

Molecular genetic analysis of the *GJB1* gene: a study of six mutations

Andrzej KOCHAŃSKI, Dagmara KABZIŃSKA

Neuromuscular Unit, Medical Research Centre, Polish Academy of Sciences, Warszawa, Poland

Abstract: Charcot-Marie-Tooth type X1 disease (CMTX1) is an X-dominant peripheral neuropathy caused by mutations in the *GJB1* gene. Molecular genetic analysis of the *GJB1* gene is crucial for CMTX1 diagnosis and for genetic counselling. To date, molecular genetic analysis of the *GJB1* gene revealed 264 mutations in the *GJB1* gene. In spite of the rising number of *GJB1* gene mutations, family history was documented in only a few CMTX1 cases. The aim of this study was a molecular genetic analysis of the *GJB1* gene in 7 families, performed in 19 CMTX1-affected patients and 13 healthy family members. Moreover, we attempted to report evidence of effects of 6 amino-acid substitutions described in this study. To the best of our knowledge, the G110D, V152D and K167E mutations are novel substitutions, which have not been reported so far.

Key words: *GJB1* gene, CMTX1 disease.

Introduction

Charcot-Marie-Tooth disease (CMT) affects about 1/3000 individuals (SKRE 1974). CMT is characterized by progressive sensory-motor neuropathy, distal muscle weakness and atrophy, sensory loss, and decreased or absent deep tendon reflexes. In 10-20% of CMT patients, an X-chromosome-linked type (CMTX) may be diagnosed (IONASESCU et al. 1996). The X-dominant form of CMTX1 (MIM # 304040) accounts for 90% of all CMTX cases. The *CMTX1* locus has been assigned to the proximal long arm of the X-chromosome (GAL et al. 1985). In 1993, six different point mutations were detected in the *GJB1* gene in CMTX1-affected families (BERGOFFEN et al. 1993). In the period between 1993 and 2003, over 260 mutations were reported in CMTX1 disease

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Correspondence: A. KOCHAŃSKI, Neuromuscular Unit, Medical Research Centre, Polish Academy of Sciences, ul. Pawińskiego 5, 02-106 Warszawa, Poland. e-mail: andko@cmdik.pan.pl

(Website-database). However, the number of mutations in the *GJB1* gene is still rising, and 23 of them (9%) are designated as 'status unknown' due to lack of sufficient clinical descriptions. For only 26 mutations (10%) in the *GJB1* gene, family history of CMTX1 disease is documented. Molecular genetic analysis of the *GJB1* gene is crucial for CMTX1 diagnosis and genetic counselling for probands and their relatives. Even amino-acid changing substitutions found in the *GJB1* gene may represent silent mutations not associated with the CMTX phenotype. Some mutations in the *GJB1* gene were detected in many CMTX1 patients or families, but many CMTX1-causing mutations were reported in one CMTX1 patient without any familial history. The aim of this study was to analyse 6 mutations in the *GJB1* gene found in 19 CMTX1-affected patients.

Material and methods

The clinical and electrophysiological characteristics of CMTX-affected patients were reported previously by us (KOCHAŃSKI et al. 2002). DNA was isolated from white blood cells from 19 CMTX-affected patients and 13 healthy family members from 7 families. The coding sequence of the *GJB1* gene was amplified with previously published primers (BERGOFFEN et al. 1993). For single-strand conformation polymorphism analysis (SSCP), 4 μ L of the PCR products were mixed with an equal volume of formamide sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole) and heated to 95°C for 5 min. After rapid cooling on ice, samples were subjected to non-denaturing gel electrophoresis with the use of 6% acrylamide gel (acrylamide: bis-acrylamide 99 : 1) at 7 W for 20 h. After electrophoresis the gels were silver-stained. The PCR products revealing abnormal SSCP migration pattern were directly sequenced on an ABI PRISM 377 automated fluorescent DNA sequencer (Applied Biosystems). Restriction enzyme analysis was performed in 30 μ L of total volume. Ten microlitres of PCR products were mixed with 3 μ L of 10 \times digestion buffer (as supplied by the manufacturer) and 5 U of respective enzyme. Reactions with TaqI, AclI, and BsuRI were incubated overnight at 37°C. Digested fragments were separated on 3% agarose gel at 50 V for 3 h and visualised with ethidium bromide in UV light.

Results

In family JAN1 an altered SSCP analysis was not performed. Direct sequencing of *GJB1* part 2 revealed a c.329G > A base change causing a G110D substitution. The G110D mutation was found in heterozygous form in the proband's mother and as a hemizygote in the proband. The transition at position 329 destroys

the endonuclease restriction site *Bsu* RI, resulting in presence of an additional 165 bp allele in the hemizygous proband and his heterozygous mother.

Sequencing performed in family JUM2 revealed a heterozygous c.455T > A base change replacing Val by Asp (V152D) at codon 152 in the CMTX1-affected proband's mother (heterozygote) and two her sons (hemizygotes). The sequence of the proband's father was not changed at codon 152. The SSCP analysis performed in the proband of the KT5 family revealed an aberrant shift suggesting a mutation in the second part of the *GJB1* gene. Sequencing of the *GJB1* gene performed in the proband and his mother disclosed a c.499A > G transition, which results in the K167E substitution. In family NIM3, a SSCP analysis of part 2 of the *GJB1* gene revealed an altered migration pattern in the proband and his mother, suggesting that this part of the *GJB1* gene is most probably mutated. A sequencing analysis revealed a c.548G > A base change resulting in the R183H mutation. In family BK4, SSCP analysis showed an altered pattern in the proband and his mother. No altered pattern was found in his father and grandfather. Again, the sequencing analysis detected a heterozygous c.548G > A substitution resulting in the R183H mutation in the proband and his mother. SSCP analysis in family SB6 revealed an altered migration pattern in 5 CMTX-affected family members and a normal pattern in 4 healthy individuals. Direct sequencing of part 2 of the *GJB1* gene showed a hemizygous (three affected males) and heterozygous (two affected females) c.623A > G mutation resulting in an E208G substitution. In the last family MA7, SSCP revealed an altered migration pattern of the PCR product corresponding to part 2 of the *GJB1* gene in the proband, his mother and grandfather. No altered migration pattern was detected in the proband's healthy sister, his father, and 2 negative controls. The automated DNA sequencing revealed a hemizygous c.622G > A substitution resulting in the E208K amino acid change in the proband and his brother and a heterozygous E208K mutation in the proband's mother and grandfather.

Discussion

The mutations in the Cx32 protein were found in intracellular, second extracellular parts, and at the boundary between the transmembrane part and C-terminus of the Cx32 protein (Figure 1).

The E208G substitution was the first *Cx32* mutation found in the Polish population (KOCHAŃSKI et al. 2001). In a large group of healthy controls and DNA samples of CMT-affected patients analysed in our laboratory, no E208G substitution was found.

In contrast to the E208G mutation, the E208K substitution found in 4 members of family MA7 was previously reported in other CMTX families from Russia, Spain and Great Britain (FAIRWEATHER et al. 1994, HAITES et al. 1998, HAHN et al. 1999, MERSIYANOVA et al. 2000).

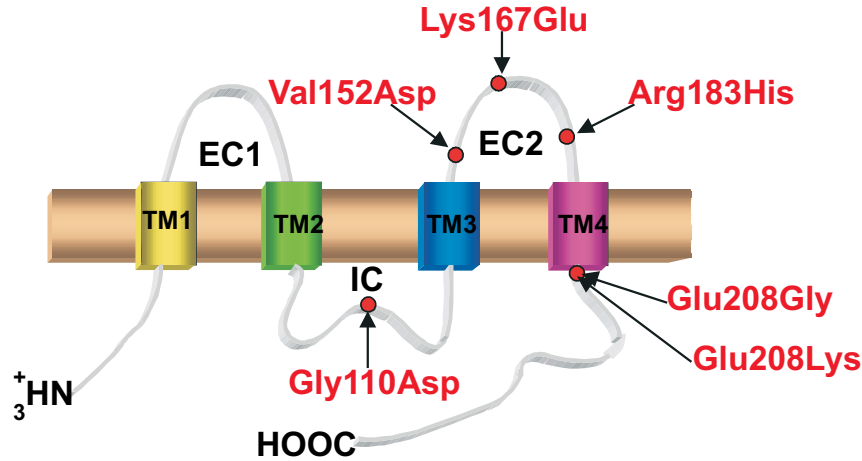


Figure 1. Model of connexin 32 in the cell plasma membrane. The red closed circles represent mutations found in this study. The TM1-4 show the transmembrane regions, IC represents intracellular region, and EC1-2 are the two extracellular domains.

The E208K and E208G substitutions are located in codon 208 of the *Cx32* gene, which is highly conserved, suggesting a critical function in gap junction assembly or functionality (Figure 2). The E208K mutant protein's ability to oligomerise into connexons was studied by using a cell-free translation system. The E208K mutant oligomerisation efficiency was similar to that of wild-type *Cx32*, but its trafficking to the plasma membrane was restricted (MARTIN et al. 2000). The E208K substitution detected in family MA7 fulfils 4 criteria of pathogenic mutation: (i) presence in other CMTX families, (ii) segregation with

	100	*	120	*	140	*	160	*		
Homo sapiens	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feavfmyvfyll	ypgyamvrlvkcd	vypcpntvdc	179
Mus musculus	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feavfmyvfyll	ypgyamvrlvkceaf	pcpntvdc	179
Rattus norvegicus	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feavfmyvfyll	ypgyamvrlvkceaf	pcpntvdc	179
Bos taurus	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feafmyvfyll	ypgyamvrlvkcd	aypcpntvdc	179
Cavia porcellus	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feavfmyvfyll	ypgyamvrlvkceah	pcpntvdc	179
Cricetulus griseus	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feavfmyvfyll	ypgyamvrlvkceay	pcpntvdc	179
Ovis aries	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feafmyvfyll	ypgyamvrlvkcd	aypc	163
Equus caballus	:	hgghiekkmlrleg	hgdp	hleevkrhkvhis	gtlw	wtyvisvfrll	feafmyvfyll	ypgyamvrlvkcd	ay	161
	180	*	200	*	220					
	:	fvsrptektvftv	flaasgicil	nvaevvyl	liiracarr					220
	:	fvsrptektvftv	flaasgicil	nvaevvyl	liiracarr					220
	:	fvsrptektvftv	flaasgicil	nvaevvyl	liiracarr					220
	:	fvsrptektvftv	flaasgicil	nvaevvyl	liiracarr					220
	:	fvsrptektvftv	flaasgicil	nvaevvyl	liiracarr					220
	:	fvsrptektvftv	flaasgicil	nvaevvyl	liiracarr					220

■ G110D
■ V152D
■ L167E
■ R183H
■ E208K E208G

Figure 2. Amino-acid sequence comparison of gap junction protein *Cx32* of 9 species. The arrows indicate mutated codons reported in this study. All the mutations are conserved within 8 species.

a CMTX trait, (iii) localization in the highly conserved Cx32 protein residue, and (iiii) aberrant Cx32 function shown in functional studies. The R183H and R183S mutants were retained in the Golgi body and only part of the mutant Cx32 protein was expressed in the cell membrane. The R183H mutations were described in families coming from China, Spain, Italy, and Russia, suggesting that codon 183 of the *Cx32* gene is frequently mutated (BONE et al. 1997, BORT et al. 1997, MOSTACCIUOLO et al. 1999, HAHN et al. 2001). The G110D, V152D and K167E substitutions were found in JUN1, JUM2 and KT5 families, respectively. Those mutations were found in CMTX-affected individuals and were not observed in healthy family members. The SSCP analysis of the 50 healthy controls did not reveal the altered migration pattern, suggesting that G110D, V152D and K167E substitutions are not polymorphisms. Up to now no mutations have been found in codons 110, 152, and 167 of the *Cx32* gene. The V152D substitution is located in codon 152 of the *Cx32* gene, which is conserved in 8 species (Figure 2). The K167E substitution is located close to the cysteine residue at codon 168, which forms a disulphide bond between the two opposite extracellular loops of the Cx32 protein (DAHL et al. 1992). Due to the Cx32 protein misfolding caused by the K167E mutation, the formation of the disulphide bonds may be disturbed. The R183H mutation was found in two families.

For G110D, V152D and K167E mutations, further functional studies are needed to understand their pathogenicity.

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