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Three novel mutations and genetic epidemiology analysis of the *Gap Junction Beta 1 (GJB1)* gene among Hungarian Charcot-Marie-Tooth disease patients

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Abstract

Pathogenic variants of the *gap junction beta 1* (*GJB1*) gene are responsible for the Charcot-Marie-Tooth neuropathy X type 1 (CMTX1). In this study, we report the mutation frequency of *GJB1* in 210 Hungarian CMT patients and the phenotype comparison between male and female CMTX1 patients. Altogether, 13 missense substitutions were found in the *GJB1* gene. Among them, 10 have been previously described as pathogenic variants (p.Arg15Trp, p.Val63Ile, p.Leu89Val, p.Ala96Gly, p.Arg107Trp, p.Arg142Gln, p.Arg164Trp, p.Arg164Gln, p.Pro172Ala and p.Asn205Ser), while 3 were novel, likely pathogenic alterations (p.Val13Glu, p.Glu186Gly, p.Met194Ile). These variants were not present in controls and were predicted as disease causing by *in silico* analysis. The frequency of the variants was 6.7% in our cohort which refers to a common cause of hereditary neuropathy among Hungarian patients. In addition to the classical phenotype, CNS involvement was proved in 26.1% of the CMTX1 patients. *GJB1* pathogenic alterations were found mainly in males but we also detected them in female probands. The statistical analysis of CMTX1 patients revealed a significant difference between the two genders regarding the age of onset, Charcot-Marie-Tooth neuropathy and examination scores.
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1. Introduction

Charcot-Marie-Tooth disease (CMT) is a clinically and genetically heterogeneous group of inherited neurological disorders with an overall prevalence of 1 per 1214 to 6250 [1–3]. Over 70 genes have been linked to hereditary neuropathies in the last two decades [4] (http://neuromuscular.wustl.edu/), and the large phenotypic description has been replaced by a genetic classification.

CMT can be classified by the inheritance pattern as autosomal dominant, autosomal recessive and X-linked (CMTX) [5]. The X-linked dominant form (CMTX1) is caused by pathogenic alterations within the *gap junction beta 1* (*GJB1*) gene, which encodes Connexin 32 (Cx32) protein. The Cx32 proteins form gap junction channels, which is involved in the transport of small molecular weight substances [6]. Cx32 is localized in many different cell types, including the paranodal region and the Schmidt–Lantermann incisures of Schwann cells. It has a crucial role in maintaining normal myelination in the peripheral nervous system [7,8]. Most of the *GJB1* mutations cause CMT through loss of normal Connexin function [9] but it is also suspected that the mutant protein has a negative dominant effect and suppresses the function of other gap junction proteins [10].

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To date, more than 400 *GJB1* pathogenic variants have been identified as the cause of CMTX1 [11]. These variants are responsible for 8.3 to 12.8 percent of all CMT cases, and after PMP22 gene duplication, are the second most common cause of hereditary motor and sensory neuropathy [12–14]. CMTX1 shows a wide spectrum of sensory and motor symptoms, and the nerve conduction studies reveal all forms of sensorimotor neuropathy. Clinical phenotype is characterized by distal muscle weakness and atrophy, absent tendon reflexes and sensory impairment of the upper and lower limbs. Pes cavus, foot drop, gait disturbances, sensory ataxia and tremor may also be associated features [5]. Though CMTX1 is considered to show X-dominant inheritance, female carriers usually show milder clinical symptoms than males with the same genotype [15,16]. In contrast to other CMT forms, certain *GJB1* pathogenic alterations were in association with central nervous system involvement and sensorineural hearing loss as well [17–19].

In this study, we determined the frequency of *GJB1* mutations among Hungarian CMT patients and the importance of novel alterations by *in silico* analysis and tested it in the healthy control cohort. We also investigated the phenotypical correlation between male and female CMTX1 patients.

2. Patients and methods

2.1. Studied cohorts

210 patients (63 females and 147 males; mean age 45.18 ± 18.87; CI95% (0.426 to 0.477)) were investigated with the suspected diagnosis of CMT from January 2006 to December 2015. The novel alterations were tested in 350 healthy control individuals (209 female, 141 male; mean age 39.88 ± 14.87; CI95% (38.32 to 41.44)) as well. All individuals were born in Hungary and descended from Hungarian ancestors.

Patients and controls were collected from NEPSY Biobank of the Institute of Genomic Medicine and Rare Disorders at Semmelweis University. Written informed consent was obtained from patients and control subjects before sample collection and molecular genetic testing. The study was approved by the Ethical Committee of Semmelweis University. Molecular genetic analysis was performed for diagnostic purposes in all investigated patients.

2.2. Clinical assessment

Detailed neurological examinations and laboratory investigations were performed in all patients. The family history was taken in all cases and age of onset was determined by asking the patients about their first neuropathy related symptoms such as paretic gait or sensory disturbances. The severity of the disorder was assessed using CMT neuropathy score (CMTNS) as follows: mild (≤10), moderate (11–20) or severe (≥20) [20].

2.3. Nerve conduction studies

Nerve conduction studies (NCS) were performed by standard techniques (Dantech Keypoint, Denmark) with superficial recording and stimulation of sensory, motor and mixed nerves. Routinely investigated nerves include: sural sensory, peroneal and tibial motor nerve conduction and F-waves, ulnar and median nerves sensory and motor conduction inclusive F-waves. Demyelinating neuropathy was diagnosed if the distal latency and/or F-latency was prolonged and/or the conduction velocity was reduced. Increased temporal dispersion was taken as a sign of demyelination as well [21]. Focal conduction block was ruled out. Diffuse amplitude-reduction and no evidence of demyelination indicated axonal loss. Nerve lesion was diagnosed as intermediate type if amplitude-reduction was present with decreased nerve conduction velocity, but the criteria of primary demyelination have not been fulfilled [5]. Normal values in reference to patients' height and gender were calculated using the Dantec Keypoint Software (Keypoint Software v.3.03).

2.4. Molecular genetic analysis of GJB1 gene

DNA was extracted from blood using the QIAamp DNA blood kit, according to the manufacturer's instructions (QIAgen, Hilden, Germany). The total coding region of *GJB1* gene was tested with Sanger sequencing using ABI Prism 3500 DNA Sequencer (Applied Biosystems, Foster City, USA). The genetic sequence was compared with the human reference genome (ENST00000361726; NM_000166.5) using NCBI's Blast® application. Novel variants were tested with PCR-RFLP methodology (c.38 T>A – Hpy166II (New England Biolabs® R0616S); c.557 A>G – NciI (New England Biolabs® R0196S); c.582 G>C – BmtI (New England Biolabs® R0658S)) in the control cohort.

2.5. In silico analysis

In silico analyses were performed with PolyPhen2, MutationTaster and SIFT softwares. The significance of detected alterations was tested with HGMD (www.hgmd.cf.ac.uk), dbSNP (www.ncbi.nlm.nih.gov/SNP/), ClinVar (www.ncbi.nlm.nih.gov/clinvar/) and CMT database (http://www.molgen.ua.ac.be). The nature of novel alterations was established according to the ACMG guideline [22].

2.6. Statistical analysis

The group comparisons were performed with independent sample t-test and Mann–Whitney U test regarding means. Percentages were compared with Chi square test. p values of <0.05 was considered statistically significant. Odds ratio (OR) in case control studies and 95% confidence intervals (CI95%) for proportions and means were calculated using standard formulas.

3. Results

3.1. Clinical assessments and nerve conduction studies

In all patients, symptoms indicated sensorimotor polyneuropathy with varying severity. Etiology of acquired neuropathy, such as diabetes mellitus, vitamin B12 deficiency, hypothyreosis, autoimmune disorders and paraneoplastic

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