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Clinical, In Silico, and Experimental Evidence for Pathogenicity of Two Novel Splice Site Mutations in the *SH3TC2* Gene

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Abstract: Charcot-Marie-Tooth (CMT) neuropathy is the most common inherited neuromuscular disorder. CMT is genetically very heterogeneous. Mutations in the *SH3TC2* gene cause Charcot-Marie-Tooth neuropathy type 4C (CMT4C), a demyelinating form with autosomal recessive inheritance. In this study, two novel splice site mutations in the *SH3TC2* gene have been studied (c.279G→A, c.3676–8G→A). Mutation c.279G→A was detected on one allele in two unrelated families with CMT4C in combination with a known pathogenic mutation (c.2860 C→T in one family, c.505T→C in the other) on the second allele of *SH3TC2* gene. Variant c.3676–8G→A was detected in two patients from unrelated families on one allele of the *SH3TC2* gene in combination with c.2860C→T mutation on the other allele. Several in silico tests were performed and exon trap experiments were undertaken in order to prove the effect of both mutations on proper splicing of *SH3TC2*. Fragments of *SH3TC2* were subcloned into pET01 exon trap vector (Mobitec) and transfected into COS-7 cells. Aberrant splicing was predicted in silico for both mutations, which was confirmed by exon trap analysis. For c.279G→A mutation, 19 bases from intron 3 are retained in cDNA. The mutation c.3676–8G→A produces a novel splice acceptor site for exon 17 and complex changes in splicing were observed. We present evidence that mutations c.279G→A and c.3676–8G→A in the *SH3TC2* gene cause aberrant splicing and are therefore pathogenic and causal for CMT4C.

Keywords: exon trapping, peripheral neuropathy, *SH3TC2* gene, splice site mutation

INTRODUCTION

Charcot-Marie-Tooth (CMT) disease is a heterogeneous group of disorders, called also hereditary motor and sensory neuropathy (HMSN). With an incidence of 1:2500, it is the most common inherited neuromuscular disorder (Pareyson & Marchesi, 2009). Neuropathy is at the core of the problem and causes typical symptoms of the disease (Reilly & Hanna, 2002). Depending on the primary cause, myelin sheet of the peripheral nerve or axon itself is affected. Accordingly, electrophysiology is able to distinguish between these two primary disease mechanisms and two groups are defined: demyelinating neuropathy with nerve conduction velocities (NCVs) ≤38 m/s (HMSN I), and axonal neuropathy with NCVs ≥38 m/s (HMSN II) (Dyck & Lambert, 1968; Harding & Thomas, 1980).

CMT is genetically very heterogeneous. Mutations in more than 50 genes can cause CMT at the present www.molgen.ua.be; www.neuromuscular.wustl.edu).

Mutations in the *SH3TC2* gene cause demyelinating Charcot-Marie-Tooth (HMSN I) with autosomal recessive (AR) inheritance. This subtype is referred to as CMT4C (Senderek et al., 2003; Azzedine et al., 2006). Clinically, patients present with early-onset neuropathy, foot deformities, and severe scoliosis (Azzedine et al., 2006).

As we have recently shown (Lassuthova et al., 2011) CMT4C is a relatively common type of CMT and it might be the most frequent AR form of CMT overall. The prevalent mutation in many populations was proved to be c.2860C→T (Senderek et al., 2003; Azzedine et al., 2006; Gosselin et al., 2008; Houlden et al., 2009). Some additional mutations in the *SH3TC2* gene were shown to be pathogenic and causal for CMT4C http://www.ncbi.nlm.nih.gov/sites/varvu?gene=79628). We recently described (Lassuthova et al., 2011) two novel mutations in the *SH3TC2* gene (c.279G→A and c.3676–8G→A) that could possibly cause aberrant splicing, but we did not previously have experimental evidence to support this hypothesis. Each of these mutations was detected in two unrelated families, always only on one allele in combination with a known pathogenic mutation on the second allele of the *SH3TC2* gene.

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RNA splicing, which is a series of steps in the process of transcription, operates on several rules. The majority of exons in the human genome follows the AG/GT principle (Strachan & Read, 2003). However, this regulation itself would not be sufficient to recognize exons and introns apart and additional mechanisms are necessary. One of these is a branch sequence, localized 30–40 bp downstream the 3′ end on the intron, which carries an invariable adenine fundamental for lariat formation. This structure enables cleavage at the 5′ end, formation of a loop, and afterwards cleavage at the 3′ end and ligation of exons (Wolfe, 1993). In addition, a series of pyrimidines at the 3′ end is important. The whole process is possible due to the presence of snRNA types U1, U2, U4, U5, and U6 (Wolfe, 1993), which form spliceosome, and bind and draw near the 5′ and 3′ ends of the intron. Several other sequences are considered important in the process of RNA splicing, especially splicing enhancers and silencers (Berget, 1995). In humans and other vertebrates, this process is extremely complex. Moreover, it is believed that rather than the sequences of splice donor and acceptor themselves, their interaction is important for proper splicing (Lim & Burge, 2001). The first and the last exon of the gene are spliced based on even more complex mechanisms.

Alternative splicing enables high recombination and therefore genome variability. On the other hand, mutations affecting splice sites are a relatively frequent cause of genetic disorders in humans (Wang & Cooper, 2007; Luco et al., 2011). Aberrant splicing can lead to at least four basic different outcomes. Exon skipping is the most prevalent. Activation of a cryptic splice site is the second. Intron retention and creation of a pseudo-exon are less probable mechanisms (Berget, 1995). Furthermore, splicing mutations are frequently associated with milder phenotypes than would be expected from the predicted effect of the mutation and compared with other types of mutations due to the phenomenon of leaky splicing (Vezain et al., 2011).

Alternative splicing can be studied by a variety of methods. The *SH3TC2* gene is not ubiquitously expressed. Tissue from which we would have been able to isolate RNA was not available in any of the families studied. We therefore decided to adopt an alternative, well-established experimental approach and have been studying these mutations and their possible effect on *SH3TC2* splicing with exon trapping.

PATIENTS AND METHODS

Patients

Four families without known relationship affected by CMT4C were studied. Patients were examined clinically and electrophysiologically. Three of the studied families have been clinically described before (families K, M, N) (Lassuthova et al., 2011); one family has not been described before (D). All patients signed informed consent and the study was approved by the ethical committee of the University Hospital Motol. Genotypes of the patients are summarized in Table 1.

Methods

PCR Amplification

DNA was isolated from peripheral blood. Exons 3 and 17 of the *SH3TC2* gene were polymerase chain reaction (PCR) amplified using primers published earlier (Senderek et al., 2003).

Because our target vector (pET01) (MoBiTec, Gottingen, Germany) and gained PCR products did not match in two available restriction sites, we opted for subcloning of PCR fragments into pCR 2.1-TOPO vector (Invitrogen, Life Technologies, Grand Island, NY, USA) and only afterwards cloning into pET01 vector.

TOPO T/A Cloning

PCR fragments were subcloned into pCR 2.1-TOPO vector. All procedures followed recommendations from the manufacturer. For chemical transformation, One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen, Life Technologies) were used. Constructs were verified by DNA sequencing. Plasmid DNA was isolated with QIAprep Spin Miniprep Kit (Qiagen, Duesseldorf, Germany).

Directional Cloning Into pET01 Vector

Products and pET01 vector (10 µg each) were digested with FastDigest Enyzme NotI and SpeI (5 µL each) (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Dephosphorylation of pET01 vector was done

Table 1. Genotypes of the patients with splicing mutations in the *SH3TC2* gene.

Family	Genotype
K	(c.2860C→T)[a] + (c.279G→A)[c]
N	(c.505T→C)[b] + (c.279G→A)[c]
M	(c.2860C→T)[a] + (c.3676-8G→A)[c]
D	(c.2860C→T)[a] + (c.3676-8G→A)[c]

[a]Mutation has been described as pathogenic in a paper by Senderek et al. (Senderek et al., 2003).
[b]Mutation has been described as pathogenic in a paper by Lupski et al. (Lupski et al., 2010).
[c]Splicing mutation, described in a paper by Lassuthova et al. (Lassuthova et al., 2011).

with shrimp alkaline phosphatase (SAP) (0.5 unit SAP per mole DNA ends) (Promega, Madison, WI, USA). GeneJET Gel Extraction Kit (Fermentas, Thermo Fisher Scientific) was used for plasmid DNA fragment isolation from 2% agarose gel.

Ligation reaction was performed with Rapid DNA Ligation Kit (Roche, Indianapolis, IN, USA). Reactions were prepared according to the manufacturer's recommendations; 5 μL of linearized vector was mixed with 5 μL of insert DNA after restriction.

Transformation was performed using standard protocol.

Selected colonies were harvested in Luria broth (LB) medium and after overnight incubation plasmid DNA was isolated with QIAprep Spin Miniprep Kit (Qiagen, Duesseldorf, Germany). Plasmids were verified by DNA sequencing.

Cell Culture, Transfection, RNA Isolation, and RT-PCR

COS-7 cells (American Type Culture Collection [ATCC] CRL-1651; Rockville, MD, USA) were maintained at 37°C in complete Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin. To obtain subconfluent cultures (~80% confluency), cells were seeded at 1.5×10^4 cells/cm in a 6-cm culture dish. Cells were transiently transfected in serum-free medium with constructs prepared in the previous step using X-tremeGENE HP (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions using 2–4 mg plasmid and a 1:3 ratio of DNA to X-tremeGENE reagent. The cells were incubated with the transfection complexes for 24 hours before changing to complete DMEM and harvested after an additional 24 hours. Total RNA was isolated from the transfected cells using TriReagent (Invitrogen Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions.

cDNA was PCR amplified and PCR products were bidirectionally sequenced with BDTv3.1 (Applied Biosystems, Life Technologies) on an ABI3130 capillary sequencer. Gained sequences were compared with a reference sequence NM_024577.3 and a published pET01 sequence.

In Silico Analyses

Alamut v.2.1 (Interactive Biosoftware, Rouen, France) was used for computer analyses of possible splicing effects of the two mutations. Several algorithms are included in this GeneSplicer algortihm (Pertea et al., 2001); for splicing mutations these include SpliceSite Finder, MaxEntScan, NNSplice, Human Splicing Finder, and ESEfinder.

RESULTS

cDNA Analyses

cDNA amplification revealed marked differences between amplification of a wild-type sequence and a mutant sequence for both mutations studied. Results are summarized in Figure 1.

Exon 3 of the *SH3TC2* gene is normally 134 bp long, exon A of the pET01 vector is 49 bp long, and exon B of the pET01 vector is 23 bp long. We have observed that for a wild-type construct, the length of the cDNA is 206 bp (134 + 49 + 23). For a mutant-type construct with c.279G→A mutation, we have observed cDNA of a size of 225 bp. This size is due to 19 bp from intron 3 being retained in the cDNA, which was also confirmed by sequencing (Figure 2). A mutation leads to an aberrant splicing; a new splice site 19 bp upstream is activated by the mutation and the classical splice site is deactivated by the mutation.

Wild-type exon 17 of the *SH3TC2* is not trapped in this experiment. Mutant-type exon 17 carrying the c.3676–8G→A mutation is trapped and several products are gained. These are products with size 492, 697, and > 1000 bp. Sequences are summarized in Figure 2.

In Silico Analyses

Results are shown in the Figure 3. These results are concordant with cDNA analyses.

Clinical Study

Families K and N (With c.279G→A Mutation)

These two families have been described in detail clinically in a previous paper (Lassuthova et al., 2011).

Briefly, family K: The patient is a sporadic case in the family. The disease manifested itself with foot deformities at the age of 11, which is why she also underwent surgery. At the last examination at the age of 50 years, she was not able to walk independently, only with crutches. No scoliosis was noticeable.

Family N: see figure 4.

Families M and D (With c.3676–8G→A Mutation)

Family M has been described in detail clinically earlier (Lassuthova et al., 2011).

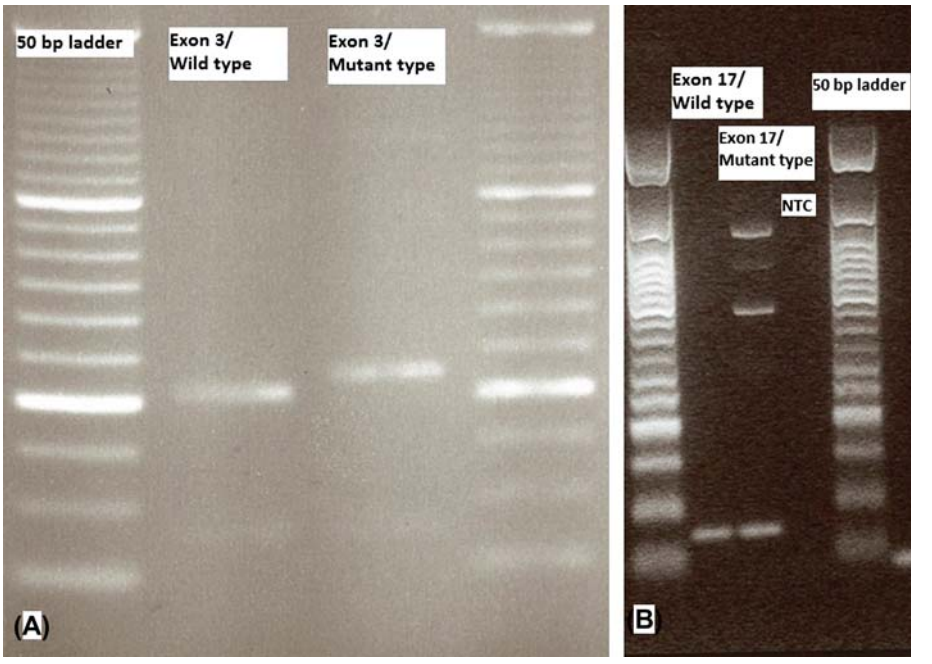

Figure 1. **cDNA amplification.** Gel electrophoresis with amplified cDNA. DNA 50-bp ladder was used. (**A**) Mutation c.279G → A. Wild-type cDNA reveals product of a size of 206 bp (49 + 23 + 134), mutant-type cDNA reveals product of a size of 225 bp (49 + 23 + 134 + 19). 49 bp = pET01 exon A; 23 bp = pET01 exon B; 134 bp = exon 3 SH3TC2 gene; 19 bp = 19 bases from the intron 3 that are retained in the mutated cDNA. (**B**) Mutation c.3676–8G → A. Wild-type cDNA reveals product 72 bp in length (49 + 23), mutant-type cDNA reveals four products—the first 72 bp in length (49 + 23), the second with size 424 bp (49 + 23 + 6 + 192 + 97 + 57), the third with size 697 bp (49 + 23 + 6 + 192 + 97 + 330), and the fourth over 1000 bp, which we were not able to sequence. 49 bp = pET01 exon A; 23bp = pET01 exon B' 192 bp = exon 17 SH3TC2 gene; 6 bp = 6 bases from the intron 17 that are retained in the mutated cDNA; 97 bp = 97 bp from the 3' UTR; 57 bp = 57 bp from the vector sequence; 330 bp = 330 bp from the vector sequence.

Family D: The patient is a sporadic case in the family. The disease manifested itself during childhood with scoliosis and foot deformities and the patient underwent foot surgery in childhood. The diagnosis of CMT was stated for the first time at the age of 15 years. At the last examination, at the age of 33 years, he presented with foot deformities, profound muscle atrophy in the lower limbs, and slight muscle atrophy in the upper limbs as well. Severe scoliosis was present. He walks only with crutches. Motor nerve conduction velocity (MNCV) at the age of 18 was 23 m/s (n. medianus), with prolonged compound muscle action potential (CMAP). Sensory nerve action potential (SNAP) was not detectable; F-wave has prolonged latency—all results are consistent with the primary demyelinating polyneuropathy.

DISCUSSION

Combined evidence that novel mutations c.279G → A and c.3676–8G → A in the *SH3TC2* gene cause aberrant splicing and are therefore pathogenic and causal for CMT4C was provided, which further broadens the spectrum of causal mutations in the *SH3TC2* gene.

For c.279G → A mutation, we were able to show that this mutation causes aberrant splicing of *SH3TC2*. In the mutated cDNA, 19 bp from intron 3 have been retained. This is in accordance with the in silico predictions. This change will result in a frameshift and a stop codon is expected to occur at position c.380 (TAA). This mutation was detected in two families with CMT4C phenotype.

In family K, the mutation is present on one allele, with c.2860C → T mutation on the second allele of the *SH3TC2* gene. In family N, mutation c.279G → A is present on one allele, with c.505C → T mutation on the second allele of the *SH3TC2* gene. In both families with c.279G → A mutation, patients presented with classical CMT4C phenotype, but without scoliosis, which is rather unusual. This might be due to the character of c.279G → A mutation and effect of leaky splicing.

For c.3676–8G → A mutation, we have shown that the mutation causes aberrant splicing in cell culture. The wild-type DNA was not trapped in the experiment as was expected; the last exon of a gene might not be trapped because of a lack of a splice donor site at the 3′ end of the exon. Therefore, only pET01 exon A and pET01 exon B were ligated together in the exon trap experiment for a wild type.

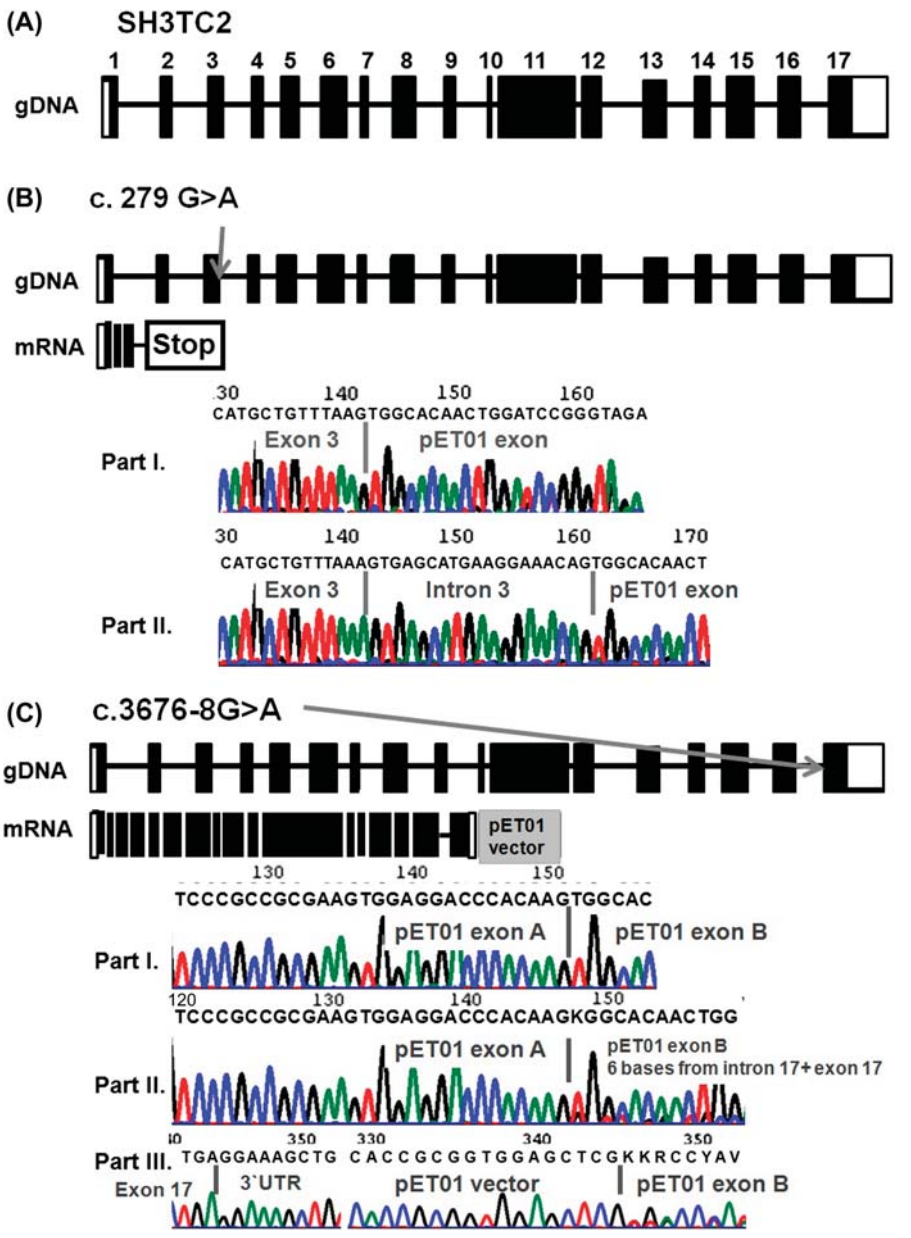

Figure 2. A comparison of sequences from cDNA amplification for reference (wild-type) sequence and mutated sequence. (**A**) A schematic representation of the *SH3TC2* gene. Translated regions are represented as black bars, UTRs are represented as white bars, intronic parts are represented as horizontal lines. (**B**) Part I—Reference (wild-type) transcript is spliced in a normal pattern, exon 3 of the SH3TC2 gene is joined with pET01 exon B. Part II—Mutated transcript is spliced in an aberrant pattern, 19 bp from the intron 3 are retained in the cDNA, cryptic splice site is activated, and this sequence is ligated with pET01 exon B. Site of the mutation is indicated with an arrow. (**C**) Part I—Reference (wild-type) transcript is spliced in a normal pattern; exon 17 of the SH3TC2 gene is not trapped by exon trap analysis, because it is the last exon of the SH3TC2 gene and lacks splice donor site at the 3′ end. Therefore, as expected, only pET01 exon A and pET01 exon B are ligated. Parts II and III—Mutated transcript is spliced in an aberrant pattern. Four products originated from cDNA amplification by PCR. This is compatible with the phenomenon of leaky splicing. The first product consists only of pET01 exon A and pET01 exon B, which is the same as wild-type sequence. The second product consists of pET01 exon A, 6 bp from the intron 17, exon 17 and 97 bp from the 3′ UTR and 57 bp from the vector sequence and finally 23 bp from the pET01 exon B. The third and the fourth products (Parts C and D) are similar to the second, with the only difference in the length of the vector sequence, which is longer.

Secondly, for a mutated DNA we have shown complex changes in splicing arising from c.3676–8G → A mutation. A new cryptic splice acceptor site is activated by the mutation, 6 bp downstream the 5′ end. This is consistent with the AG/GT rule. Since other regulatory mechanisms are not affected by the mutation, namely invariable adenine located 40 bp downstream and a series of pyrimidines, the new splice acceptor site is recognized and spliced

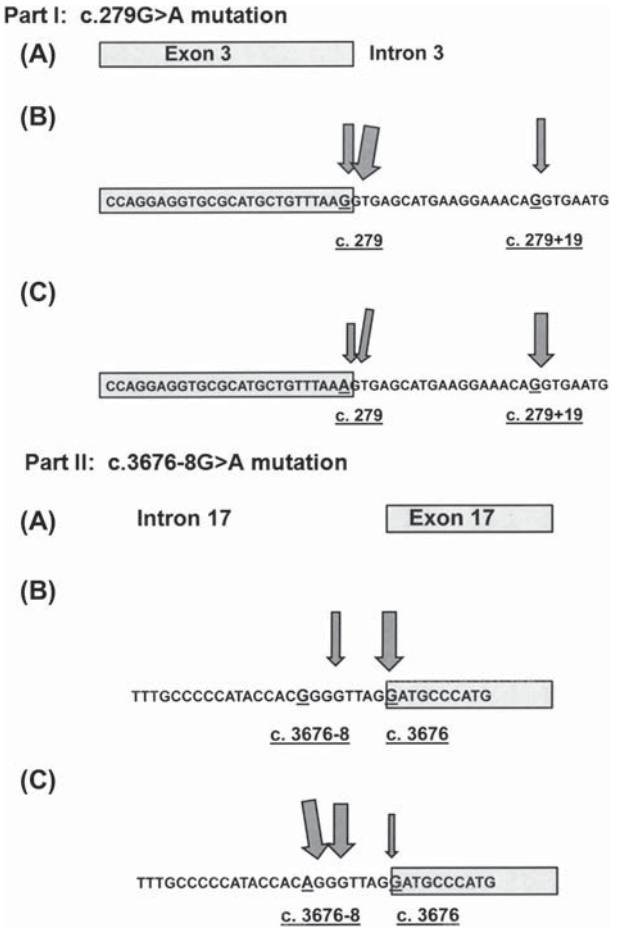

Figure 3. **PREDICTIONS.** Exonic parts are highlighted with bars. Arrows indicate the predictions for a strength of splicing; size of the arrows correspond to a strength of a relationship. (**A**) A schematic representation of the sequence (exonic and intronic parts); (**B**) wild-type sequence; (**C**) mutated sequence. Splicing window predictions for mutations c.279G→A (Part I) and c.3676–8G→A (Part II). Part I shows that for c.279G→A mutation, the cryptic splice donor site is localized 19 bp upstream the 5′ end of the exon 3. This splice site might be activated by the mutation. Also, the values representing the hits of binding of splicing molecules toward classical splice site are lower for mutated sequence compared with those for the reference sequence. Part II shows that for c.3676–8G→A mutation, the cryptic splice acceptor site is activated by the mutation. This new splice site is located 6 bases downstream the 3′ end of the exon. Also, there is a difference in the site of a classical splice site.

accordingly. However, the last exon of a gene is spliced based on even more complicated mechanisms and the interaction between splice donor site and 3′ UTR region is necessary. And although in our situation a splice donor site has been changed, we nonetheless propose that the interaction relationship has changed as well. This is what has given rise to a variety of products—all with variable length, which is due to the unsatisfactory recognition of the stop codon of the *SH3TC2* gene.

Moreover, for c.3676–8G→A mutation we also propose that in according to the theory of leaky splicing, we were able to detect also the wild-type product, the product consisting only of the form pET01 exon A and pET01 exon B. This shows that the classical splice site is not completely inactivated but only suppressed by the mutation and some nonmutated SH3TC2 RNA might be present. However, this RNA level is not adequate to retain the function of the *SH3TC2* gene and patients carrying this mutation manifest CMT4C phenotype.

We have also focused on the severity of the phenotype of patients carrying c.3676–8G→A mutation. However, we are not able to state that these two patients are affected less severely.

Moreover, this mutation has been recently described (Yger et al., 2012). We think that the fact that the mutation was detected in other CMT4C patient further supports the hypothesis that the mutation is causal and pathogenic for CMT4C.

Aberrant splicing has been already described as a mechanism that could lead to CMT (Taioli et al., 2011). In this paper the authors describe a silent change, p.Gly137Gly, and show the effect of this change on MPZ splicing. We also describe a silent change, p.Lys93Lys, that affects proper mRNA splicing. Both these reports suggest that even silent changes have to be considered and are important factors of alternative splicing.

SH3TC2 is expressed in nerve cells and is important for proper peripheral myelination. Exon trapping is a well-established method for proving alternative splicing in vitro. We decided to use the Exon Trapping System to study the in vitro effect of *SH3TC2* splice site mutations and their effect because no nerve biopsy material was available from the patients. This method is the only available noninvasive possibility to study splice site mutation if no expressing tissue is available.

Despite the limitations due to the method, we believe that the results of the experiments are valid based on the fact that our results are in accordance with in silico analyses, are in agreement with clinical study, the mutations do segregate in the pedigrees, and the results are supported by other published papers.

We suggest that "knock-in mouse," which would carry these mutations, would be the best proof of pathogenic effect and the process of myelination could have then been studied much more precisely. However, we think that the cost-benefit ratio in this case would be very unfavorable.

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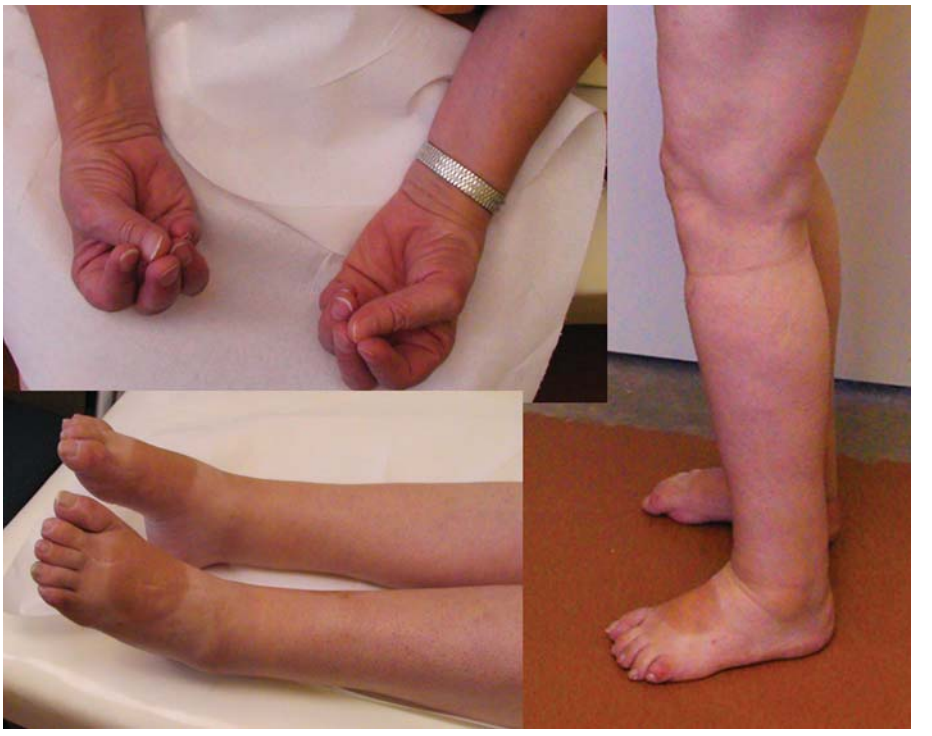

Figure 4. Patient from family N. The patient presented with distal muscle atrophy, slightly noticeable hammer toes, and shortened Achilles tendons. Hypoesthesia from knees down was present. Only faint atrophies were present on upper extremities. No scoliosis was noticeable. She is able to walk only with crutches.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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