

## Two pathogenic mutations located within the 5'-regulatory sequence of the *GJB1* gene affecting initiation of transcription and translation

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In contrast to mutations in the coding sequences of a genes involved in the pathogenesis of Charcot-Marie-Tooth disease (CMT), little is known about CMT phenotypes resulting from a DNA variants located in regulatory sequences of a given "CMT gene". Charcot-Marie-Tooth type X1 disease (CMTX1) is caused by mutations in the *GJB1* gene coding for an ion channel known as connexin, with a molecular mass of 32 kDa (Cx32). Only 0.01% of the *GJB1* gene mutations have been reported in its 5' regulatory sequence. Pathogenic mutations occurred in the internal ribosome entry site (IRES) are extremely rarely reported in human genetic disorders. To the best of our knowledge, in this study we report for the first time in an Eastern European population, two CMTX1 families in which two pathogenic mutations in the 5' regulatory sequence of the *GJB1* gene (c.-529T>C and -459C>T) have been found. The two mutations identified in our study disturb the 5' UTR sequence in two different ways, by affecting the transcription factor SOX10 binding site (c.-529T>C) and by the disrupting IRES element of *GJB1* gene (c.-459C>T). These regions are responsible for transcription (SOX10) and initiation of translation (IRES), respectively. On the basis of our findings that, in contrast to the most DNA sequence variants reported in untranslated regulatory regions of genes, the c.-459C>T and c.-529T>C mutations remain pathogenic in the context of different ethnic background.

**Keywords:** mutations in regulatory elements, *GJB1* gene, IRES element, transcription, translation

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### INTRODUCTION

Charcot-Marie-Tooth disease (CMT) is one of the most heterogeneous human heritable neuromuscular disorders with a very complex classification. CMT prevalence has been estimated at 1 in 2500 (Skre, 1974). Recent investigations in the Norwegian population estimated CMT prevalence at 1 per 1214, while other epidemiological studies in Spain, Italy, Iceland and Japan gave estimates ranging from 1 in 3500 to 1 in 9200. Charcot-Marie-Tooth type X-linked disease (CMTX1) linked to mutations in the *GJB1/Cx32* gene (gap junction protein, beta 1/connexin 32) belongs to the most frequent CMT forms with the prevalence of 4.1% to 9.8%, depending on particular CMT patient population (Braathen *et al.*,

2011). CMT1X is an X-linked dominant disease, with males being generally more affected than females. Phenotype in males ranges from moderate to severe, while women are in most cases only mildly affected or even asymptomatic (Kleopa & Scherer, 2006).

Since the initial linkage studies of the CMT1X in 1978 and 1982 (de Weerd, 1978; Iselius & Grimby, 1982) and description of families with mutations in the *GJB1* gene (Bergoffen *et al.*, 1993) over 300 different pathogenic mutations in this gene have been reported (IPNMD, PubMed). Most mutations in the *GJB1* have been found in the coding region of the gene, including nonsense and missense mutations, deletions, insertions and frameshifts. Merely eight sequence variants including six pathogenic mutations and one unclear variant have been described in a non-coding region comprising gene promoter 2 (Table 1) [IPNMD, PubMed]. The *GJB1* gene contains two alternative promoters, P1 and P2, responsible for expression in different tissues. The Cx32 transcript (variant 1) in the peripheral nervous system is mainly initiated by the P2 promoter, while promoter P1 is responsible for expression of transcripts (variant 2) in the liver, pancreas, oocytes and embryonic stem cells. On the other hand, transcripts initiated in the central nervous system use both P1 and P2 promoters (Fig. 1) (Neuhaus *et al.*, 1995; 1996; Söhl *et al.*, 2001). The most prominent transcription factors of the *GJB1* gene expressed in myelinating Schwann cells are EGR2 and SOX10 (Kuhlbrodt *et al.*, 1998; Warner *et al.*, 1998). EGR2 and SOX10 mutations have prominent influence on the expression of the *GJB1* gene (Bondurand *et al.*, 2001) and these mutations are associated with CMT1D and Waardenburg syndrome type 4C, respectively (Pingault *et al.*, 1998; Warner *et al.*, 1998). Such non-coding mutations can strongly affect translation of the *GJB1* gene via an IRES-dependent mechanism, which is the next level of expression modulation. The internal ribosome entry site (IRES) is a

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**Abbreviations:** GJB1/Cx32, gap junction protein beta 1/connexin 32; IRES, internal ribosome entry site; CMT, Charcot-Marie-Tooth disease; CMTX1, Charcot-Marie-Tooth type X1; SOX10, SRY-box 10; IPNMD, Inherited Peripheral Neuropathies Mutation Database; EGR2, early growth response 2; CMT1D, Charcot-Marie-Tooth type 1D; 5' UTR, 5' untranslated region of RNA; ITAFs, IRES, specific cellular trans-acting factors; RQ-PCR, real-time PCR; SSCP, single strand conformation polymorphism; HA, heteroduplex analysis; PMP22, peripheral myelin protein 22; FANCC, Fanconi anemia, complementation group C; FA, Fanconi anemia; IVS4, intron 4; LITAF, lipopolysaccharide-induced TNF factor; CMT1C, Charcot-Marie-Tooth type 1C; PubMed, database of references and abstracts on life sciences and biomedical topics

**Table 1. Mutations in the promoter region of the *GJB1* gene reported in 1996–2010**

Mutations in the <i>GJB1</i> gene promoter region		Phenotype	Citations
Original paper numbering	Standardized numbering		
c.-215 G>A	c.-713 G>A	CMTX1/polymorphism	Wang <i>et al.</i> , 2000, Bergmann <i>et al.</i> , 2001
c.-529 T>C	c.-529 T>C	CMTX1	Beauvais <i>et al.</i> , 2006
c.-528 T>G	c.-529 T>G	CMTX1	Ionasescu <i>et al.</i> , 1996
c.-526 G>C	c.-527 G>C	CMTX1	Houlden <i>et al.</i> , 2004
c.-458 C>T	c.-459 C>T	CMTX1	Ionasescu <i>et al.</i> , 1996
c.-458 G>A	c.-458 G>A	polymorphism	Bergmann <i>et al.</i> , 2002
c.-373 G>A	c.-373 G>A	CMTX1	Murphy <i>et al.</i> , 2010
c.-215 G>A	c.-215 G>A	CMTX1	Wu <i>et al.</i> , 2004

CMTX1, Charcot–Marie–Tooth disease X-linked type 1. Original paper numbering, mutation numbers used in original papers based on previous reference sequence. Standardized numbering, mutation numbers according to current reference sequence.

highly structured mRNA sequence in the 5' untranslated region of RNA (5' UTR), which recruits, among others, IRES-specific cellular *trans*-acting factors (ITAFs), RNA-binding proteins needed to start or enhance translation. The presence of IRES elements within the 5'-regulatory sequence of a gene indicates that translation of its mRNA may be conducted *via* an alternative molecular mechanism that does not depend on the cap structure on the 5'-end of the mRNA (Baird *et al.*, 2006).

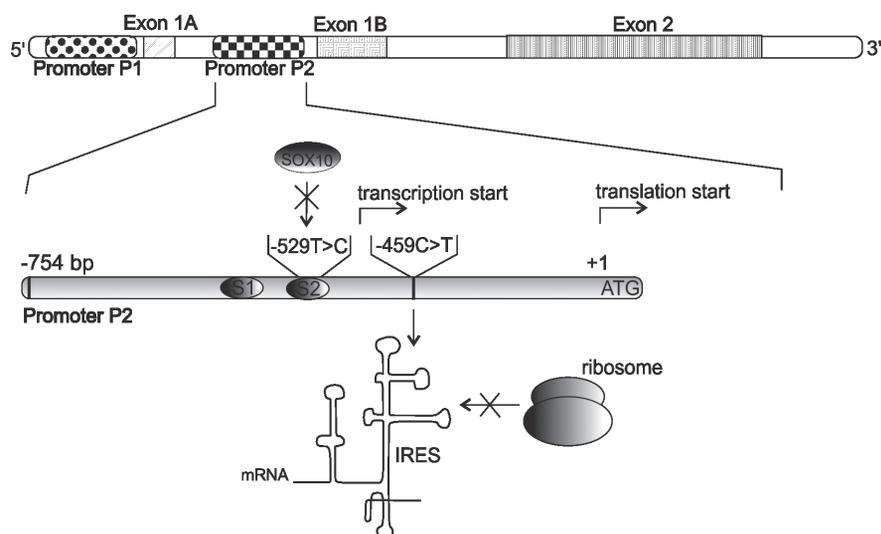
In this study, we report two Polish CMTX families in which two pathogenic mutations, c.-529T>C and c.-459C>T, were found for the first time in an Eastern European population.

## MATERIALS AND METHODS

**Molecular genetic studies.** DNA from peripheral blood from six family members (II:6, III:1 from fam-

ily 1; and I:1, III:4, III:5 and IV:2 from the family 2) was screened for duplication and deletion of the *PMP22* (peripheral myelin protein 22) gene using real-time quantitative polymerase chain reaction (RQ-PCR) (Aarskog & Vedeler, 2000). Reference values for the dosage of *PMP22* gene of healthy individuals (two copies of *PMP22* gene) range between 0.700 and 1.090. Values below 0.600 indicate a heterozygous deletion of the *PMP22* gene, and those above 1.180 indicate duplication (Kabzińska *et al.*, 2009). The whole coding sequence of *PMP22* gene was screened using single strand conformation polymorphism (SSCP) and heteroduplex analysis (HA), and the coding sequence plus 754 nucleotides upstream of ATG codon of the *GJB1* gene was sequenced directly. The primers used, were designed by Bergoffen *et al.* (1993).

Sequencing of the promoter of the *GJB1* gene in those families was done after a detailed family data analysis on the basis of the following: (i) pedigree analysis



**Figure 1. Two mutations located in the 5' regulatory sequence of the *GJB1* gene (P2 promoter) act in two independent ways**

The -529T>C mutation in the S2 binding site for the transcription factor SOX10 abolishes SOX10 binding, which impairs transcription of the *GJB1* gene. The -459C>T mutation in the IRES element impairs binding of the small subunit of the ribosome with IRES and in this way leads to impaired translation of the *GJB1* mRNA. The region analyzed encompasses 754 bp in the 5' regulatory region of the *GJB1* gene.

indicated possible X-linked mode of inheritance, (ii) the clinical course of CMT was milder in affected women than in affected men and (iii) no mutations were found in the coding sequence of the *GJB1* gene.

## RESULTS

### Family reports

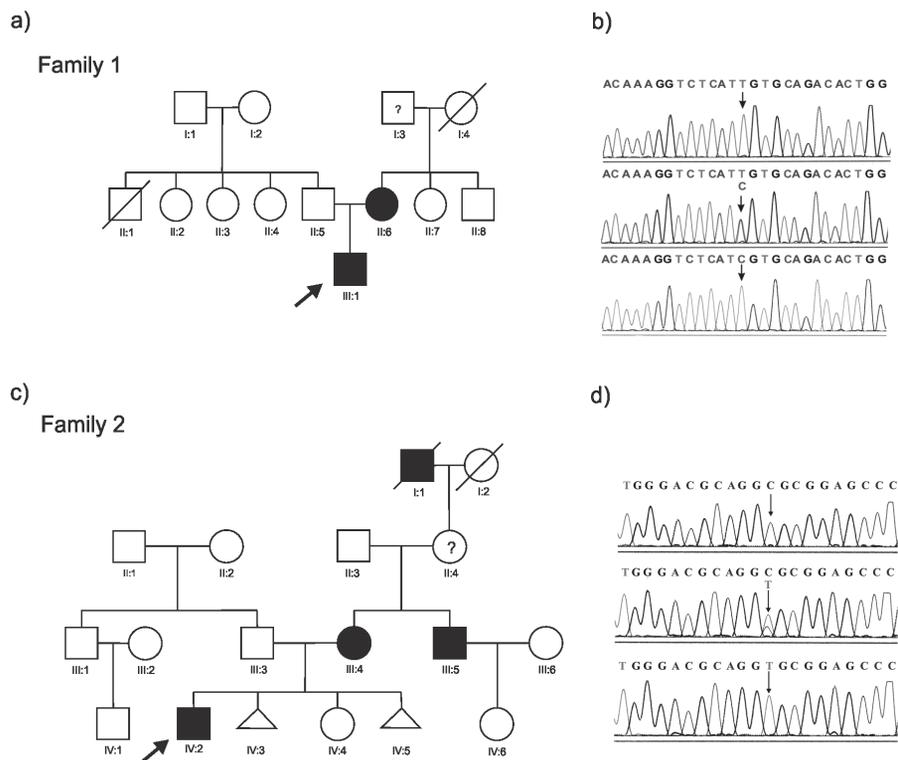
The affected members of the first family included the proband (14-year-old boy) and his 33-year-old mother (Fig. 2). The proband (III:1) was born by cesarean section following a normal pregnancy, with the birth weight of 2600 g, and scored 9 points in the Apgar scale. He could sit at 6 months and walk at about 18 months. Walking disturbances and progressive foot deformity appeared at approximately 7 years of age. He was admitted to our neurological department in 2006. We found distal muscle atrophy, absent knee and ankle jerks, slight distal paresis in legs without any sensory deficit and feet deformity. The mother of the proband (II:6) was considered to be healthy but she showed slight foot deformity and ankle jerks. ENG was performed in the patient and his mother. In the proband, we found axonal changes with CV slowing (about 31–36 m/s in the median and peroneal nerves) and low response amplitude (below 1.0 mV), and chronic neurogenic changes in the tibial muscle. Similar changes were found in the ENG in the mother of the proband. In both examinations, slight demyelinating changes were present, especially distal latency prolongation.

The second family consisted of four generations in which three individuals have been found to be as af-

fected (Fig. 2): the proband, his mother and his uncle. The status of individual II:4 could not be verified by us, however, due to the rules of X-dominant inheritance, the grandmother of the proband had to be affected. No male to male transmission of CMT disease was observed in this family. Clinical, electrophysiological and genetic evaluation was available in proband (IV:2) and his mother (III:4). The proband, 21-year-old university student, had slight distal paresis of the small hand muscles. He was unable to walk on his heels. The ankle jerk was weak on the left side and absent on the right side. He also showed foot drop on the right side. Additionally, he showed mild sensory disturbances in the distal parts of the lower and upper limbs. The mother of the proband, aged 44 years, was previously considered a healthy woman. In neurological examination, she was able to walk on her toes but could not walk on her feet. She had mild *pes cavus* deformity, and the ankle jerks were weak. The uncle of the proband (III:5), aged 45 years, was also affected with CMT. He showed symmetrical distal weakness and atrophy in the upper and lower limbs (Fig. 2).

### Molecular genetic findings

Duplication or deletion of the *PMP22* gene as well as mutations in the coding regions of the *PMP22* and *GJB1* genes were excluded. The values of the *PMP22* gene dosage in the DNA samples from individuals examined ranged from 0.799 to 0.874. Direct sequencing of the 754 bp promoter region of the *GJB1* gene in family 1 revealed a T to C transition at the nucleotide -529 (relative to the ATG start codon) in the proband (III:1) and his mother (II:6), located in the S2 binding site of SOX10 within the P2 promoter region. In family 2, a



**Figure 2.** Family trees for the -529T>C (Family 1) and -459C>T (Family 2) mutations in the 5' regulatory sequence of the *GJB1* gene. The probands are marked with arrows. Open symbols indicate healthy males (squares) and females (circles). Filled symbols correspond to affected individuals. Deceased individuals are marked with diagonal lines. Triangles represent spontaneous miscarriages. a) pedigree of the first family, b) chromatograms showing the base pair -529 (marked with an arrow) of a healthy individual (top), heterozygote (middle) and hemizygote (bottom) c) pedigree of the second family, d) chromatograms showing the base pair -459 (marked with an arrow) of a healthy individual (top), the heterozygote (middle) and hemizygote (bottom).

C to T transition at the nucleotide -459 (relative to the ATG start codon) was found in the three individuals (proband, mother and uncle) in the IRES (Internal Ribosome Entry Site) element in the P2 region.

## DISCUSSION

In the standard molecular genetic approach to CMT, molecular analysis is limited to the coding region of a gene. Thus, due to the lack of a systematic analysis of the regulatory sequences of the CMT genes it is impossible accurately to estimate the impact of mutations located in the regulatory region of the *GJB1* gene on the etiology of CMT1X disease. In fact, of the hundreds of mutations reported to date in the *GJB1* gene, only eight have been identified in its regulatory sequence (IPNMD).

In our study, we decided to analyze the 5' regulatory sequence of the *GJB1* gene due to the absence of any mutations in the coding region of the *GJB1* gene in the two families with a mode of inheritance typical for X-dominant disorders, and an observed discrepancy between the classical CMTX1 phenotype in affected males and a mild clinical course of CMTX1 in affected females. Thus, our study illustrates the importance of the phenotype-genotype correlations in the direction of molecular diagnosis of CMT.

To the best of our knowledge, this is the first report of two mutations in the 5' regulatory sequence of the *GJB1* gene in an Eastern European population. We found a c.-459C>T mutation in three related members of the CMTX1 family that segregated with the disease phenotype. Interestingly, the c.-459C>T mutation which was previously reported in ethnically divergent populations (Italy, United States and Hong Kong) also segregated with the CMTX1 phenotype (Ionasescu *et al.*, 1996; Flagiello *et al.*, 1998; Li *et al.*, 2009). The results of our study show that the pathogenic status of the c.-459C>T mutation does not depend on the ethnic background. Notably, the IVS4+4 A to T transition in the *FANCC* gene was initially thought to be limited to the Ashkenazi Jewish population and to segregate with a severe phenotype of the Fanconi anemia (FA) (Whitney, *et al.*, 1993). The same IVS4+4 A>T mutation in the *FANCC* gene occurring in the Japanese population is not associated with a severe phenotype of Fanconi anemia, and the clinical symptoms of FA in patients harboring IVS4+4 A>T mutation do not differ from those in patients with other mutations in the *FANCC* gene (Futaki *et al.*, 2000).

The second mutation in the 5' regulatory region of the *GJB1* gene reported by us, c.-529T>C, also seems not to be dependent on the ethnic background, since it has been previously reported in the French population (Beauvais *et al.*, 2006). In contrast to the -459C>T mutation, the c.-529T>C mutation cosegregated with the T49M mutation in the *LITAF* gene which may be causative for the CMT1C disease (Saifi *et al.*, 2005; Beauvais *et al.*, 2006). Thus, there is evidence that the c.-459C>T mutation is more pathogenic than the -529T>C sequence variant.

The two *GJB1* promoter mutations reported in this study act *via* two independent molecular mechanisms. The -529T>C mutation is located within a binding site (S2) of the transcription factor SOX10. Transcription efficiency of the *GJB1* gene has been reported to be regulated by the SOX10 and EGR2 transcription factors. In an elegant study, Houlden and colleagues (2004) provided evidence for decreased transcription of the *GJB1* gene harboring the -529T>C sequence variant. The molecular

mechanism of the -459C>T mutation affects expression is extremely rarely encountered in human pathology. Internal ribosome entry sites (IRES) are well characterized in bacteria and viruses, but so far no mutations within IRES elements have been detected in human disorders. Therefore, the presence of mutations in the IRES elements of the *GJB1* gene suggests that similar pathogenic mutations in other human genes are likely to be discovered (Macejak & Sarnow, 1991). Hudder and Werner (2004) provided evidence that the -459C>T mutation located in the IRES region of the *GJB1* gene abolishes its translation at an early phase (initiation).

To conclude, in our study we reported two CMTX1 families in which two mutations in the 5' regulatory sequence of the *GJB1* gene were found. Due to the segregation of the *GJB1* sequence variants with the CMTX1 phenotype and complete penetrance of these mutations in different populations, we assume that these mutations located in the regulatory region of the *GJB1* gene may be classified as pathogenic.

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