

## ONLINE MUTATION REPORT

## Allelic variants in the 5' non-coding region of the connexin32 gene: possible pitfalls in the diagnosis of X linked Charcot-Marie-Tooth neuropathy (CMTX)

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Charcot-Marie-Tooth neuropathy (CMT) or hereditary motor and sensory neuropathy (HMSN) comprises a group of inherited disorders of the peripheral nervous system. With a prevalence of about 1 in 2500 persons it represents the most common inherited neuromuscular disease. The clinical features of CMT include progressive distal muscle weakness and atrophy, foot deformities, a steppage gait, distal sensory loss, and decreased or absent tendon reflexes. Variation in age of onset and clinical presentation in CMT is wide. It ranges from minimal adult onset pes cavus to severe distal atrophy and foot deformity starting in childhood and resulting in early wheelchair dependence. CMT is genetically heterogeneous with dominant gene defects on chromosomes 17, X, 1, and 10.<sup>1</sup> The most common molecular subtype is CMT1A associated with a 1.5 Mb duplication of the locus for the peripheral myelin protein 22 (PMP22) in the chromosomal region 17p11.2.<sup>2</sup> Mutations in the connexin32 gene (*Cx32*) on chromosome Xq13.1 cause the X linked dominant type of CMT.<sup>3</sup> *Cx32* mutations are now considered to be the second most common molecular cause of hereditary sensorimotor neuropathy, accounting for at least 10% of cases. *Cx32* is widely expressed in human tissues,<sup>4</sup> but only nervous tissue is affected with peripheral neuropathy and electrophysiological abnormalities in central nervous system pathways.<sup>5-6</sup> In man, at least two different *Cx32* mRNA transcripts exist that are transcribed from two alternate tissue specific promoters, P1 and P2.<sup>7-8</sup> Transcripts initiated from promoter P1 make up the most abundant *Cx32* mRNA in any other tissue except for the nervous system and contain the non-coding exon 1a separated by a large intron from exon 2, which encompasses the complete coding region. Promoter P2 used in nervous tissue is located within the large intron and primary transcripts consist of exon 1b, separated by a 355 bp intron from exon 2. Thus, these two mRNAs differ in their 5' untranslated region (5' UTR) while containing the same coding region. *Cx32* mutations reported so far are spread throughout the entire protein.<sup>1</sup> Moreover, some patients who did not exhibit mutations of the coding sequence were found to have allelic variants of the nerve specific *Cx32* 5' non-coding region.<sup>9-10</sup> Here we report two additional alleles within this region initially assumed to be of phenotypic relevance. One of these (G-713A) has been recently reported as a disease causing mutation,<sup>11</sup> whereas in our series it has been identified as a harmless polymorphism.<sup>12</sup>

## MATERIALS AND METHODS

## Patients

A molecular analysis of the *Cx32* 5' non-coding region was performed in 57 unrelated CMT patients with possible X linked inheritance showing neither the PMP22 duplication nor micromutations in the coding region of CMT related genes (*Cx32*, *P0*, *PMP22*). The pathophysiology of CMTX includes defective myelination as well as axonal dysfunction.<sup>3-13</sup> Therefore, we selected 39 subjects diagnosed with axonal CMT

(CMT2)<sup>6</sup> and 18 patients with demyelinating CMT (CMT1) based on electrophysiological and nerve biopsy results. Twenty-one patients were females and 36 were males. Families compatible with X linked transmission were seen in 22 index patients, whereas 17 were isolated patients, and in 18 patients there were no conclusive family data available (table 1). The absence of the CMT1A duplication was confirmed by analysis of the short tandem repeat DNA markers D17S122 (RM11-GT), D17S921 (AFM192xh12), D17S955 (AFM317yg1), and D17S1357 (103B11ac1) residing within band 17p11.2. Single strand conformation polymorphism analysis (SSCP) of the coding region of the *Cx32*, *P0*, and *PMP22* genes did not identify any PCR fragment of abnormal electrophoretic mobility making it unlikely that a mutation is present in these sequences.

## Mutation analysis

To test for mutations in the promoter P2/exon 1b region, we amplified four overlapping DNA segments spanning about 750

## Key points

- Mutations in the connexin32 gene (*Cx32*) on Xq13.1 cause X linked dominant Charcot-Marie-Tooth neuropathy (CMTX). While most patients harbour mutations in the coding region at least two allelic variants have been reported in the 5' non-coding region. We analysed the nerve specific *Cx32* promoter P2 and 5' untranslated region (5' UTR) in 57 unrelated German patients with possible CMTX.
- Neither the common 17p11.2 CMT1A duplication nor micromutations in the coding sequences of *Cx32* or other CMT genes could be identified. A G-713A transition, previously reported to cause CMTX (*Brain Res Mol Brain Res* 2000;78:146-53), was found in one out of two chromosomes tested, both from the series of CMT patients and from the series of healthy controls. Conclusively, -713A does not represent a significant risk factor for the development of CMTX, but is a common polymorphism in the general German population (*Brain Res Mol Brain Res* 2001;88:183-5).
- Another single nucleotide substitution (G-458A) was found exclusively in a familial CMT patient and was not detected in the remaining CMT cases or in normal controls. However, investigation of further family members showed that the mutation did not segregate with the phenotype, excluding *Cx32* as the disease locus.
- Our data suggest that allelic variants in the nerve specific 5' non-coding region of *Cx32* do not necessarily cause CMTX, but this region is variable with at least four harmless alleles.

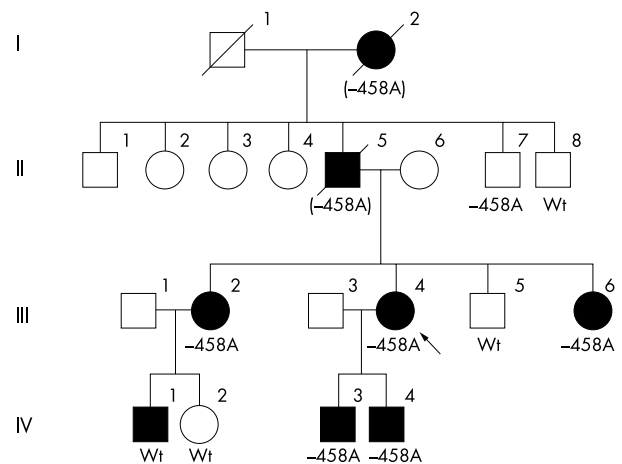
**Table 1** The cohort of CMT patients

| Family data       | Axonal CMT | Demyelinating CMT | Total |
|-------------------|------------|-------------------|-------|
| Familial patients |            |                   |       |
| Female            | 8          | 3                 | 22    |
| Male              | 10         | 1                 |       |
| Isolated patients |            |                   |       |
| Female            | 1          | 0                 | 17    |
| Male              | 8          | 8                 |       |
| Insufficient data |            |                   |       |
| Female            | 7          | 2                 | 18    |
| Male              | 5          | 4                 |       |
| Total             | 39         | 18                | 57    |

bp upstream of coding exon 2. PCR was performed through 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 60 seconds at 72°C with oligonucleotide primers corresponding to nucleotide positions -746 to -726 and -525 to -504 (P2-1); -565 to -544 and -334 to -314 (P2-2); -376 to -355 and -132 to -113 (P2-3); -181 to -163 and +59 to +77 (P2-4). The first nucleotide of the ATG start codon was numbered +1. Our nucleotide numbering differs from that in Neuhaus *et al*<sup>8</sup> by 1 bp as an additional thymine base is present at position -21. PCR products were screened for nucleotide changes by SSCP. Fragments P2-1, P2-2, and P2-4 were electrophoresed on a 1 × Mutation Detection Enhancement (MDE) gel (FMC Bioproducts, Rockland, ME, USA) at 15 W for 20 hours, and fragment P2-3 on an 8% polyacrylamide gel with 10% glycerol at 8 W for 18.5 hours. After electrophoresis, gels were silver stained. Automated sequencing was performed with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the same primers as for SSCP analysis were used as sequencing primers. The samples were run and analysed on an ABI PRISM 373A automated fluorescent DNA sequencer (Applied Biosystems). Restriction enzyme analysis was performed as recommended by the manufacturers. The resulting restriction fragments were separated on 2.0% agarose gels.

## RESULTS

Two novel alleles within the *Cx32* non-coding region were found. A G-458A transition was exclusively present in a single



**Figure 1** The -458A *Cx32* promoter P2 allele does not segregate with CMT in the pedigree of family AC11. Filled symbols: family members affected by CMT; open symbols: unaffected subjects; arrow: index patient. The -458 alleles of the *Cx32* promoter P2 are given below the symbols. The genotypes of deceased subjects I.2 and II.5 were deduced from the results obtained in their offspring and are given in brackets.

family (family AC11). Affected family members displayed moderate to severe sensorimotor neuropathy with males more severely affected at a given age and symptomatic at an earlier age (table 2). There was no male to male transmission and all three daughters of deceased male patient II.5 were affected while his only son was healthy (fig 1). Electrophysiological findings were compatible with the diagnosis of a predominantly axonal form of CMT with more prominent alterations in men. Male patients IV.1, IV.3, and IV.4 displayed median motor nerve conduction velocities (NCV) below the cut off value of 38 m/s for CMT1. However, the values were still intermediate (between 30 and 40 m/s). It is likely that marked loss of large, fast conducting axons (as seen in the nerve biopsy of patient IV.4) can be held responsible for NCV slowing in these patients.

Considering the inheritance pattern and the clinical phenotype, the disease was initially considered to be X linked dominantly inherited justifying *Cx32* mutation analysis. SSCP screening showed a band with altered mobility in fragment P2-2 in the index patient III.4. Sequencing disclosed a G→A

**Table 2** Molecular genetic, clinical, and electrophysiological data of family AC11

| Patient | Sex | Genotype* | Age of onset | Age at last examination | Foot deformity† | Muscle atrophy‡ | Gait disturbance§ | Tendon reflexes¶ |     | Sensory disturbance** | NCV (m/s)    |              |       |
|---------|-----|-----------|--------------|-------------------------|-----------------|-----------------|-------------------|------------------|-----|-----------------------|--------------|--------------|-------|
|         |     |           |              |                         |                 |                 |                   | ATR              | PTR |                       | Motor median | Motor tibial | Sural |
| I.2     | F   | (-458A/G) | 25-30        | 40                      | +               | +               | +                 | ND               | ND  | ND                    | ND           | ND           | ND    |
| II.5    | M   | (-458A)   | 10-15        | 50                      | +++             | +++             | +++               | -                | -   | ++                    | ND           | ND           | ND    |
| II.7    | M   | -458A     | Unaffected   | 65                      |                 |                 |                   |                  |     |                       |              |              |       |
| II.8    | M   | -458G     | Unaffected   | 63                      |                 |                 |                   |                  |     |                       |              |              |       |
| III.2   | F   | -458A/G   | 20-25        | 42                      | +++             | ++              | ++                | -                | -   | ++                    | 39.4         | 30.5         | NR    |
| III.4   | F   | -458A/G   | 20           | 40                      | +++             | +++             | ++                | -                | -   | +++                   | 40.1         | 29.2         | NR    |
| III.5   | M   | -458G     | Unaffected   | 37                      |                 |                 |                   |                  |     |                       |              |              |       |
| III.6   | F   | -458A/G   | 20           | 34                      | +++             | ++              | ++                | -                | -   | ++                    | 41.7         | 32.3         | NR    |
| IV.1    | M   | -458G     | 5-10         | 18                      | +++             | +++             | ++                | 5-               | -   | +++                   | 32.2         | ND           | NR    |
| IV.2    | F   | -458G/G   | Unaffected   | 23                      |                 |                 |                   |                  |     |                       |              |              |       |
| IV.3    | M   | -458A     | 10           | 16                      | +++             | ++              | ++                | -                | -   | ++                    | 33.8         | 25.4         | NR    |
| IV.4    | M   | -458A     | 5            | 13                      | +++             | +++             | ++                | -                | -   | +++                   | 32.2         | 24.7         | NR    |

\*The haplotypes of deceased subjects I.2 and II.5 were deduced from the haplotype data of their offspring and are given in brackets.

†Foot deformity: mild foot deformity (+); needs orthopaedic shoes (++); requires surgery (+++).

‡Muscle atrophy: mild (+); moderate (++) ; severe (+++).

§Gait disturbance: difficulties in running (+); steppage gait (++) ; wheelchair dependent (+++).

¶Tendon reflexes: absent (-); diminished (+); normal (++) .

\*\*Sensory disturbance: mildly reduced sensitivity (+); severely reduced sensitivity (++) ; sensory loss (+++).

ND: not determined; NR: not recordable.

transition at nucleotide position -458. This mutation resides within the non-coding exon 1b. The sequence change abolishes a restriction site for enzyme *HhaI* (Promega, Madison, WI, USA) in the 252 bp PCR fragment. To test whether the mutation segregated with the disease in family AC11, we analysed DNA samples from further available family members. *HhaI* digestion disclosed that the same nucleotide substitution was present in both affected sisters of patient III.4 and in both her affected sons. However, the allele was absent in patient AC11.IV.1 who suffered from disabling symptoms at the age of 18. His phenotype was similar to those displayed by other affected subjects from family AC11 so that another cause for his disease seemed unlikely. Conversely, proband II.7 was clinically normal but DNA analysis disclosed the G-458A transition in the *Cx32* gene. As a consequence, the G-458A exchange could be ruled out as the underlying defect in this family (fig 1).

The -458A allele could neither be detected in the remaining 56 patients included in the present study nor in any of 100 normal controls (59 females and 41 males) when their DNA was subjected to *HhaI* digestion. Moreover, this sequence was not detected in the studies of others who used *HhaI* digestion (in 100 patients with unspecified sex)<sup>9</sup> and SSCP analysis (in 50 patients with unspecified sex)<sup>10</sup> to screen for the C to T transition affecting nucleotide -459. Altogether, testing of at least 385 independent X chromosomes failed to show the -458A allele in the general population, suggesting an uncommon familial DNA variant without clinical phenotype.

PCR products generated with primers for fragment P2-1 produced mobility shifts in 31 out of 57 patients. Sequencing showed a G→A transition at nucleotide position -713, 215 bp upstream from the transcription start point of the nerve specific transcript 2. The sequence change creates a unique site for *NcoI* restriction endonuclease (Boehringer, Mannheim, Germany) in the PCR product, removing 32 bp from the 243 bp wild type fragment. The distribution of the -713A allele in the CMT group was not distinct from that in the control group and the distribution of genotypes showed no major deviation from Hardy-Weinberg equilibrium in either cohort. In particular, in the CMT group, respective SSCP and restriction patterns were present in the hemizygous state in 17 men, heterozygously in eight women including the index case of family AC11 (III.4), and in the homozygous state in six women (37 out of 78 independent chromosomes,  $q=0.47$ ). The calculated proportion of heterozygosity in women (2pq) was 50%. We did not notice any correlation between disease severity and genotype. Segregation with the phenotype was excluded in family AC11 simultaneously harbouring the G-458A substitution. Analysis of the healthy controls showed that the polymorphism occurred in 21 males, 30 women were heterozygous for position -713, and 15 women were homozygous (81 out of 159 chromosomes,  $q=0.51$ ). The calculated heterozygosity rate in women also reached 50%.

## DISCUSSION

Mutation analysis of the human *Cx32* 5' non-coding region showed two sequence changes in patients with CMT. A G-458A transition was exclusively present in a single family, but the altered allele did not segregate with the disease. The second DNA variant, G-713A, was found in one out of two chromosomes tested and was also detectable in an equal proportion of healthy controls.

The -458A allele was initially supposed to be the disease causing mutation in family AC11 as it was found in the index patient and in both her sons. However, by screening further family members the G-458A transition turned out to be a non-pathogenic allele. This example illustrates that careful pedigree studies are required to avoid diagnostic pitfalls of mutation analysis. This is especially warranted when analysing non-coding regions of genes in which variant sequences

are more frequent than in coding regions. Family AC11 is not linked to the *Cx32* locus, whereas clinical and genealogical findings were suggestive of an X chromosomal trait. However, these data might have been misleading and the disease might actually be autosomally inherited. We could exclude mutations in the coding regions of the *PMP22* and *P0* genes that were previously reported not only in demyelinating forms of CMT but also in a minor proportion of patients with axonal CMT.<sup>14-16</sup> However, there are at least five chromosomal loci for CMT2 for which only two candidate genes have been reported so far.<sup>17-22</sup> The differential diagnosis of CMTX includes disorders such as Friedreich's ataxia (FA) and distal spinal muscular atrophy (distal SMA or hereditary motor neuropathy (HMN)). The diagnosis of FA seems unlikely considering the vertical transmission in our pedigree and the absence of prominent ataxia as well as typical features such as cardiac abnormalities. In distal types of SMA one would not expect sensory disturbances or electrophysiological and severe morphological alterations in sensory nerves such as seen in the sural nerve of patient IV.4.

The G-458A transition affects a nucleotide that is conserved among the *Cx32* of man, mouse, and rat.<sup>8, 23, 24</sup> Interestingly, mutation of the neighbouring C-459 nucleotide leads to CMTX.<sup>9, 10</sup> The *Cx32* 5' UTR has been reported to act as an internal ribosome entry site (IRES) and the -459T allele has been shown to disrupt IRES function.<sup>25</sup> In contrast, the distribution of the -458A allele in pedigree AC11 suggests a DNA variant without phenotype. There is increasing evidence that not every mutation of an IRES element results in loss of function; even gain of function mutations have been recently reported.<sup>25</sup> Taken together, these data imply that -458A does not interfere with initiation of the translation machinery and, thus, does not impair *Cx32* synthesis in the cell.

Recently, a *Cx32*-P2 G-713A mutation was reported to segregate with CMTX in a Taiwanese family.<sup>11</sup> In contrast, we found the same -713A allele in about 50% of chromosomes of CMT patients and in an equal proportion of chromosomes derived from healthy volunteers. Our data imply that the -713A allele represents a harmless polymorphism that neither acts as a significant risk factor for developing peripheral neuropathy nor modifies the course of the disease in the white German population. A possible interpretation of these divergent results would be that the -713A allele by itself does not cause the disease but the detrimental mutation in the Taiwanese family could be physically linked to the *Cx32*-P2 variant. *Cx32* gene function might be impaired by mutation of additional upstream or downstream untranslated sequences or by other mechanisms that cannot be detected by standard PCR methods. Alternatively, clinical findings and molecular genetic data suggesting an X linked trait might be coincidental and the disease might actually be autosomal dominantly inherited.

In addition, the authors showed that the -713A sequence greatly reduced expression of a *Cx32*-P2-luciferase fusion gene in transfected cells suggesting that a reduced amount of *Cx32* sets off the CMTX phenotype. On the one hand, this seems reasonable to assume in light of data from functional analysis of several *Cx32* mutants as well as from *Cx32* knockout mice.<sup>26-28</sup> On the other hand, it is curious that the -713A allele is not associated with CMTX in our German cohort. It is conceivable that the results of the *Cx32*-P2-luciferase assay do not accurately reflect the situation in tissues affected in CMTX. One could hypothesise that the G-713A mutation of the P2 sequence might disrupt a binding site for a not yet known activator of transcription. Moreover, it might be suggested that there is a repressor recognition site outside the sequence present in the P2-Luc constructs. This repressor might not interfere with P2 activity by itself, but probably acts via negative regulation of the proposed activator. The G-713A mutation would then not lead to a dramatic decrease of *Cx32* expression with phenotype in allele carriers, whereas the wild

type construct would give an overestimate of P2 activity in the reporter assay. The -713A allele is common in the German population but was not found in the chromosomes of over 100 Taiwanese controls.<sup>11</sup> However, these findings are not necessarily contradictory since some single nucleotide polymorphisms (SNP) are specific to particular ethnic groups while others can be found with similar allele frequencies throughout the world.<sup>29,30</sup> The sequence variation creates a unique *NcoI* restriction site allowing a convenient genetic test using PCR amplified DNA. Thus, the -713 *Cx32*-P2 SNP could provide a reliable tool for allele tracking, at least in the white German population. This might be useful in cosegregation studies when a complex mutation is suspected (for example, gene inversion or major rearrangements<sup>31</sup>) that can be missed by sequencing or SSCP.

The existence of two different promoters for the human *Cx32* gene may allow tissue specific regulation of gene expression. It is likely that the transcriptional or translational machinery in Schwann cells and in central nervous system oligodendrocytes and neurones requires specific 5' sequences for correct initiation of transcription or to determine rates of translation or stability of the mRNA. Integrity of these non-coding sequences is presumably vital for directing gene expression and at least two deleterious sequence variations have been recognised.<sup>9,25</sup> Nevertheless, the nucleotide sequence of the nerve specific *Cx32* 5' UTR seems to tolerate some minor changes such as the single nucleotide substitutions reported here without resulting in a clinical phenotype.

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