Clumsiness hands, osteomyelitis in the foot	50y	Distally severely reduced for all modalities, LL>UL	Ulcerations	+ (fingers and toes)	Osteomyelitis in the foot	–
CMT-451.04	*HSN2*	Pro85ProfsX14 + Gln364SerfsX16	Italy	HSAN2	IC	<1y	Difficulties in hand manipulation	33y	Distally severely reduced for all modalities, LL>UL	Ulcerations in hands and feet	+ (progressive spontaneous amputations hands, and LL to knees)	Painless pathologic fractures LL	Unk
CMT-179.01	*NTRK1*	Gly181GlyfsX16	Belgium	HSAN4/ CIPA	IC	cong	Delayed motor milestones?	12y	Insensitivity to pain	Thickening and ulcerations LL	–	–	Anhidrosis
CMT-197.01	*NTRK1*	c.359+ 5G>T aberrant splicing	Belgium	HSAN4/ CIPA	IC	<8m	Fever of unknown cause	14y	Insensitivity to pain	Unk	–	–	Anhidrosis, fever episodes
CMT-366.01	*NTRK1*	Arg761Trp	The Netherlands	HSAN4/ CIPA	IC	cong	Anhidrosis, hyperthermia, hypotonia	3y	Insensitivity to pain	Unk	–	Painless fracture right calcaneus	Anhidrosis, fever episodes
CMT-826.01	*NTRK1*	c.2046+ 3 A>C aberrant splicing	Spain	HSAN4/ CIPA	AR	5m	Skin ulcerations	20y	Insensitivity to pain and temperature	Hyperkeratosis, ulcerations	+ (toes)	Osteomyelitis	Anhidrosis, fever episodes

(continued)

Table 1 Continued

Patient	Gene	AA change	Origin	Diagnosis	Inheritance	AAO	SAO	ALE	Sensory loss	Skin changes	Amputations	Bone complications	Autonomic symptoms
CMT-841.01	*NTRK1*	Arg565Gln	The Netherlands (Moroccan origin)	HSAN4/ CIPA	AR (cons)	4 yrs	Painless tibial fracture, poor healing	35y	Insensitivity to pain	–	–	Multiple factures (pelvic bone, upper and lower limbs and foot bones), osteomyelitis	Anhidrosis
PN-1192.03	*NTRK1*	Gln626GlnfsX7	The Netherlands (Moroccan origin)	HSAN4/ CIPA	IC (cons)	cong	Hypotonia, recurrent episodes of fever	2y	Insensitivity to pain	Keratodermatitis, xerodermia, selfmutilation of fingers and tongue, necrotizing fasciitis of right hand	–	–	Anhidrosis, fevers
CMT-886.01	*NTRK1*	c.354_359+3 delTCGCCTGAA aberrant splicing	Turkey	HSAN4/ CIPA	IC	5m	Fever, recurrent infections	7y	Insensitivity to pain	Hyperkeratosis, ulcerations, nail dystrophy, automutilations, neck abscess	–	Avascular necrosis left talus	Anhidrosis

AA = amino acid; AAO = age at onset (years); SAO = symptoms at onset; ALE = age at last exam (y); cong = congenital; inf = infancy; ad = adulthood; IC = isolated case; AD = autosomal dominant; AR = autosomal recessive; Fam = familial; cons = consanguineous; LL = lower limb; UL = upper limb; + = present; – = absent; unk = unknown; dist = distal; prox = proximal; R = right; L = left.; y = year; m = month.

secondary to hypogammaglobulinemia, a feature not previously known to be associated with CIPA.

Six additional known mutations were found in six index patients. Homozygous splice site mutations were found in Patients CMT-197.01 (c.359+5G>T) and CMT-826.01 (IVS15+3A>C). Homozygous frameshift mutations were present in Patients PN-1192.03 (c.1877-1878insA; p.Gln626GlnfsX7) and CMT-179.01 (c.543delG; p.Gly181GlyfsX16). Finally, we identified a homozygous splice site mutation in Patient CMT-197.01 (c.359+5G>T) and a homozygous missense mutation (c.2281C>T; p.Arg761Trp) in Patient CMT-366.01 (Verpoorten *et al*., 2006*b*). Haplotype analysis suggested a common founder effect with previously described patients carrying this missense mutation (Indo *et al*., 2001).

Discussion

In this study, we investigated a cohort of 100 HSAN patients and determined the relative contribution of mutations in six genes known to be involved in various forms of HSAN (*SPTLC1, RAB7, WNK1/HSN2, NTRK1, NGFB* and *CCT5*). In addition, we studied the functional candidate gene *NGFR*. The known subforms of HSAN and their most important clinical characteristics have been summarized in Table 3. In four genes (*SPTLC1, RAB7, WNK1/HSN2, NTRK1*), we identified disease-causing mutations in 19 index patients representing a mutation frequency of 19%. Only nine of these patients had a clear familial history suggestive of HSAN with the remaining 10 being isolated patients. This results in a relative mutation frequency of 31% (9/29) for familial patients and 14% (10/71) for isolated patients. In the group of dominantly inherited HSAN (*RAB7* and *SPTLC1*), the mutation frequency is 33% (7/21) and for recessive HSAN (*WNK1/HSN2* and *NTRK1*), the frequency is 25% (2/8). Conversely, 20 familial patients in our screening cohort (14 dominant and 6 recessive) remain unsolved. These findings clearly indicate that additional genes must be involved in the pathogenesis of HSAN. *RAB7* and *NTRK1* were the most frequently mutated genes in our cohort (both 7%), followed by *WNK1/HSN2* with 3% and *SPTLC1* with 2%.

The three mutations previously described in *SPTLC1* (Cys133Trp, Cys133Tyr and Val144Asp) are associated with an ulcero-mutilating sensory neuropathy with a spectrum of clinical and electrophysiological features that is variable within and between families (Bejaoui *et al*., 2001; Dawkins *et al*., 2001; Auer-Grumbach, 2004; Houlden *et al*., 2006). A fourth *SPTLC1* mutation (Gly387Ala) was recently shown not to be disease causing (Verhoeven *et al*., 2004; Hornemann *et al*., 2009).

In the present study, we describe a novel *SPTLC1* mutation (Ser331Phe) in a patient who presented with a severe congenital phenotype. This mutation occurred *de novo*, was absent from 600 control chromosomes and affected a highly conserved amino acid. So far, all known missense mutations are located within a 12-amino acid segment encoded by exons 5 and 6 of *SPTLC1*. The Ser331Phe mutation is located downstream of this segment (Fig. 2). It is possible that the Ser331Phe mutation, due to its

Table 2 Clinical features of HSAN patients with proven mutation

Patient	Gene/AA change	Foot deformities	Walking difficulties	Weakness	Atrophy	Reflexes	Mental retardation	Nerve conduction studies + electromyography		Additional features	Reference
								Sensory	Motor		
CMT-186.05	*SPTLC1* Ala352Val	Mild pes cavus	Steppage	Distal LL	Peroneal	+, Ach absent	–	Axonal loss UL, absent responses LL	Absent responses LL	Severe hypesthesia and spontaneaous lancinating pain LL	Novel family
CMT-791.01	*SPTLC1* Ser331Phe	Pes cavus/ equinovarus	+	+ (global)	Global amyotrophy UL and LL	↓/–	+, microcephaly	Absent responses UL/LL	Absent responses UL/LL	Severe growth retardation, hypotonia, joint hyperlaxity, vocal cord paralysis, bilateral cataract, respiratory involvement with sleep apnea requiring non-invasive ventilation	Novel family
PN-626.01	*RAB7* Val162Met	–	Steppage L>R	Distal and proximal LL	Unk	↓ (L<R)	–	Axonal loss UL, absent responses LL	Normal UL, absent responses LL	Brown-Séquard syndrome and quadriplegia due to bleeding cervical spinal angioma, IgA nephropathy	(Verhoeven *et al.*, 2003)
CMT-90.01	*RAB7* Val162Met	Multiple toe amputations	Steppage	Distal in LL	Peroneal	↓ to –	–	Axonal loss UL, absent responses LL	UL normal, absent responses LL	–	(Verhoeven *et al.*, 2003)
CMT-126.01	*RAB7* Leu129Phe	Pes cavus	+ (severe)	Distal LL>UL	Peroneal	↓	–	Axonal loss UL, absent responses LL	Axonal loss UL, absent responses LL	–	(Verhoeven *et al.*, 2003)
CMT-140.01	*RAB7* Leu129Phe	Pes cavus	Mild steppage gait	Distal UL and LL, mild	Mild in UL and in LL	↓	–	Normal UL, axonal loss LL	Axonal-demyelinating NCVs in UL and LL	–	(Verhoeven *et al.*, 2003)
CMT-186.26	*RAB7* Leu129Phe	Charcot foot deformity	Mild steppage gait	Distal LL, mild	Distal LL, small foot muscles	↑	–	Normal UL, axonal loss LL	Normal UL, axonal loss LL	–	(Verhoeven *et al.*, 2003)
CMT-186.28	*RAB7* Val162Met	Toe deformities	Steppage, after amputation, wheelchair dependent	Distal LL>UL	Peroneal and hands	+ to ↑	–	Absent responses LL	Absent responses LL	–	(Verhoeven *et al.*, 2003)
CMT-195.01	*RAB7* Val162Met	Pes cavus, hammer toes	Steppage	Distal LL	Unk	↓	–	Axonal loss	Axonal loss	–	(Verhoeven *et al.*, 2003)
CMT-178.01	*HSN2* Ile355AsnfsX7	–	–	–	–	–	–	Absent responses UL/LL	Normal	–	(Coen *et al.*, 2006)
CMT-260.01	*HSN2* Gln184X	Deformed due to recurrent infections	Disturbed due to amputations	–	–	↓ to –	–	Absent responses UL/LL	UL normal, LL axonal loss	–	(Coen *et al.*, 2006)
CMT-451.04	*HSN2* Pro85HisfsX14 + Gln364SerfsX16	–	Disturbed due to amputations	–	–	–	–	Absent responses UL/LL	Normal	Sural nerve biopsy: complete loss of myelinated fibers, endoneuronal fibrosis	(Coen *et al.*, 2006)

(continued)

Table 2 Continued

Patient	Gene/AA change	Foot deformities	Walking difficulties	Weakness	Atrophy	Reflexes	Mental retardation	Nerve conduction studies + electromyography		Additional features	Reference
								Sensory	Motor		
CMT-179.01	*NTRK1* Gly181GlyfsX16	–	–	–	–	+	+	Unk	Unk	Henoch-Schönlein vasculitis, pseudotumor cerebri, tooth abscesses	(Verpoorten *et al.*, 2006*b*)
CMT-197.01	*NTRK1* c.359+5G>T	Unk	Unk	–	–	↓	+	Unk	Unk	Skin biopsy: innervation of blood vessels and eccrine sweat glands absent	(Verpoorten *et al.*, 2006*b*)
CMT-366.01	*NTRK1* Arg761Trp	Charcot joint on the right ankle	Normal	–	–	+	Unk	Unk	Unk	–	(Verpoorten *et al.*, 2006*b*)
CMT-826.01	*NTRK1* c.2046+3A>C	Charcot joint on both ankles and knees	Disturbed due to Charcot joint	–	–	↓	+ (mild)	Axonal loss LL	Axonal loss UL/LL	Corneal opacities	Novel family
CMT-841.01	*NTRK1* Arg565Gln	Charcot deformity right ankle and knee	Walks with rollator	–	–	↓ to –	– (younger affected sister has learning difficulties)	Unk	Unk	Sural nerve biopsy: loss of thinly myelinated fibers, partial caudacompression syndrome age 34 yrs. traumatic cataract right eye, hearing loss due to ear drum perforation	Novel family, clinical description previously reported (Kruyt *et al.*, 2007)
PN-1192.03	*NTRK1* Gln626GlnfsX7	–	–	–	–	+	+	Normal	Normal	Skin biopsy: absent innervation of capillaries and sweat glands, loss of myelinated and unmyelinated axons	(Verpoorten *et al.*, 2006*b*)
CMT-886.01	*NTRK1* c.354_359+3 delTCGCCTGAA	–	Delayed motor milestones	–	–	+	+	Normal	Normal	Recurrent infections respiratory and gastrointestinal tract due to hypogammaglobulinemia, recurrent hip dislocation left	(Kilic *et al.*, 2009)

AA = amino acid; LL = lower limb; UL = upper limb; + = present; – = absent; ↓ = decreased; ↑ = increased; unk = unknown; R = right; L = left; Ach = Achilles tendon.

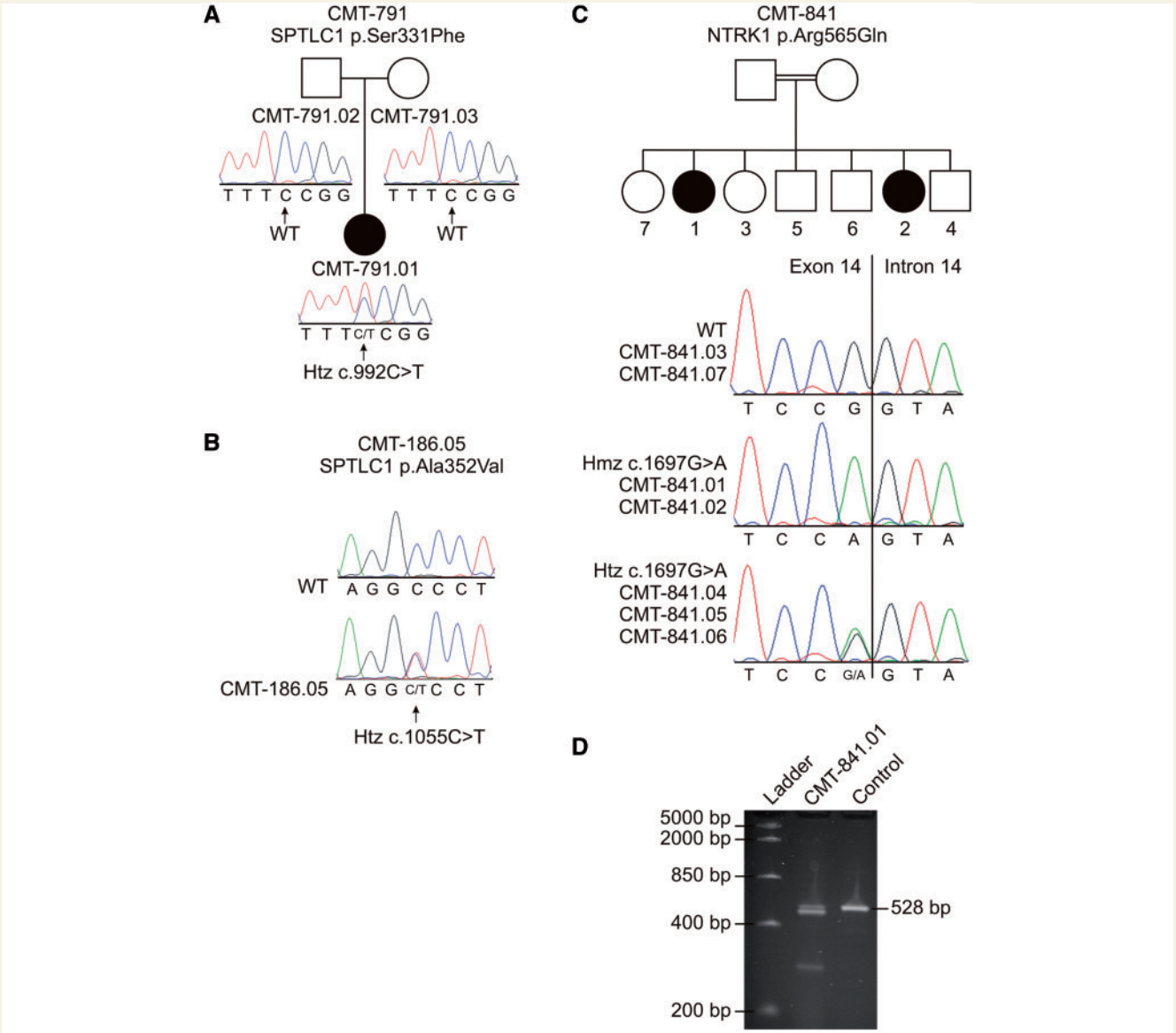

Figure 1 Segregation of p.Ser331Phe and p.Ala352Val missense mutations in SPTLC1 (**A** and **B**) and segregation and cDNA analysis of the p.Arg565Gln missense mutation in *NTRK1* (**C** and **D**). Segregation analysis of the p.Ser331Phe missense mutation in SPTLC1 reveals that this mutation occurred *de novo* (**A**). Panel B shows the sequence trace file of the p.Ala352Val missense mutation found in SPTLC1 in an isolated Patient CMT-186.05. Segregation of the p.Arg565Gln mutation in NTRK1 is shown in panel C. Two CIPA patients in family CMT-841 (CMT-841.01 and CMT-841.02) had a homozygous Arg565Gln mutation in NTRK1. The healthy siblings of these patients had either the wild-type allele (CMT-841.03 and CMT-841.07) or carried the Arg565Gln mutation in the heterozygous state (CMT-841.04, CMT-841.05 and CMT-841.06). The parents of the patients were first cousins. The mutated nucleotide (c.1697G > A) was the last nucleotide of exon 14, which could affect proper splicing of this exon. cDNA analysis of CMT-841.01 showed the absence of the expected band (528 bp), which was present in the control and confirmed by direct DNA sequencing (**D**). We could not determine the sequence of the three lower bands present in the patient. square = male, circle = female, black filled symbol = affected, empty symbol = unaffected.

different location in the protein, exerts a different effect on SPT activity leading to a more severe phenotype. These results suggest a broadening of the phenotype associated with HSAN type I. We identified a second sequence variant (Ala352Val) in *SPTLC1*, in an isolated patient with a sensory neuropathy. However, the pathogenicity of this sequence variant could not be verified because DNA of the family members was not available for segregation analysis. Furthermore, the amino acid targeted is not well-conserved in evolution and the change from alanine to valine is mild considering their similar chemical properties.

We found mutations in *SPTLC1* in only 2% of our patients confirming the rare occurrence of *SPTLC1* mutations in isolated and familial HSAN, as found in previous studies (Klein *et al*., 2005; Houlden *et al*., 2006).

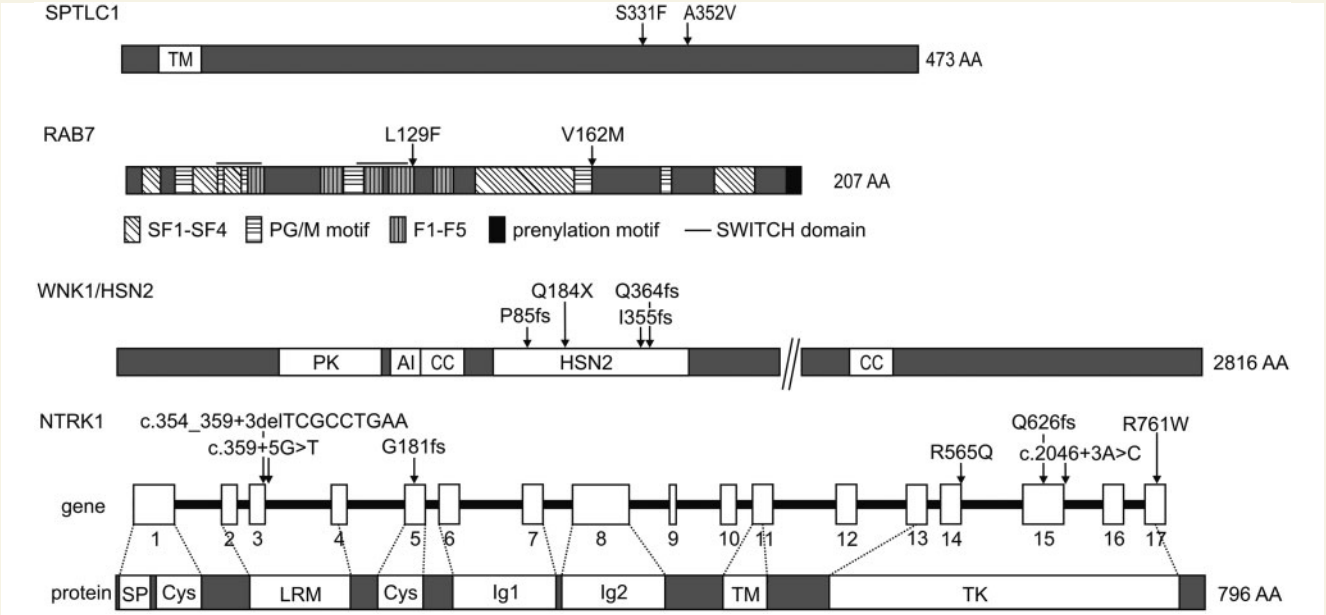

Figure 2 Schematic presentation of protein structures of SPTLC1, RAB7 (Pereira-Leal and Seabra, 2000), WNK1/HSN2 and NTRK1 (Indo, 2001) with mutations identified in this study causing HSAN.

Table 3 Overview of HSAN types with corresponding gene/locus, inheritance pattern, cardinal phenotypic features and references to the Online Mendelian Inheritance in Man (OMIM) database and literature

Type	Gene	Locus	Inh	Clinical features	AAO	OMIM	Reference
HSAN I	*SPTLC1*	9q22.2	AD	Predominant loss of pain and temperature sensation, preservation of vibration sense, lancinating pain, variable distal motor involvement[a]	Adult[a]	162400	Bejaoui *et al*., 2001; Dawkins *et al*., 2001
HSAN IB	unknown	3p24-p22	AD	Predominant sensory neuropathy with cough and gastroesophageal reflux, rarely foot ulcerations	Adult	608088	Kok *et al*., 2003
CMT2B	*RAB7*	3q21.3	AD	Prominent distal motor involvement, sensory loss of all qualities, acro-mutilating complications	Adult	600882	Verhoeven *et al*., 2003
HSAN II	*WNK1/HSN2*	12p13.3	AR	Prominent sensory loss and mutilations in hands and feet, acropathy	Childhood	201300	Lafreniere *et al*., 2004
HSAN III (Riley–Day syndrome)	*IKBKAP*	9q31	AR	Familial dysautonomia, prominent autonomic disturbances and complications, absence of fungiform papillae of the tongue, alacrimia, excessive sweating	Congenital	223900	Slaugenhaupt *et al*., 2001
HSAN IV (CIPA)	*NTRK1*	1q21-22	AR	No or reduced response to painful stimuli, anhidrosis, episodic fever, mild mental retardation, skin and cornea lesions, joint deformities, hypogammaglobulinemia in one patient (this study)	Congenital	256800	Indo *et al*., 1996
HSAN V	*NGFB* (*NTRK1* in rare cases)	1p13.1 (1q21-22)	AR	Congenital insensitivity to pain, severe loss of deep pain perception, painless fractures, joint deformities, normal intelligence	Congenital	608654	Einarsdottir *et al*., 2004 (Houlden *et al*., 2001)
HSAN with spastic paraplegia	*CCT5*	5p15-p14	AR	Prominent sensory neuropathy with sensory loss of all qualities, mutilating acropathy, spastic paraplegia.	Early childhood	256840	Bouhouche *et al*., 2006*a*

a Congenital onset in one patient with hypotonia, cataract, microcephaly and vocal cord paralysis (this study).
Inh = inheritance; AAO = age at onset; (updated from Auer-Grumbach *et al*., 2006 and Verhoeven *et al*., 2006).

To date, four missense mutations (Leu129Phe, Lys157Asn, Asn161Thr and Val162Met) have been reported in *RAB7* (Verhoeven *et al*., 2003; Houlden *et al*., 2004*b*; Meggouh *et al*., 2006). In our cohort, we found the Leu129Phe mutation in two families (CMT-126 and CMT-140) and one isolated Patient (CMT-186.26), and the Val162Met mutation in families CMT-90 and CMT-195 and in two additional patients with a positive family history (CMT-186.28 and PN626.01) (Verhoeven *et al*., 2003). The patients carrying the Leu129Phe mutation were all of Austrian descent and shared a common disease haplotype,

indicating a founder effect. However, the patients with the Val162Met mutation did not share a common haplotype, suggesting independently arising mutations in the same residue, possibly pointing towards a mutational hotspot for *RAB7*. This mutation is located in a highly conserved domain, important for the formation of the nucleotide-binding site of RAB7 (Fig. 2). The functional importance of this domain in peripheral neuron integrity is further underlined by the identification of mutations in adjacent amino acids (Lys157Asn and Asn161Thr) in patients with CMT2B (Houlden *et al*., 2004*b*; Meggouh *et al*., 2006).

In our cohort, *RAB7* mutations were exclusively found in patients diagnosed with HSMN II, with ulcero-mutilations also known as CMT2B. Apart from the rare exception of a patient with a typical HSAN type I phenotype carrying the Asn161Thr *RAB7* mutation (Houlden *et al*., 2004*b*), the phenotype associated with *RAB7* mutations seems to be largely confined to HMSN II (CMT2B). Because of the marked motor involvement, *RAB7*-neuropathy was originally classified as hereditary motor and sensory neuropathy type 2B (CMT2B) (Kwon *et al*., 1995). Due to the prominent presence of ulcerations, however, CMT2B should be considered part of the spectrum of HSAN (Vance *et al*., 1996).

The mutation frequency in the CMT2B-subgroup of our study cohort was very high (7 out of 13 CMT2B patients). The high mutation frequency of *RAB7* found in the present study contrasts with a previous report (Klein *et al*., 2005), where the known *RAB7* mutations were shown to be absent from a group of 25 families with adult-onset HSAN I or HMSN II with prominent sensory involvement and from an additional 92 idiopathic patients. The frequency of the *RAB7* mutation in our cohort remained high, even when the founder effect of the Leu129Phe mutation was taken into account.

The phenotype associated with mutations in *WNK1/HSN2* is a severe AR ulcero-mutilating sensory neuropathy with mild autonomic disturbances beginning in early childhood (Axelrod and Gold-von Simson, 2007). So far, 11 different non-sense and frameshift mutations in *WNK1/HSN2* have been reported in the literature, all resulting in a complete loss of protein. Recently, it was shown that *HSN2* is not a separate gene residing in intron 8 of *WNK1* but in fact is a neuron-specific exon of *WNK1* itself, with high expression in dorsal root ganglia (DRG) and sciatic nerves (Shekarabi *et al*., 2008). In our cohort, we identified four loss-of-function mutations in *WNK1/HSN2*, in three patients, all of which resided in the *HSN2* exon (Fig. 2) (Coen *et al*., 2006). From the first large-scale screening of *WNK1* in HSAN patients performed in this study, we can conclude that mutations outside of the *HSN2* exon are likely to be rare.

The most frequently mutated gene in this study, together with *RAB7*, was *NTRK1*. The NTRK1 protein is a receptor tyrosine kinase, which is phosphorylated in response to nerve growth factor (NGF), supporting survival of sympathetic ganglion neurons and nociceptive sensory neurons in DRG (Levi-Montalcini, 1987). To date, more than 40 different missense, non-sense, frameshift and splice site mutations in *NTRK1* have been described in families from various ethnic origins (http://www.molgen.ua.ac.be/CMTMutations/). The corresponding syndrome, CIPA, consists of characteristic features: recurrent episodic fevers due to anhidrosis, absence of reaction to painful stimuli, self-mutilating behaviour and mental retardation (Axelrod and Gold-von Simson, 2007).

In our cohort of 100 HSAN patients, seven different mutations were identified in *NTRK1* (Fig. 2), of which six were previously reported (Verpoorten *et al*., 2006*b*; Kilic *et al*., 2009). We identified a previously unreported splice site mutation (Arg565Gln), which comprised the last nucleotide of exon 14 and further broadens the genetic spectrum of *NTRK1* mutations. This mutation resides in the tyrosine kinase domain of the NTRK1 receptor, which regulates autophosphorylation of NTRK1 in response to NGF. Both the index patient (CMT-841.01) and her affected sister (CMT-841.02) were diagnosed with CIPA and carried the same homozygous mutation. However, Patient CMT-841.01 had a normal intelligence whereas the affected sib (CMT-841.02) had learning difficulties. The phenotype of these patients has been described previously (Kruyt *et al*., 2007).

Interestingly, a 9-bp deletion (c.354_359 + 3delTCGCCTGAA), resulting in skipping of exon 3 and of exons 2 and 3, was found in a CIPA patient of Turkish origin (CMT-860.01). This patient presented with a multisystem involvement including recurrent infections due to immunological abnormalities as hypogammaglobulinemia (Kilic *et al*., 2009). The same 9-bp deletion was recently reported in an unrelated Turkish HSAN type IV patient (Tuysuz *et al*., 2008). Therefore, this mutation is likely to be a founder mutation in the Turkish population. The phenotype in our patient broadens the clinical spectrum of CIPA.

It has previously been suggested (Indo *et al*., 2001) that the current literature reveals little to no genetic and clinical heterogeneity in HSAN IV. Although some degree of phenotypic variability was observed in our CIPA-patients with proven *NTRK1* mutations, overall the clinical presentation seems to correspond to a readily recognizable syndrome that is indeed genetically homogenous.

The phenotype caused by *NGFB* mutations (HSAN type V) is similar to the CIPA phenotype (HSAN type IV). So far, only one homozygous missense mutation has been reported, in a recessive Swedish family (Einarsdottir *et al*., 2004; Minde *et al*., 2004). Detailed clinical, neurophysiological and genetic analysis of this family revealed that the heterozygous carriers presented with a variable but mild phenotype (Minde *et al*., 2009). The main difference with CIPA was the absence of obvious mental retardation and less-pronounced anhidrosis. However, one family with the HSAN type V phenotype was described with pathogenic mutations in *NTRK1* indicating the overlap between HSAN types IV and V (Houlden *et al*., 2001, 2004*a*). These findings underscore the relevance of genetic screenings outside of the known phenotypes and modes of inheritance. In our study cohort, no heterozygous or homozygous sequence variations were found in *NGFB* confirming the rare occurrence of *NGFB* mutations in HSAN patients.

Recessive mutations in *CCT5* were identified in a consanguineous Moroccan family presenting with HSAN with spastic paraplegia (Bouhouche *et al*., 2006*a*). In our cohort, only two HSAN patients presented with an associated spastic paraplegia. However, we did not identify mutations in *CCT5*. Additional screening of 25 unrelated index patients with hereditary spastic

paraplegia with sensory involvement did not reveal any mutations in *CCT5* (data not shown). Our results suggest that mutations in *CCT5* are a rare cause for HSAN and make it unlikely to find any mutations outside of the known phenotype.

Because of the phenotypical resemblance among Ntrk1$^{-/-}$, Ngfb$^{-/-}$ and Ngfr$^{-/-}$ knockout mice, the patient cohort was screened for *NGFR* (*p75/NTR*) (Lee *et al*., 1992). No mutations were found in this gene making its contribution to the pathogenesis of HSAN uncertain.

In summary, we examined the distribution of mutations in genes associated with AD and AR forms of HSAN in a large group of familial and sporadic patients. The genotype–phenotype correlations in this study revealed little variability when compared with previous reports, with the sole exception of a *de novo SPTLC1* mutation in a severe phenotype with congenital onset. Taken together, these results show that the relevant clinical phenotypes are recognizable and should be used to orient molecular diagnosis. Screening of *NTRK1* should be confined to patients presenting with a CIPA phenotype (both AR and isolated patients) as no *NTRK1* mutations were found in other HSAN phenotypes. *RAB7* screening is mandatory in patients presenting with an AD axonal sensory-motor neuropathy with ulcerations (CMT2B) as we found a high mutation frequency in this subgroup (54% or 7/13). Mutations in *WNK1/HSN2* and *SPTLC1* seem to be rare in HSAN. We found no mutations in *NGFB* and *CCT5* indicating that these genes are only rarely involved in HSAN. No pathogenic sequence variations were identified in the functional candidate gene *NGFR*, making its contribution to the pathogenesis of HSAN uncertain. The overall mutation rate was relatively low (19%) suggesting that other genes must be involved in the pathogenesis of HSAN.

At the present time, the precise nature of the mechanism underlying the pathogenesis of the various HSAN forms remains unclear. Although it is particularly challenging to link the different genes, some preliminary disease pathways may already take form. Of special interest are disturbances of vesicular transport. Both RAB7 and SPTLC1 have a function in endocytotic membrane trafficking. In addition, the NGFB/NTRK1 signalling complex, which is critically important in the development and function of nociceptive neurons, is also dependent upon retrograde transport through signalling endosomes (Verhoeven *et al*., 2006). Future research is needed to improve our still very incomplete understanding of these mechanisms.

Additional descriptions of HSAN families and patients with known or novel genetic defects are needed to further refine the existing classification and to get a better insight into the molecular basis of these disorders.

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