Biochem Genet (2010) 48:248–256
DOI 10.1007/s10528-009-9314-7

Two Novel Missense Mutations in the Connexin 26 Gene in Turkish Patients with Nonsyndromic Hearing Loss

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Received: 27 November 2008 / Accepted: 21 August 2009 / Published online: 26 November 2009
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Abstract Most nonsyndromic hearing losses are caused by mutations in the *GJB2* gene, and studies have revealed that the forms and frequencies of these mutations are largely dependent on ethnic origin. In the present study, we aimed to characterize the mutation profiles of 151 patients with hearing loss in Turkey. The entire coding region of the *GJB2* was directly sequenced in all patients. We found 35 (23.2%) individuals carrying *GJB2* mutations. Seven different mutations were identified, five of which were previously known (35delG, delE120, R184P, M163V, L90P), the remaining two being novel variants (M34V, L205V). The most common mutation was 35delG followed by delE120. The 35delG mutation was homozygous in 22 cases (14.5%) and heterozygous in 4 cases (2.6%). Compound heterozygosity for 35delG was also observed. The delE120 mutation was found in three patients in homozygous form. A homozygous L90P and heterozygous mutations M163V and M34V were found in single cases.

Keywords Connexin 26 · Mutation · Hearing loss · Turkish

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Introduction

Congenital deafness occurs in approximately 1 in 1,000 live births and 50% of these cases are hereditary. The majority of them (70%) are nonsyndromic, and the remaining cases (30%) show syndromic hearing loss. Approximately 80% of nonsyndromic hereditary hearing loss is inherited in an autosomal recessive manner, whereas 15–20% of cases show autosomal dominant inheritance, and the remaining cases show X-linked or mitochondrial inheritance (Schrijver 2004).

In the field of nonsyndromic hearing loss, about 120 loci and 40 genes have been identified (Van Camp and Smith 2008). One of these genes, *GJB2*, encodes for a gap junction protein connexin-26 (Cx26), which is important for intercellular communication (Lefebvre and Van De Water 2000). In the cochlea, Cx26 proteins form connexons, and one of the important roles of these complexes is the recirculation of potassium ions. During the mechanosensory transduction process, they function in recycling potassium ions from hair cells to strial marginal cells. Thus mutations in the *GJB2* gene eliminate normal protein synthesis and result in hearing loss due to impaired potassium cycling (Kikuchi et al. 2000).

More than 100 different mutations have been identified in association with autosomal dominant and recessive hearing loss (Ballana et al. 2008). Some of these mutations have been detected at a relatively high frequency in patients with nonsyndromic hearing loss in many populations (Rabionet et al. 2000). Among these mutations, 35delG is the most frequent in Caucasians, 235delC in Asians (Abe et al. 2000; Liu et al. 2002; Guo et al. 2008), 167delT in Ashkenazi Jewish (Morell et al. 1998; Sobe et al. 1999; Lerer et al. 2000), and R143W in Africans (Brobby et al. 1998).

The aim of this study was to investigate the spectrum of *GJB2* mutations among 151 patients with profound hearing loss in Turkey.

Materials and Methods

Patients

The study included 151 patients with profound hearing loss (>90 dB), and all patients had genetic nonsyndromic hearing loss. Diagnostic workup included history (including perinatal history), physical examination (including otorhinolaryngologic examination), audiologic assessments (pure tone and speech audiometry, behavioral audiometry, evoked response auditometry, and otoacoustic emission testing as indicated), and computed tomography of the temporal bone as indicated. The study protocol was approved by the local ethical committee.

Molecular Analysis

Genomic DNA was isolated from samples of peripheral blood of the patients using a DNA extraction kit (Heliosis; Metis Biotechnology, Ankara, Turkey) according to the manufacturer's instructions. An 845-bp DNA fragment containing the coding

exon of the *GJB2* gene (exon 2) and its flanking intron sequence was amplified with PCR using the following primers: forward 5′-GCTTACCCAGACTCAGAGAAG-3′ and reverse 5′-TGAGCACGGGTTGCCTCATC-3′ (Gene Bank NG_008358.1). PCR was performed using the following steps: after initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 60 s, extension at 72°C for 60 s, and 5 min of final extension at 72°C. The total volume of 50 µl PCR mixture contained 50 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 µM each primer, and 1.0 U *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). PCR products were purified with an Invisorb Spin PCRapid Kit (Invitek, Berlin, Germany). The purified PCR products were sequenced in both directions using the same primers and BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Cycle sequencing products were then further purified using ethanol/sodium acetate precipitation and run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). All sequence variations were verified by resequencing.

Sequence variations in *GJB2* are described according to the nomenclature recommendations of den Dunnen and Antonarakis (2001). Numbering of *GJB2* nucleotides starts with the A of the ATG start codon in exon 2 as position number +1.

The evolutionary conservation of the amino acids affected by novel mutations was evaluated by multiple sequence alignment. The human connexin 26 amino acid sequence (acc. no. P29033) was aligned with connexin 26 sequences of the Norway rat (P21994), mouse (Q00977), gibbon (Q7JGL3), lowland gorilla (Q8MHW5), orangutan (Q8MIT9), rhesus monkey (Q8MIT8), cow (A2VE67), sheep (P46691), and human connexins 30 (O95452), 30.3 (Q9NTQ9), 31 (O75712), 31.1 (O95377), 32 (P08034), and 43 (P17302). Amino acid sequences were compared using an on-line ClustalW2 program from the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Results

Sixty-three mutations were detected in 35 of the 151 (23.2%) patients enrolled in the study (Table 1). Seven different mutations were identified, five of which were previously known mutations (35delG, delE120, R184P, M163V, L90P), the remaining two being the novel variants M34V and L205V. The most common mutation was 35delG. A total of 22 homozygous and 4 heterozygous carriers of 35delG were detected. Compound heterozygosity for 35delG was also observed (35delG/R184P in two patients and 35delG/L205V in one patient). The delE120 mutation was found in three patients in homozygous form. A homozygous L90P mutation was found in only a single case. Heterozygous mutations M163V and M34V were also found in single cases.

Two novel *GJB2* mutations were found, M34V and L205V (Fig. 1). M34V is the result of a c.100A>G transition, changing an ATG codon for Met to a GTG codon for Val (M34V). The second novel missense mutation, L205V, is the result of a c.613C>G transversion, changing a CTG codon for Leu to a GTG codon for Val

Table 1 Genotypes of the *GJB2* gene observed in 151 patients with nonsyndromic hearing loss

Genotype	Patients (% of 151)
35delG/35delG	22 (14.5%)
35delG/WT	4 (2.6%)
delE120/delE120	3 (2.0%)
35delG/R184P (c.551G>C)	2 (1.3%)
35delG/L205V (c.613C>G)	1 (0.7%)
M163V (c.487A>G)/WT	1 (0.7%)
L90P/L90P (c.269T>C)	1 (0.7%)
M34V (c.100A>G)/WT	1 (0.7%)
WT/WT	116 (76.8%)

WT wild type

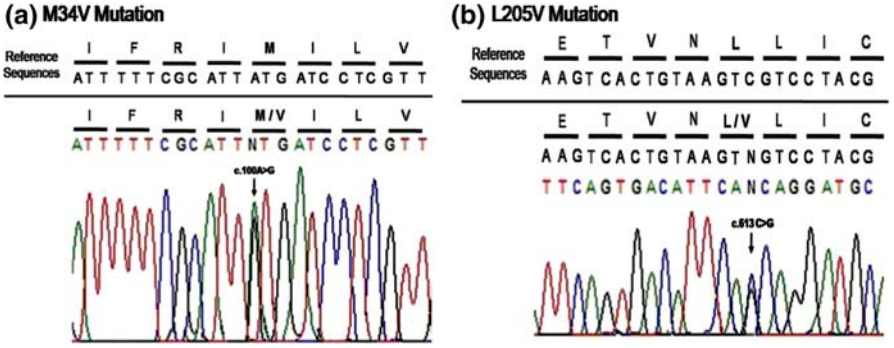


Fig. 1 Electropherograms of the sequence surrounding the M34V (**a**) and L205V (**b**) mutations in the *GJB2* gene and comparison with reference sequences. The M34V mutation is shown in forward strand, L205V in reverse strand. *Arrow* indicates mutated nucleotides

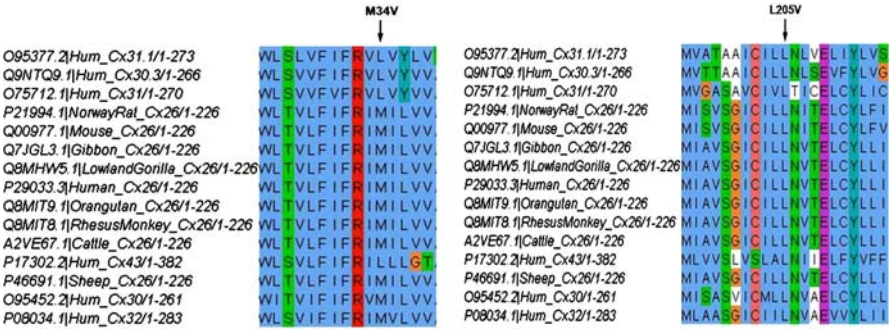


Fig. 2 Alignment of connexin 26 amino acid sequences. *Arrow* indicates position of the amino acids affected by novel mutations M34 and L205

(L205V). These two novel variants were analyzed further in terms of multiple sequence alignment to assess the evolutionary conservation of the amino acids affected by these mutations. The human Cx26 amino acid sequence was aligned

Table 2 Frequency and proportions of all identified *GJB2* mutations

Mutation	Number of alleles (% of 302)	Proportion of 63 mutations (%)
35delG	51 (17%)	80.9
delE120	6 (2%)	9.5
R184P	2 (0.7%)	3.2
L205V	1 (0.3%)	1.6
M163V	1 (0.3%)	1.6
L90P	1 (0.3%)	1.6
M34V	1 (0.3%)	1.6
Total mutation	63 (20.9%)	
WT	239 (79.1%)	

WT wild type

with Cx26 proteins from the Norway rat, mouse, gibbon, lowland gorilla, orangutan, rhesus monkey, cow, sheep, and human Cx30, Cx30.3, Cx31, Cx31.1, Cx32, and Cx43 protein sequences (Fig. 2). The evolutionary conservation of the amino acid M34 was observed when comparing the amino acid sequence of the human Cx26 protein with Cx26 from the Norway rat, mouse, gibbon, lowland gorilla, orangutan, rhesus monkey, cow, sheep, and human Cx30 and Cx32. The amino acid L205 was also conserved across all aligned connexin 26 proteins and human connexin proteins (Cx30, 30.3, 31, 31.1, 32, and 43).

No mutations were found in the coding region of the *GJB2* gene in 116 out of 151 patients, but the V153I polymorphism was found in 5 patients. The V27I and G160S polymorphisms were found in single cases. These polymorphisms were not encountered in patients with the *GJB2* gene mutations.

The total frequency of the *GJB2* gene mutations in patients with nonsyndromic hearing loss was 20.9% among the 302 choromosomes analyzed (Table 2). Of the seven different mutations detected, the most common was 35delG, with an allele frequency of 17%, followed by delE120, with an allele frequency of 2%. The R184P mutation was observed in two alleles (0.7%). The remaining four mutations (L205V, M163V, L90P, and M34V) had an allele frequency of 0.3%. The total number of mutations found was 63 in 151 patients with nonsyndromic hearing loss. The proportion of 35delG among all mutant alleles was 80.9%, followed by delE120 at 9.5%.

Discussion

Our results confirm that 35delG and delE120 are the most common mutations causing nonsyndromic hearing loss in Turkey, as reported previously (Uyguner et al. 2003; Kalay et al. 2005; Hismi et al. 2006; Tekin and Arici 2007; Evirgen et al. 2008; Baysal et al. 2008).

The 35delG mutation (80.9%) constituted the majority of the *GJB2* mutations in our study group (Table 2). Similar findings have been observed in other populations. The 35delG mutation accounts for 95.2% of mutations in Greece (Pampanos et al.

2002), 93.1% in Slovakia (Minarik et al. 2005), 86.7% in Finland (Löppönen et al. 2003), and 82.8% in the Czech Republic (Seeman et al. 2004). Tekin et al. (2005) reported that delE120 is the second most common mutation in Turkey. Similarly, in our study the second most common mutation was delE120 (9.5%), and three patients homozygous for this mutation were identified.

In a previous study from Turkey, Kalay et al. (2005) showed that the *GJB2* gene mutations result in approximately 25% of autosomal recessive nonsyndromic hearing losses. In addition, Bayazit et al. (2003) revealed that 21.4% of families had *GJB2* mutations in Turkey. Furthermore, it was reported that *GJB2* mutations are responsible for about 22.4% of cases of deafness (Tekin and Arici 2007). Consistent with these findings, our results showed that *GJB2* mutations were present in about 23% of patients with nonsyndromic hearing loss in Turkey.

The two novel sequence changes identified in this study, M34V and L205V, are located in the first and fourth transmembrane domain, respectively. The M34V mutation was identified in the heterozygous form in a patient with profound hearing loss. The M34T mutation was identified at the same position in several other studies. Although the affected amino acid residue was the same in the M34T and M34V mutations, the nucleotide changes were different. The M34V mutation is the result of a c.100A>G transition, and the M34T mutation results from a c.101T>C transition. The role of the M34T mutation in hearing impairment remains controversial. It was first reported as a pathogenic mutation with a dominant effect (Kelsell et al. 1997). In other studies, it was suggested that M34T acts in a recessive mode (Wilcox et al. 2000; Houseman et al. 2001) or is a nonpathogenic polymorphism (Denoyelle et al. 1998; Kelley et al. 1998; Scott et al. 1998a, b; Green et al. 1999; Prasad et al. 2000; Feldmann et al. 2004; Roux et al. 2004). Pollak et al. (2007) showed that the M34T mutation was associated with hearing impairment and had reduced penetrance compared with other *GJB2* mutations.

The other novel mutation, L205V, which was located in the fourth transmembrane domain of *GJB2* (TM4), was found as a compound heterozygote with the 35delG mutation in a patient with profound hearing loss. A C to G transversion at nucleotide 613 (c.613C>G) results in a leucine to valine change at amino acid 205 (L205V). L205 is conserved across all species and other human connexin proteins (Fig. 2). This may reflect the possible pathogenic nature of the L205V mutation. Moreover, the L205P (614T>C) mutation was also found in homozygous form at the same position in a Georgian Jewish patient (Leshinsky-Silver et al. 2005). Although the affected amino acid residue was the same in the L205V and L205P mutations, the nucleotide changes were different (c.613C>G and c.614T>C, respectively). Furthermore, hearing-loss-associated mutations have already been described in the TM4 domains Cys202Phe (Morlé et al. 2000) and Asn206Ser (Kelsell et al. 1997), but functional assays for these novel mutations are needed to identify the exact effects on hearing loss.

Heterozygous carriers of the 35delG, M163V, D50Y, and M34V mutations had no other mutations detected in the coding region of *GJB2* (exon 2). A second mutation may be located in the promoter, untranscribed regions, or exon 1. It is also possible that another gene causing hearing loss is involved in these patients. Further

analysis is needed to solve the problem of patients with monoallelic mutations in the coding region of *GJB2*.

In summary, these data support and extend previous findings concerning the contributions of *GJB2* to hearing loss. Identification of all genes involved in genetic hearing loss will help in our understanding of the fundamental mechanisms underlying normal hearing, both in early diagnosis and therapy.

Acknowledgments This study was supported by the Gazi University Research Found, with the project code number 01/2005-82.

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