Charcot-Marie-Tooth type X: A novel mutation in the Cx32 gene with central conduction slowing

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Abstract. Charcot-Marie-Tooth disease (CMT) is characterized by distal muscle weakness and wasting, often resulting in foot deformities and gait disturbances, distal sensory impairment and by more or less typical changes in sural nerve biopsy. CMT type 1 is also characterized by reduced nerve conduction velocities. For these demyelinating subtypes, most frequently a 1.5 Mb tandem duplication in chromosome 17p11.2-12 comprising the gene for the peripheral myelin protein 22 (PMP22) is observed (CMT1A), but point mutations in PMP22 have also rarely been reported. X-linked, dominant CMTX1 disease is the second most common type of these hereditary motor and sensory neuropathies (HMSN). Mutations in the X chromosomal gene Connexin32 (Cx32) synonymous gap junction β-1 (GJB1) are detectable in most X-linked CMT families. We report a novel nonsense mutation - Tyr65His - in the first extracellular domain of the Cx32 gene in a Czech CMTX1 family. The mutation was not detectable in 50 healthy controls. The clinical phenotype in both the male proband and his mother was moderate with pronounced peroneal weakness and foot drop. Nerve conduction velocities were immediately decreased (31-38 m/s) in both patients and slowing of central acoustic conduction (BAEP) was found in the both son and the mother whereas visual central conduction slowing (VEP) was detectable only in the son.

Introduction

Charcot-Marie-Tooth disease (CMT) is a clinically and genetically heterogeneous group of inherited disorders of the peripheral nervous system causing progressive neuromuscular impairment in both children and adults (1,2).

The main clinical features are progressive distal muscle weakness and atrophy starting usually at the lower limb particularly at peroneal muscles with later progression to the arms. Foot deformities like pes cavus are often present as well as distal sensation impairment. There is a wide phenotypic variability. The onset of this disease is in most cases during the first or second decade of life (3-5).

In general, the demyelinating type 1 CMT is accompanied by reduced nerve conduction velocities (NCV), whereas the axonal type 2 results in normal or nearly normal NCV but decreased amplitude of action potential (6). Genetically, the group is highly heterogeneous with at least 30 different chromosomal gene localisations (7). The most common mode of inheritance among CMT disorders follows an autosomal dominant pattern, which is also typical for CMT type 1A, resulting from a 1.5 Mb tandem duplication in chromosome 17p11.2-12 including the PMP22 gene or from rare point mutations in the same gene.

Mutations in 9 different genes are known to affect peripheral myelin or axons of the peripheral nerves - peripheral myelin protein 22 (PMP 22), myelin protein zero (MPZ), connexin32 (Cx32), early growth response 2 gene (EGR 2), myotubularin-related protein-2 gene (MTM2), neurofilament 2 (NFL 2), N-myc downstream-regulated gene 1 (NDRG1), Neurotrophic tyrosine kinase receptor type 1 (PTK1), and gigaxonin (GAN) (7,8,10).

X-linked dominant mode of inheritance is typical for Charcot-Marie-Tooth type X1 (CMTX1) (OMIM 302 800), resulting from mutations in the connexin32 gene (Cx32) located at Xq13.1. This mode of inheritance is rather rare, however it is the second most common one in the entire group of inherited peripheral neuropathies (9). Charcot-Marie-Tooth type X1 (CMTX1) patients account for 10-20% of demyelinating type 1 CMT cases (5,6). Neurological findings are not distinguishable from that of proven CMT1A patients, but in family studies males are usually more severely affected and have slower NCV than females of the same CMTX1 family. NCVs in affected CMTX1 males are on average 10 m/s higher than NCVs in CMT1A patients (11,12). Findings in sural nerve biopsies in patients with proven Cx32 mutation are clearly distinguishable from biopsies from CMT1A patients (13).

More than 220 mutations in the Cx32 gene located at Xq13.1 were reported in CMTX1 families. These include mostly missense, but also frameshift, deletion or nonsense
mutations spread over the whole coding region without any hotspots. Deletion of the entire coding region of Cx32 was also reported (14,15). Genotype - phenotype studies showed that most missense mutations result in a mild clinical phenotype, whereas most nonsense, deletion and frameshift mutations in Cx32 result in severe CMT phenotype (16).

Cx32 (GenBank acc. nr. 1176889) encodes a gap junction protein of the Schwann cell membrane. Such gap junctions function as channels for fast radial transport of ions and small molecules between the perinuclear and adaxonal cytoplasmatic compartment of myelinating Schwann cell, but the Cx32 protein is also present in the central nervous system (CNS), where it is mainly detectable in oligodendrocytes but also in neurons (17-19). Clinically evident dysfunctions in CMTX1 patients generally arise only from PNS abnormalities, but these patients may have subclinical lesions also in the CNS. Recent reports describe abnormal brain stem evoked potentials in patients with Cx32 mutations (7,20), but also visual evoked potentials and central motor pathway abnormalities were recently reported (21). Such findings were not detectable in CMT1A patients (21). Therefore electrophysiological testing of subclinical CNS involvement is very useful for distinguishing CMTX patients from other CMT1 patients.

In this report we describe the clinical and electrophysiological phenotype of a novel mutation 193 T>C (Tyr65His) in the connexin32 gene in a Czech CMTX1 family.

Patients and methods

Genealogy. Distal muscle weakness and atrophy with moderate foot drop and gait disturbances were reported in at least three generations of the reported family - in male proband (P), his mother (M) and maternal grandmother of this family (Fig. 1). The proband's affected grandmother died at the age of 50 years of cancer. She had a pes cavus, foot drop and gait disturbances. The proband (P) as well as his affected mother (M) have no siblings.

Clinical neurological findings. Male proband (P); during preschool age, he was without any symptoms. Psychomotorical development was normal. At the age of 9 years he...
had left ankle injury, distorsion. From the age of 10 years he suffered from slowly progressive muscle weakness particularly distal. He had less strength during school sport lessons. From 12 years of age a slowly progressive foot deformity - bilateral pes cavus is reported. At present, he complains about distal paraesthesias in the upper and lower limbs and nocturnal crampi.

On examination at the age of 16 years, we found a moderate pes cavus with hammer toes and foot varusity bilaterally (Fig. 2) and slight calf atrophies (Fig. 3). Heel walk was not possible. There were almost no visible atrophies of hand muscles. Deep tendon reflexes C5-7 are diminished and in C8 are absent. We found hyporeflexia L2-S4 but areflexia L5-S2. He shows a very limited dorsiflexion in the ankle (Fig. 4) with resulting pronounced steppage gait, similar to his mother. Hypoesthesia to ankles and wrist was detected and vibration sensation was slightly impaired on upper as well as on lower extremities - 6/8. At present no visual or hearing loss was noted. Orthopedic surgery correction of foot deformity and varusity is planned for him this year.

Proband's mother (M): From high school age she noticed bilateral footdrop, she reports worsening of her problems during pregnancy at 18 years of age. There is continuous slow progression of her distal muscle weakness and atrophy. On examination at her present age of 35 years, we found
slight calf muscle atrophies, more pronounced on the right side (Figs. 5 and 6). Atrophies of small hand muscles (Fig. 7), pronounced footdrop with only minimal active dorsiflexion (Fig. 8) and pronounced steppage gait were also present. She is unable to walk on heels. Patellar deep tendon reflexes are diminished and there is areflexia on L2-S2. We have found hyposthesia only on distal phalanges of toes and fingers. Vibration sensation was only slightly impaired 7/8 on upper limbs and 6/8 on the lower limbs.

**DNA analysis.** After exclusion of the CMT1A duplication by use of a set of 8 microsatellite markers (22), direct sequencing of the entire Cx32 coding region was performed in the affected male proband according to standard protocols (23). The complete coding region of Cx32 is located in exon 2 and was amplified by 3 overlapping PCR fragments. The resulting PCR products were purified using columns (Qingene, USA) and subsequently used as a template for a sequencing reaction with Big Dye Terminator sequencing kit (Applied Biosystems, USA). Analysis of the resulting reaction products was performed on a capillary automated sequencer ABI 310 (Applied Biosystems, USA). The resulting sequence was then compared with the published Cx32 sequence (24). The finding of 193 C>T in the male proband was confirmed by sequencing of the complementary DNA strand.

**Electrophysiological evaluations**

*Nerve conduction study and EMG.* Motor and sensory NCV measurements were performed according to standard procedures.
Brainstem reflex - blink reflex - was performed by stimulation of supranuclear nerve unilaterally and responses were recorded from both orbicularis oculi muscles. Latencies of early response R1 and late responses R2 ipsi and contralaterally to the site of stimulation were evaluated.

Evoked potentials. Visually evoked potentials (VEPs) and brainstem auditory evoked potentials (BAEPs) were recorded with standard techniques. VEPs were obtained by stimulating the 16th angle to specifically assess demyelination of visual pathways, BAEPs in response to 1024, 10 and 50 Hz click stimuli were measured bilaterally focusing on interpeak latencies I-III and III-V.

Results

A Czech CMT family known to be affected in at least 3 generations was investigated on clinical, electrophysiological and DNA levels.

A novel missense mutation in the Connexin32 gene was detected for the affected family members by direct sequencing. The mutation is a T to C substitution in nucleotide 193 of Cx32 gene (Fig. 9), which predicts an amino acid exchange Tyr65His in the first extracellular domain. According to the CMT mutation database (14), this mutation has not previously been reported. This mutation was not found in 50 DNA control samples of Czech and German origin.

Both affected family members from two generations have a similar, moderate CMT phenotype with pronounced footdrop and minimal dorsiflexion, pes cavus and diminished to absent reflexes. The age of onset of the affected male was earlier and the phenotype is more severe compared to his mother.

Electrophysiological study in the male proband showed diffuse neurogenic lesion of the peripheral nerves of the extremities but also of the cranial nerves. The results are summarised in Table I. The lesion affected sensory as well as motor nerve fibres and showed signs of combined myelin and axonal lesion. SNAP was absent at the sural and ulnar nerves.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years), sex</th>
<th>Nerve</th>
<th>Motor NCS Distal CMAP amplitude (mV)</th>
<th>Distal Latency (ms)</th>
<th>NCV (m/s)</th>
<th>Sensory NCS SNAP amplitude (uV)</th>
<th>NCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>17, M</td>
<td>Median</td>
<td>7.9</td>
<td>4.9</td>
<td>39</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tibial</td>
<td>2.5</td>
<td>6.5</td>
<td>36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No response</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>36, F</td>
<td>Median</td>
<td>1.9</td>
<td>5.8</td>
<td>31</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tibial</td>
<td>1.6</td>
<td>6.1</td>
<td>34</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No response</td>
<td>-</td>
</tr>
</tbody>
</table>

*The results showed slowing of conduction velocity, prolongation of distal motor latencies and reduction of amplitude of CMAP on upper and lower limb. These abnormalities were more pronounced in sensory fibers (inexcitability of sural nerve) and in lower extremities. The conduction abnormalities were found in both subjects, slightly pronounced in probands mother M.*
Table II. Summary of results from brainstem auditory evoked potentials (BAEP) studies in male proband P and his mother M compared to the normal reference values.a

<table>
<thead>
<tr>
<th>Proband</th>
<th>Side</th>
<th>I</th>
<th>II</th>
<th>III-V</th>
<th>I-V</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>L</td>
<td>2.03</td>
<td>4.50</td>
<td>Not present</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.74</td>
<td>4.52</td>
<td>Not present</td>
<td>2.78</td>
</tr>
<tr>
<td>M</td>
<td>L</td>
<td>1.92</td>
<td>4.00</td>
<td>5.96</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.87</td>
<td>4.19</td>
<td>6.58</td>
<td>2.32</td>
</tr>
<tr>
<td>Normal</td>
<td>L or R</td>
<td>2.04</td>
<td>4.16</td>
<td>6.06</td>
<td>2.16</td>
</tr>
</tbody>
</table>

*aBottom line, L is for left ear, R is for right ear, I, III, V is for each wave I-V. Probands P BAEPs showed a peripheral (prolonged I-III interpeak latency IPI) and a central auditory pathway pathology (wave V was not present). In probands mother (M), BAEPs were slightly abnormal bilaterally in central acoustic pathway. These abnormalities were pronounced on the right side.

Table III. Results from brainstem reflex - blink reflex - abnormality was detected only in male proband P.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stimul. side</th>
<th>R1 Latency Ipsilateral (ms)</th>
<th>R2 Latency Ipsilateral (ms)</th>
<th>R2 Latency Contralat. (ms)</th>
<th>R2 Latency Difference (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Right</td>
<td>13.4</td>
<td>46.6</td>
<td>40.2</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>12.0</td>
<td>41.8</td>
<td>39.2</td>
<td>2.6</td>
</tr>
<tr>
<td>M</td>
<td>Right</td>
<td>9.5</td>
<td>34.2</td>
<td>33.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>11.0</td>
<td>32.5</td>
<td>30.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>10.5-12</td>
<td>36-40</td>
<td>36-40</td>
<td></td>
</tr>
</tbody>
</table>

Table IV. Summary of results from visually evoked potentials (VEP) studies in male proband P and his mother M compared to the normal reference values.a

<table>
<thead>
<tr>
<th>Proband</th>
<th>Stimulation</th>
<th>Visually evoked potentials</th>
<th>Visually evoked potentials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Latencies (ms)</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amplitudes (μV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>P</td>
<td>Pattern-reversed transient</td>
<td>125</td>
<td>119</td>
</tr>
<tr>
<td>M</td>
<td>Pattern-reversed transient</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>Normal</td>
<td>Pattern-reversed transient</td>
<td>114</td>
<td></td>
</tr>
</tbody>
</table>

*aBottom line, L is for left eye, R is for right eye. The proband P (male) had prolonged VEP latencies bilaterally, his mother M had normal VEPs.

Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) at the median nerve were 39 and 38 ms/s respectively (Table I).

Electromyography of the tibialis anterior muscle performed with a concentric needle electrode showed abnormal spontaneous activity as well as an abnormal motor unit potentials, morphologies and recruitment pattern. Chronic neurogenic changes with reinnervation and discrete spontaneous activity were found in both family members. Abnormalities of blink reflex were found in the male proband.
only, but not in his heterozygous mother (Table III). Peripheral conduction slowing, as well as a central brainstem pathway lesion, were detected by this test.

The male patient (P) showed also abnormally prolonged latencies and an absent wave V in brainstem evoked potentials (BAEP) - (Table II). Slightly abnormally prolonged latencies were shown also in visually evoked potentials (VEP); wave P 100 was 119 on the right side and 125 on the left side (Table IV).

The affected mother of the proband showed diffuse neurogenic lesion of peripheral nerves on the extremities, but without any measurable abnormality at cranial nerves in blink reflex (Table III). MNCV at the median nerve was 31 m/s and SNCV at the same nerve 33 m/s. SNAP was absent at the sural and ulnar nerves. There was a detectable lesion of sensitive as well as motoric nerve fibres with combined signs of demyelination and axonal damage. The reduction of CMAP amplitude as a result of axonal damage were more pronounced in the mother, probably resulting from a longer course of the disease (Table I).

No abnormality was detected at VEP (Table IV) for the mother, but there were also prolonged latencies in BAEP, however less then in her affected son (Table II).

Discussion

We describe a detailed clinical, neurological and electrophysiological phenotype observed in CMTX1 patients bearing a novel mutation (Tyrv5His) in the 1st extracellular domain of the Cx32 protein. The disease clinically started earlier in the male proband compared to his mother. The muscle weakness and wasting are relatively severe and progressive in both affected members of this family despite their young age and are more pronounced in the male proband. Electrophysiological findings in peripheral nerves showed signs of a more progressed axonal damage in the mother than in her son. In contrast, the central nerve pathway in the brainstem were more impaired in the son than in his mother.

In general, our electrophysiological findings in both affected male and female are similar to previous results reported by Nicholson et al (12,20) and recently by Bhat et al (21).

Our report further confirms the necessity of careful evaluation of CNS pathways in CMT 1 patients, particularly in patients where the most common DNA mutation, the CMT1A duplication was excluded and before further search for rarer and more difficult to find point mutations in the other CMT genes are started. Subclinical CNS involvement could be a helpful indicative sign for Cx32 investigation. Observations in this Czech family with patients carrying a novel Cx32 mutation supports previous reports about CNS pathway involvement in CMTX1 patients, probably related to Cx32 expression in oligodendrocytes.

Panas et al (25) reported 6 patients with 4 different Cx32 mutations. They reported subclinical CNS involvement on electrophysiological and MRI levels in 4 patients with mutations located in extracellular domains of the gap junction β 1 (GJB1) protein in contrast to patients with mutations in the intracellular domains, who had no detectable CNS involvement. The novel mutation - Tyrv5His - described here is located in the first extracellular domain of the GJB1 protein and both affected members of the family showed a clear conduction slowing in brain stem auditory evoked responses. Our finding even if it comprises only one Cx32 mutation, supports the hypothesis made by Panas et al (25).

Analysis of more CMTX1 patients will validate this theory of CNS involvement, specifically for patients with mutations in the intracellular domain Cx32.

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References