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Original Article

High frequency of *SH3TC2* mutations in Czech HMSN I patients

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Charcot–Marie–Tooth (CMT) neuropathy type 4C (CMT4C) is an autosomal recessive (AR), demyelinating neuropathy with early spine deformities caused by mutations in the *SH3TC2* gene. To determine the spectrum of *SH3TC2* mutations in the Czech population, the entire coding region of *SH3TC2* was sequenced in 60 unrelated Czech patients. The prevalent mutation was shown to be the p.Arg954Stop. Therefore, 412 additional patients referred for CMT testing were tested for the presence of p.Arg954Stop only. Of 60 patients in whom the *SH3TC2* gene was sequenced, at least one mutation was detected in 13 (21.7%) patients and biallelic pathogenic mutations were detected in 7 (11.6%) patients. Of the 412 patients tested for p.Arg954Stop, the mutation was found in 8 patients (1.94%), 6 were homozygous and 2 were heterozygous. The second causative mutation was detected by sequencing in one of the patients but not in the other. Nine novel sequence variants were detected. Their pathogenicity was further tested in silico and in control samples. Mutations in the *SH3TC2* gene are a frequent cause of demyelinating hereditary neuropathy among Czech patients. In total, at least one mutation was found in 21 unrelated patients. CMT4C seems to be the most frequent type of AR CMT and one of the most frequent of all CMT types. Mutation p.Arg954Stop is highly prevalent in the Czech population. Patients with demyelinating neuropathy along with non-dominant mode of inheritance and negative for CMT1A/hereditary neuropathy with liability to pressure palsy should be tested for the presence of the p.Arg954Stop mutation or other mutations in the *SH3TC2* gene.

Conflict of interest

The authors have no conflicts of interest.

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Charcot–Marie–Tooth (CMT) disease is a heterogeneous group of disorders, also called hereditary motor and sensory neuropathies (HMSNs), with the main clinical features being progressive distal muscle weakness and atrophy, foot deformities and distal sensory loss (1, 2). According to clinical, electrophysiological and pathophysiological findings, these are divided into primary demyelinating (HMSN I) and primary axonal forms (HMSN II) (3). All patterns of mendelian inheritance were observed, with autosomal dominant being the most common. A large number of causative genes have been identified (www. molgen.ua.ac.be, http://neuromuscular.wustl.edu).

Recessively inherited neuropathies are less common and are referred to as CMT4 (demyelinating) or autosomal recessive (AR) CMT2 (axonal).

Mutations in the *SH3TC2* gene cause CMT type 4C (CMT4C) neuropathy. CMT4C neuropathy is characterized by an early onset, demyelinating neuropathy, early progressive scoliosis and moderate severity (non-severe handicap).

The *SH3TC2* gene is localized on chromosome 5 (4), encoding a protein with Src homology 3 domain (SH3) and tetratricopeptide repeat (TRP) domains. Several studies were taken to explain the function of the SH3TC2 protein. SH3TC2_Ex1/_Ex1 knockout mouse was generated at the University of Lausanne (5). Lupo et al. (1) showed that the SH3TC2 protein is involved in endocytic pathway of cell traffic and is anchored to the plasma membrane. Recently, the recycling endosome has been described as the precise compartment that the wild-type SH3TC2 targets and its binding with Rab11 has been shown (6).

In this study, all 17 coding exons of the *SH3TC2* were sequenced in 60 unrelated Czech HMSN I patients. Mutation p.Arg954Stop in the *SH3TC2* gene was found to be the most prevalent in the Czech population. Therefore, an additional 412 unselected, unresolved, and unrelated patients regardless their phenotype, previously sent for CMT DNA testing were tested with TaqMan SNP (single nucleotide polymorphism) genotyping assay for the presence of p.Arg954Stop mutation in the *SH3TC2* gene.

In total, 13 different *SH3TC2* sequence alterations were detected in this study, 9 of them were novel, not yet having been described.

Patients and methods

Patients

First, 60 unrelated Czech HMSN I patients were screened for mutations in the *SH3TC2* gene by direct sequencing of all 17 coding exons and exon/intron boundaries. Inclusion criteria for patient selection were as follows:

(1) All selected families have pedigrees compatible with AR inheritance; the patients were either affected siblings born to healthy parents based on family history (two families and four patients) or more often sporadic cases (58 patients). Consanguinity was not reported for any of the families.
(2) Only patients with demyelinating motor and sensory neuropathy/median motor nerve conduction velocity (MNCV) <38 m/s were included.
(3) Regarding the onset of the disease, only patients with first symptoms of the disease early in life (first- or second-life decade) were selected.

We focused particularly on reported scoliosis and patients with similarly affected siblings.

Second, screening for the prevalent mutation p.Arg954Stop with Custom TaqMan® SNP Genotyping Assay designed by Applied Biosystems (Foster City, CA) was carried out on 412 unrelated patients from our database. These patients were referred for CMT diagnostics and all previously tested negative for hereditary neuropathy with liability to pressure palsy (HNPP) deletions/CMT1A duplications in the 17p11.2 region. We had no clinical criteria for this group regarding the type of neuropathy, age at onset, and so forth, but patients with recognizable autosomal dominant inheritance were excluded.

All tested patients signed informed consent for the CMT DNA diagnostics and the study was approved by the Central Ethical Committee of the University Hospital Motol Prague.

Methods

DNA sequencing by capillary electrophoresis

DNA was extracted from peripheral blood or from saliva. All 17 exons and exon/intron boundaries of the *SH3TC2* gene were polymerase chain reaction (PCR) amplified in 19 fragments using primers published elsewhere (4). PCR products were purified (Agencourt Ampure, Beckman Coulter, Brea, CA) and then sequenced using dye-terminator reaction (BigDye Terminator v. 3.1, Applied Biosystems). After dye-terminator removal with Agencourt CleanSeq System (Beckman Coulter), the products were analyzed on an ABI 3130 automated genetic analyzer (Applied Biosystems). Sequence traces were compared with a *SH3TC2* reference sequence (NM_024577.3, http://www.ncbi.nlm.nih.gov/sites/entrez) using Sequencing analysis software (Applied Biosystems) and Mutation Surveyor Software (SoftGenetics, LLC, State College, PA).

Genotyping for p.Thr27Ala and p.Gln46Pro mutations using SnapShot method

Exon 2 of the *SH3TC2* gene was PCR amplified. PCR products were then purified with Agencourt Ampure System (Beckman Coulter).

Subsequently, a SnapShot reaction was performed with SnapShot kit (Applied Biosystems) and primers (3′-5′): TGAGGCTATACACTCACTCGATACAG for p.Thr27Ala mutation and TCTTTGGCATTTGGATACCTGGATTAATTTC for p.Gln46Pro mutation. Products of the SnapShot reaction were purified with Agencourt CleanSeq System (Beckman Coulter) and fragment analysis by capillary electrophoresis was performed on Genetic Analyzer ABI 310 (Applied Biosystems).

Genotyping for mutations p.Arg954Stop, p.Lys93Lys, p.Ser433Leu and p.Val1158Ile using restriction enzymes

Exons 3, 15, and PCR fragments 10–11 and 11–12 were PCR amplified. Restriction digest was performed under conditions recommended by the manufacturer (New England Biolabs, Ipswich, MA) and digested products were separated on a 1.5% agarose gel. For mutation p.Arg954Stop, the Taq^{a}I; for p.Lys93Lys, the DraI; for p.Ser433Leu, the MscI; and for p.Val1158Ile, the HpyCH4III were used (all: New England Biolabs, Ipswich, MA). All samples with a mark of mutation were confirmed by sequencing from a new PCR product.

Allelic discrimination assays for mutations p.Arg954Stop, p.Arg227Gln, p.Asp1229Val and p.Thr199Ile

For SNP analysis, we used Custom TaqMan® SNP Genotyping Assays designed by Applied Biosystems. List of primers and TaqManProbe sequences is provided in Table S1 (Supporting information). Amplification and end-point analysis were performed on the ABI 7000 Sequence Detection System (Applied Biosystems).

In silico analysis

Detected novel *SH3TC2* sequence alterations were analyzed in silico using these five databases and software tools.

ClustalX, a multiple alignment tool (http://www.clustal.org/download/current/, accessed on September 5, 2010) (7); sift, an application that predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html, accessed on September 10, 2010) (8); PolyPhen, a tool that predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straight forward physical and comparative considerations (http://genetics.bwh.harvard.edu/pph/, accessed on March 7, 2010) (9); Panther, which estimates the likelihood of a particular non-synonymous (amino acid changing) coding SNP to cause a functional impact on the protein. The probability that a given variant will cause a deleterious effect on protein function is estimated by $p_{\text{deleterious}}$, such that a subPSEC score of −3 corresponds to a $p_{\text{deleterious}}$ of 0.5 (10) (http://www.pantherdb.org/tools/csnpScoreForm.jsp, accessed on August 16, 2010) (11–14) and Alternative Splicing database (ASD). This project aims to understand the mechanism of alternative splicing on a genome-wide scale by creating a database of alternative splice events and the resultant isoform splice patterns of genes from human and other model species (http://www.ebi.ac.uk/asd/, accessed on August 19, 2010) (15–17).

Results

The entire coding sequence of the *SH3TC2* gene was sequenced in 60 unrelated HMSN I patients. Sequence variants (other than those SNPs already described as non-pathogenic in Pubmed, http://www.ncbi.nlm.nih.gov/projects/SNP) were detected in 13 of them (21.7%).

From 412 additional patients tested only for the presence of the p.Arg954Stop mutation, this mutation was detected in 8 (1.94%) of them.

Detected variants are listed in Table 1. Four of these variants were described earlier as causal for CMT4C: p.Arg954Stop, p.Arg658Cys, p.Tyr169His and p.Asn881Ser (4, 18–19). Nine novel variants were detected: p.Thr27Ala, p.Gln46Pro, p.Lys93Lys, p.Thr199Ile, p.Arg227Gln, p.Glu429Stop, p.Ser433Leu, p.Asp1229Val and c.3676-8G>A. One missense mutation that was detected in the course of the study after analysis turned out to be a benign polymorphism, the mutation p.Val1158Ile, recently has been reported in dbSNP (www.pubmed.com, rs55853803).

Several types of mutations were detected: two nonsense mutations (p.Arg954Stop and p.Glu429Stop), two mutations that probably affect

Table 1. Mutations detected in Czech CMT patients in this study and their relative frequency

Mutation	Nucleotide position	Number of pathogenic alleles	Relative frequency of mutations in the *SH3TC2* gene (% of alleles)
p.Arg954Stop	c.2860C>T	24	63.2
p.Lys93Lys	c.279G>A	2	5.3
p.Asp1229Val	c.3686A>T	2	5.3
p.Asn881Ser	c.2642 A>G	1	2.6
p.Arg658Cys	c.1972C> T	1	2.6
p.Tyr169His	c.505T>C	1	2.6
p.Glu429Stop	c.1285G>T	1	2.6
p.Thr27Ala	c.231A>G	1	2.6
p.Gln46Pro	c.137A>T	1	2.6
p.Thr199Ile	c.596C>T	1	2.6
p.Arg227Gln	c.680G>A	1	2.6
p.Ser433Leu	c.1450C>T	1	2.6
	c.3676-8G>A	1	2.6

Table 2. Genotypes of patients with mutations in the *SH3TC2* gene

Family/patient	Mutation
A/1	(p.Arg954Stop)+(p.Arg954Stop)
B/1	(p.Arg954Stop)+(p.Arg954Stop)
C/1	(p.Arg954Stop)+(p.Arg954Stop)
D/1	(p.Arg954Stop)+(p.Arg954Stop)
D/2	(p.Arg954Stop)+(p.Arg954Stop)
E/1	(p.Arg954Stop)+(p.Arg954Stop)
E/2	(p.Arg954Stop)+(p.Arg954Stop)
F/1	(p.Arg954Stop)+(p.Arg954Stop)
G/1	(p.Arg954Stop)+(p.Arg954Stop)
H/1	(p.Arg954Stop)+(p.Arg954Stop)
I/1	(p.Arg954Stop)+(p.Arg658Cys)
J/1	(p.Arg954Stop)+(p.Asn881Ser)
K/1	(p.Arg954Stop)+(p.Lys93Lys)
L/1	(p.Arg954Stop)+(p.Glu429Stop)
M/1	(p.Arg954Stop)+(c.3676-8G>A)
N/1	(p.Tyr169His)+(p.Lys93Lys)
O/1	(p.Thr27Ala)+(p.Ser433Leu)
P/1	(p.Arg954Stop)
R/1	(p.Gln46Pro)
R/2	(p.Gln46Pro)
S/1	(p.Asp1229Val)
T/1	(p.Asp1229Val)
U/1	(p.Thr199Ile)
V/1	(p.Arg227Gln)

splicing (p.Lys93Lys and c.3676-8G>A), and nine missense mutations.

At least one mutation was found in 21 unrelated patients in this study (Table 2). Eight of these patients are homozygous for the p.Arg954Stop mutation (families A–H), and two unrelated patients carry causal pathogenic compound heterozygous alleles (families I and J) for CMT4C. Causal mutation in combination with a novel mutation in a compound heterozygous form was detected in four patients (families K–N). The remaining six families (O, R–V) carry novel sequence alterations. Their effect on the SH3TC2 protein is difficult to estimate with any certainty. One patient (family P) carries mutation p.Arg954Stop in heterozygous state with mutation on the second-allele missing. Segregation of mutations in the families is shown in Fig. 1.

Novel missense mutation analyses

Novel missense mutations were extensively evaluated in silico and in healthy controls. A summary of analyses of novel sequence variants is shown in Table 3.

(1) Results from CLUSTALX analysis: Multiple alignments using CLUSTALX algorithm showed high conservation in several species for these sequence variants, p.Gln46Pro, p.Lys93Lys, p.Thr199Ile, p.Arg227Gln, p.Glu429Stop, p.Ser433Leu and p.Asp1229Val, suggesting that they have an important function (Table S2, Supporting information).
(2) Results from SIFT analysis: SIFT analysis results have shown that variants p.Arg227Gln and p.Asp1229Val might be pathogenic (Table S3, Supporting information). Others were evaluated as benign.
(3) Results from POLYPHEN analysis: POLYPHEN analysis results have suggested causal effect only for variants p.Gln46Pro and p.Asp1229Val (Table S4, Supporting information).
(4) Results from PANTHER analysis: PANTHER analysis has displayed probable deleterious effect for the variant p.Asp1229Val (Table S5, Supporting information).
(5) Results from Alternative Splicing Workbench analysis: Variant p.Lys93Lys in the *SH3TC2* gene was tested with Alternative Splicing Workbench. Analysis showed that in a wild-type allele, four splicing proteins bind to the site of the mutation. In the mutant-type allele, three of these binding sites diminish and only one persists.
(6) Variant c.3676-8G>A in the *SH3TC2* gene was tested with Alternative Splicing Workbench. Analysis showed that in a wild-type allele, six splicing proteins bind to the site of the mutation. In the mutant-type allele, the binding site for SR55 splicing protein diminished.

Healthy controls testing

(1) Genotyping for variants p.Thr27Ala and p.Gln 46Pro using SnapShot method: Two hundred and forty-six unrelated healthy Czech controls were tested for the presence of both these variants (p.Thr27Ala and p.Gln46Pro). Two individuals were heterozygous for the p.Gln46Pro (confirmed by sequencing; population frequency is 0.406% of alleles). Nobody from the 246 controls carried the p.Thr27Ala.
(2) Genotyping for mutations p.Arg954Stop, p.Lys93Lys, p.Ser433Leu and p.Val1158Ile using restriction enzymes: Mutation p.Arg954Stop was not found in 206 DNA samples from anonymous healthy individuals indicating that the heterozygote rate in the Czech normal population is lower than 0.5% (<1:206). The same is true for p.Lys93Lys.

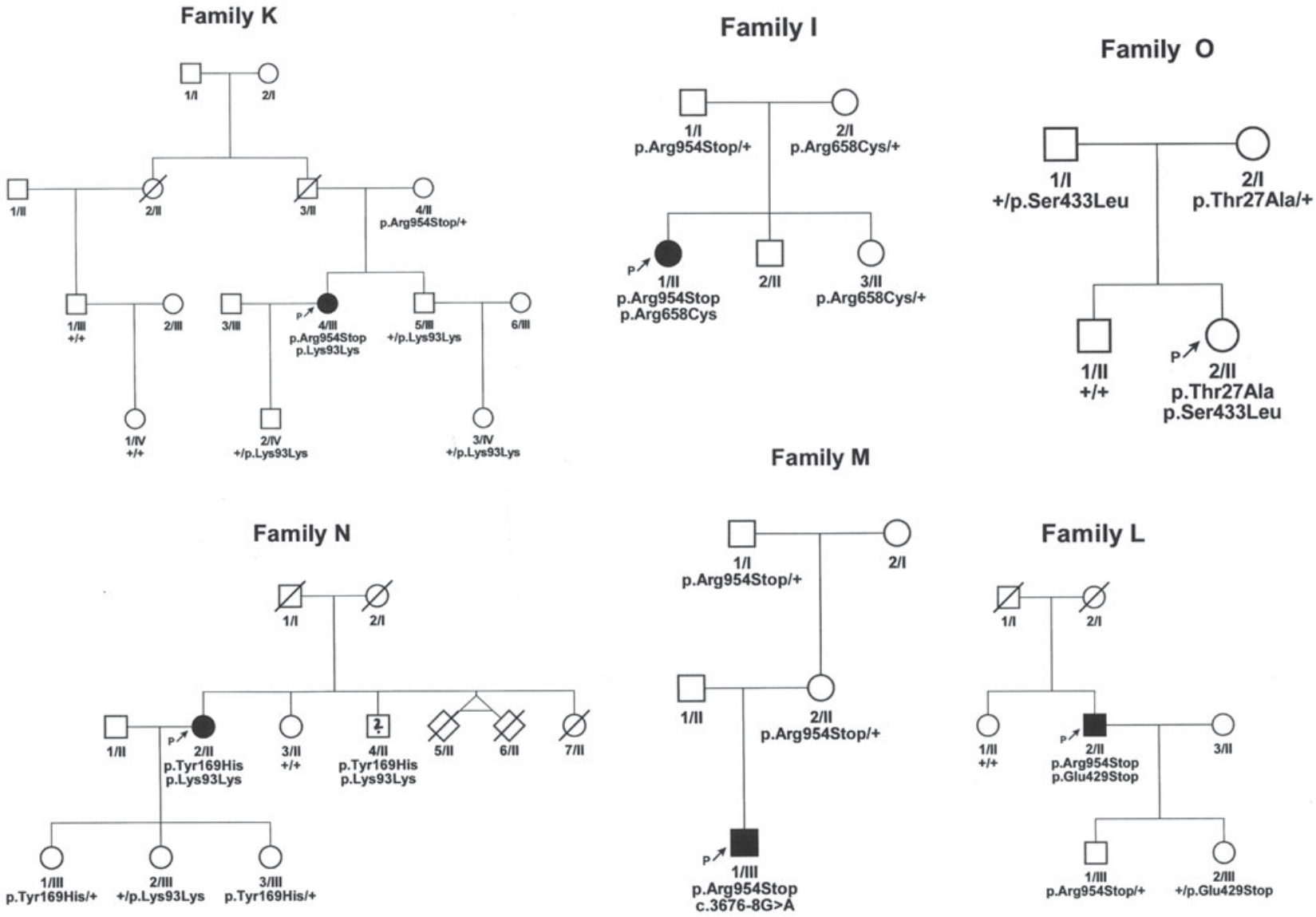

Fig. 1. Pedigrees (only families with biallelic mutations in the *SH3TC2* gene other than homozygous for p.Arg954Stop are shown). Square, male; circle, female; diamond, unknown gender; full sign, affected individual; crossed sign, deceased individual; an arrow with P, proband; ?, clinically not evaluated; and +, wild-type allele.

Table 3. Summary of analyses of novel sequence variants

Mutation analysis tool	SIFT	PolyPhen	Panther	ClustalX	Population frequency	Conclusion
p.Thr27Ala	Benign	Benign	Benign	Not highly conserved	0	Benign
p.Gln46Pro	Benign	Damaging	Benign	Conserved	0.406%	Benign
p.Thr199Ile	Benign	Benign	Benign	Conserved	0	Benign
p.Arg227Gln	Damaging	Benign	Benign	Conserved	0	?
p.Ser433Leu	Benign	Damaging	Benign	Conserved	0.167%	Benign
p.Asp1229Val	Damaging	Damaging	Damaging	Conserved	0	Probably damaging

?, unresolved.

Regarding p.Ser433Leu and p.Val1158Ile, they were detected in healthy controls with a frequency of 0.167% for p.Ser433Leu and 2.5% for p.Val1158Ile. The results are summarized in Table S6 (Supporting information).

(3) Allelic discrimination assays for mutations p.Arg227Gln, p.Asp1229Val and p.Thr199Ile: None of these variants was detected in healthy controls (for p.Asp1229Val, 260 controls; for p.Thr199Ile, 302 controls and for p.Arg227Gln, 304 controls were examined).

Clinical data

The clinical data of the Czech CMT4C patients are summarized in Table 4.

The course of the CMT4C disease is rather mild to moderate and similar in all our patients (with both clearly pathogenic mutations). All had the first symptoms of the disease in the first decade of life. These were severe foot deformities and scoliosis. Eight underwent orthopedic corrective surgery on the feet.

The ability to walk with or without walking aids was preserved in all patients. In neurological examination, the distal weakness of legs is much more pronounced than in the upper limbs, whereas arms and hands are unaffected or affected only slightly. Pronounced sensory deficit with severely diminished vibratory sensation is also very frequent.

Foot deformities and scoliosis were the main clinical symptoms. Electrophysiologically, all CMT4C patients presented with demyelinating motor and sensory peripheral neuropathy with MNCV <38 m/s.

In addition, three patients presented with late onset and progressive hearing loss, two of them (both older than 60 years) uses a hearing aid and one (aged 31) does not. Hypoacusia, but not age related, was also reported in other studies (18).

Discussion

We have shown that mutations in the *SH3TC2* gene are a common cause of HMSN I in Czech patients. Moreover, the presented data suggest that CMT4C is more frequent than was previously thought. For example, an evidence-based review published in 2008 (20) lacks any sort of information about CMT4C in the decision algorithm. We provide evidence that CMT4C is one of the most common types of inherited neuropathy, comparable with CMT due to mutations in the *MPZ* gene and it may be the most commonly known AR form at all. We compared the number of unrelated families with *SH3TC2* mutations with families detected to date in our DNA laboratory with *MPZ* mutations. We have 22 unrelated families with *MPZ* gene mutations and 21 unrelated families with at least one *SH3TC2* mutation. In 14 unrelated families, both causal and pathogenic or probable pathogenic mutations in *SH3TC2* gene were found.

The prevalent mutation in the *SH3TC2* gene in the Czech population is p.Arg954Stop (63% of pathogenic alleles), which is similar to other populations (21, 22). This simplifies DNA testing for CMT because testing will now be targeted at this mutation first.

Targeted testing of only p.Arg954Stop mutation and sequencing the entire coding region of *SH3TC2* only in patients in whom the p.Arg954Stop was detected on one allele are able to identify 92.86% of all CMT4C patients. Less than 8% of CMT4C patients would be missed using this testing protocol. However, the savings are much higher, amplifying only one PCR fragment instead of 19 fragments, greatly reducing the amount of material and labor involved.

Patients with demyelinating neuropathy, non-dominant pedigree and negative for CMT1A/HNPP should be preferably tested for the presence of the p.Arg954Stop mutation or even other mutations in the *SH3TC2* gene.

Table 4. Clinical data of CMT4C patients

Family/ patient	Age at onset (years)	Age at examination	Sensory symptoms	Motor symptoms	Strength upper limbs	Strength lower limbs	Foot deformity/ orthopedic surgery	Scoliosis	NCS (nerve conduction velocity)	Other signs
A/1	NA	—	—	—	—	—	—	—	—	—
B/1	2	24	Diminished distal sensation (vibratory + touch)	Foot drop/difficulty with buttons	Distal weakness	Distal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	—
C/1	<6	30	None	Foot drop/difficulty with buttons	Distal weakness	Distal weakness	Foot deformity/hammer toes	Yes	Demyelinating neuropathy	—
D/1	<10	11	Slightly reduced distal sensation (touch)	Foot drop	Normal	Distal weakness	Pes cavus	No	Demyelinating neuropathy	—
D/2	<6	11	Slightly reduced distal sensation (touch)	Foot drop	Normal	Distal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	—
E/1	10	31	Severely diminished distal sensation (vibratory, cold, and pinprick)	Foot drop/difficulty with buttons	Distal weakness	Distal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	Hearing loss/without aid
E/2	NA	—	—	—	—	—	—	—	—	—
F/1	<20	67	Severely diminished distal sensation	Foot drop/difficulty with buttons	Distal weakness	Distal weakness	Pes cavus/no	No	Demyelinating neuropathy	Hearing loss/with aid
G/1	<6	26	Severely diminished distal sensation	Foot drop	Slight distal weakness	Distal +proximal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	—
H/1	<10	45	Diminished distal sensation	Difficulty with buttons	Slight distal weakness	Distal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	—
I/1	4	35	Severely diminished distal sensation	Foot drop/difficulty with buttons	Normal	Distal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	—
J/1	<6	47	Diminished distal sensation (vibratory)	Foot drop	Normal	Distal weakness	Pes cavus	Yes	Demyelinating neuropathy	—
K/1	11	43	Severely diminished distal sensation	Foot drop/difficulty with buttons	Distal weakness	Distal weakness	Pes cavus/yes	—	Demyelinating neuropathy	—
L/1	3	61	Severely diminished distal sensation	Arthrodesis bilateral	Normal	Distal weakness	Pes cavus/yes	Yes	Demyelinating neuropathy	Hearing loss/with aid
M/1	4	16	Diminished distal sensation (vibratory)	Foot drop	Normal	Distal weakness	Pes cavus	Yes	Demyelinating neuropathy	—
N/1	12	53	Diminished distal sensation (vibratory + touch)	Foot drop/difficulty with buttons	Normal	Distal weakness	Pes cavus/yes	—	Demyelinating neuropathy	—
O/1	6	16	None	None	Normal	Normal	No/no	—	Demyelinating neuropathy	—

Families A–J – patients homozygous for p.Arg954Stop mutation and patients with p.Arg954Stop mutation over a known missense mutation

A strong genotype–phenotype correlation was observed, especially for patients homozygous for p.Arg954Stop mutation (families A–H, see Tables 2 and 4) and for patients heterozygous for p.Arg954Stop in combination with p.Arg658Cys or p.Asn881Ser (families I and J, see Tables 2 and 4). Clinically and electrophysiologically, these patients presented with mild to moderate neuropathy and early onset of the disease (first and second decades). Foot deformities and scoliosis were the main clinical symptoms as well as a demyelinating motor and sensory neuropathy with MNCV < 38 m/s.

Other CMT4C patients (families K–N, see Table 2) with two mutations in the *SH3TC2* gene but not homozygous for p.Arg954Stop showed very similar phenotype to those homozygous for p.Arg954Stop mutation with only little difference.

Family L – patient with biallelic inactivating mutations

Sequence variant p.Glu429Stop was detected in family L, in a patient who is compound heterozygous for p.Arg954Stop and p.Glu429Stop. We concluded that these alterations are causal for CMT4C in this patient. The p.Glu429Stop is a novel variant previously unreported. It is a nonsense mutation; therefore, there is little doubt about its pathogenicity. Additionally, we have shown that each of the p.Arg954Stop and p.Glu429Stop mutations is on separate alleles (Fig. 1). The phenotype of the proband is shown in Fig. 2. Scoliosis and foot deformities in preschool age were the first symptoms of the disease. Foot deformities led to nine orthopedic surgeries that culminated in bilateral sub talo arthrodesis. Hearing impairment is present from the age of 45. The patient recently began using a hearing aid.

Family M + K – patient with one sequence variant of uncertain pathogenicity/causality over a known inactivating mutation

Variant c.3676-8G>A was detected in a patient (family M) who is compound heterozygous for p.Arg954Stop and c.3676-8G>A (Fig. 1). Alternative splicing mechanism of the c.3676-8G>A mutation is supported by the fact that this mutation is located in a functionally important domain. The results of analysis with the ASD project also support this proposition. Further experiments may prove or disprove the mechanism of splicing. Phenotype of the patient is very similar to the phenotype of other CMT4C patients with known pathogenic mutations in the *SH3TC2* gene (families A–L).

Variant p.Lys93Lys: This novel sequence alteration changes the last nucleotide of exon 3 of the *SH3TC2* gene (c.279G>A). However, its effect is not an amino acid substitution; in our opinion, the mutation causes an alternative splicing of *SH3TC2*. The variant p.Lys93Lys was detected in family K, in a patient who is compound heterozygous for p.Arg954Stop and p.Lys93Lys, with no other mutations in the *SH3TC2* gene. The patient shows a phenotype that resembles other CMT4C patients with known pathogenic mutations. This p.Lys93Lys variant segregates in the family (Fig. 1) and was evaluated as pathogenic by Alternative Splicing Project, as shown earlier.

Two hundred and sixty-two healthy control DNA samples were tested for the presence of the p.Lys93Lys sequence alteration with restriction enzyme analysis using *Dra*I enzyme. The variant was not detected in any of these 524 chromosomes.

On the basis of the data, we believe that this alteration (p.Lys93Lys) may be pathogenic by the mechanisms of alternative splicing. Nevertheless, further experiments such as ExonTrap analysis are needed to prove or disprove this hypothesis.

Family N – patient with one sequence variant of uncertain pathogenicity/causality over a known missense mutation

The second family, where p.Lys93Lys was detected, is family N (for pedigree see Fig. 1). A patient in the family carries the compound heterozygous alleles for p.Lys93Lys and p.Tyr169His. As to her phenotype, the course of the disease is very similar to patients with biallelic causal mutations. The disease started early (at the age of 12); foot deformities were the main clinical symptom. The patient's brother was subsequently detected to be carrying both these alterations. He was never examined for the presence of signs of HMSN I and unfortunately refused any medical examination. The second mutation was already described by Lupski et al. (19) as pathogenic in one family.

Family O – patients with biallelic missense sequence alterations of uncertain pathogenicity/causality

The proband is compound heterozygous for two variants p.Thr27Ala and p.Ser433Leu. On closer examination provided at our department, no weakness and no signs of neuropathy were observed

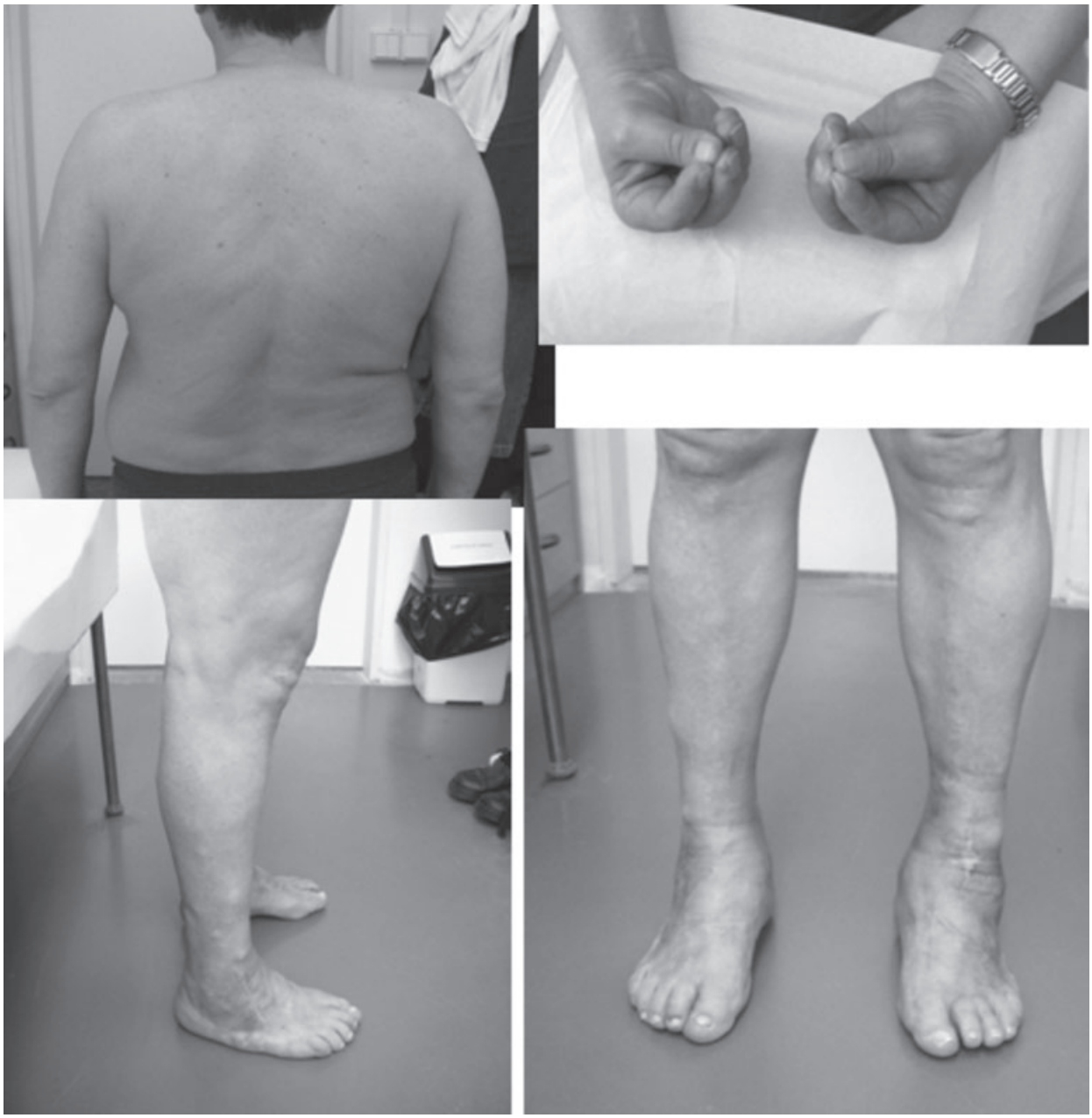

Fig. 2. The phenotype of the proband who is compound heterozygous for pArg954Stop and p.Glu429Stop (scoliosis, bilateral sub talo arthrodesis, and hand muscles almost unaffected).

at all, even on electrophysiological testing, thus showing the patient to be healthy. Moreover, variant p.Ser433Leu was found in a healthy control population with a frequency of 0.167% alleles, whereas the other alteration p.Thr27Ala was not detected in healthy controls. Variant p.Thr27Ala was evaluated as benign by both tools – PolyPhen and sift. ClustalX showed that this amino acid site is not highly conserved among species. Variant p.Ser433Leu was evaluated to be benign by sift but not PolyPhen (regarded as possibly damaging) and showed a high level of conservation among species. On the whole, we cannot exclude that one of these mutations is pathogenic, but we present here strong evidence for the non-pathogenic character of their combination at least in this particular patient.

Families R–V – patients with only one monoallelic sequence variant of uncertain pathogenicity/causality

It is difficult to characterize the remaining novel sequence alteration that is missense: p.Thr199Ile, p.Arg227Gln, and p.Asp1229Val. These were

detected in families R–V, respectively, always on one allele only, without any second mutation in the opposite allele. All these missense sequence variants may be rare benign alterations. In silico evidence for the pathogenic character is for variant p.Asp1229Val because this mutation was evaluated as pathogenic by all algorithms and was located in a functionally important domain. Variants p.Thr199Ile and p.Arg227Gln may be rare polymorphisms or not causal for CMT4C in these patients also because a second pathogenic mutation on the opposite alelle could not be detected in these patients.

As for patients with monoallelic mutations in the *SH3TC2* gene, the following was found (families from R to V, see Table 2).

Having considered the analysis of novel mutations, we concluded that variant p.Gln46Pro is a benign polymorphism and is non-causal for CMT4C in these patients. This mutation was detected in a healthy control population. Variant p.Gln46Pro was detected in family R on one allele, but no other mutation in the *SH3TC2* gene on the second allele was found. Even the phenotype of the patients in the family R is different from the other patients with true CMT4C. Patient R/1 (examined at the age of 16) presented with foot deformities from the age of 5, with only very mild muscle atrophy. Scoliosis was not reported. His sister (patient R/2, aged 9 at the time of examination) presented with walking instability and foot deformities from preschool age. Muscle atrophy was not described. We concluded that the variant p.Gln46Pro is not causal for HMSN in this family.

Two unrelated patients from families S and T with mutation p.Asp1229Val – patients had no foot deformities, but early onset scoliosis was observed. Coordination instability was the main clinical symptom.

A patient with variant p.Thr199Ile from the family U also had no foot deformities, but had worsened walking ability, mainly because of lack of stability.

Patient V with p.Arg227Gln had pedes equinovari, but the mode of inheritance was re-evaluated as being autosomal dominant.

In general, the phenotype in these patients differed from patients with biallelic pathogenic mutations.

The fact that we were not able to identify the second mutation in the *SH3TC2* gene in these patients might be because these variants are not pathogenic alterations. This is what we concluded about p.Gln46Pro. On the other hand, the second (undetected) mutation might be localized in a region of the *SH3TC2* gene that was not sequenced, or it might be a copy number variation, which was not detectable by standard methods, or crypting splicing might be involved. This relates to the mutation p. Asp1229Val in our opinion. Frameshift or other inactivating mutations even in monoallelic state would be more suspicious to be causal than missense mutations. It is not possible to make a final conclusion about pathogenicity or causal character of the remaining two variants (p. Arg227Gln and p. Thr199Ile), where further analyses are needed.

Family P – patient with a known inativating mutation on only one allele

In the patient from family P, mutation p.Arg954 Stop was found on only one allele, with mutation on the second allele missing. The patient is the only affected member in the family. He was 40 years old at the time of examination. The patient perceives muscle weakness on the lower limbs, has foot deformities and is not able to walk on his heels. Arms are also affected with muscle atrophy. Electromyography showed demyelinating pattern, with NCV on nervus medianus 30 m/s. Scoliosis was not reported. This patient was previously tested for CMT1A/HNPP and for mutations in genes *EGR2* and *NEFL* and was negative.

We were not able to identify the second causative mutation in this patient. This might be due to the fact that the second mutation is localized in the region of the *SH3TC2* gene that was not tested (non-coding regions). Or it might be a copy number variation that is not recognizable by standard sequencing methods. On the other hand, this patient might also be a heterozygous carrier of p.Arg954Stop mutation in the *SH3TC2* gene only by chance and his peripheral neuropathy might be caused by mutations in some other genes, but we feel this is less probable. It may also be possible that there could be mutations in two different genes – double heterozygozity or digenic character, but this is only speculation and further analyses are needed.

Mutations detected in the study and their relative position on the SH3TC2 protein is summarized in Fig. 3.

Summary

Mutations in the *SH3TC2* gene are a frequent cause of HMSN I in Czech patients. At least one mutation was found in 21 unrelated patients. From these 21 patients, biallelic pathogenic mutations were found in 14 of them. The prevalent mutation is p.Arg954Stop (63% of pathogenic alleles),

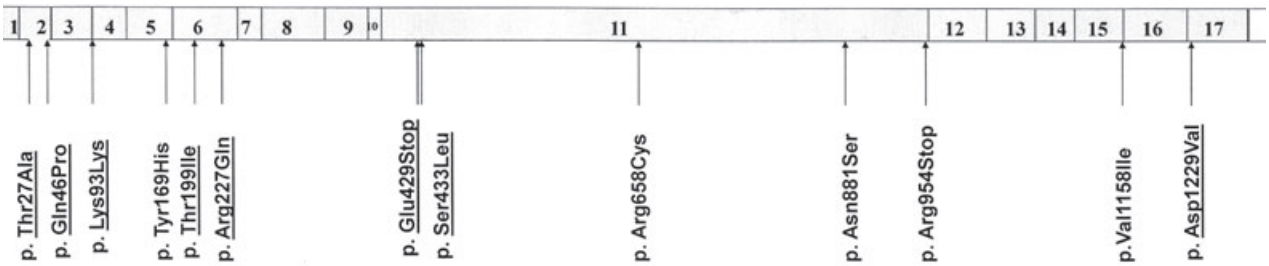

Fig. 3. Mutations in the *SH3TC2* gene detected in this study and their position relative to exons of the *SH3TC2* gene. *SH3TC2* gene contains 17 coding exons. Relative position of mutations detected in the study at the SH3TC2 gene transcript is shown. Underlined mutations are novel mutations. The rest are known mutations.

similar to other populations. CMT4C might be the most frequent AR type of CMT and belongs to the most frequent of all CMT types.

Patients with demyelinating neuropathy and negative for CMT1A/HNPP should be tested for the presence of the p.Arg954Stop mutation or even other mutations in the *SH3TC2* gene.

Supporting Information

The following Supporting information is available for this article:

Table S1. Custom TaqMan® SNP Genotyping Assays designed by Applied Biosystems (Foster City, CA). Sequences of Primers and TaqMan Probes.

Table S2. Multiple alignments using ClustalX algorithm showed conservation in several species of the mutated p.Thr27Ala, p.Gln46Pro, p.Lys93Lys, p.Thr199Ile, p.Arg227Gln, p.Glu429Stop, p.Ser433Leu and p.Asp1229Val, suggesting that they have an important function.

Table S3. sift analysis.

Table S4. PolyPhen analysis.

Table S5. Panther analysis (probably pathogenic mutation is in bold).

Table S6. Results of the restriction digest for mutations p.Arg954 Stop, p.Lys93Lys, p.Ser433Leu and p.Val1158Ile.

Additional Supporting information may be found in the online version of this article.

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