

# Coincidence of mutations in different connexin genes in Hungarian patients

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**Abstract.** Mutations in the *GJB2* gene are the most common cause of hereditary prelingual sensorineural hearing impairment in Europe. Several studies indicate that different members of the connexin protein family interact to form gap junctions in the inner ear. Mutations in different connexin genes may accumulate and, consequently lead to hearing impairment. Therefore, we screened 47 Hungarian *GJB2*-heterozygous (one mutation in coding exon of the *GJB2* gene) patients with hearing impairment for DNA changes in two further connexin genes (*GJB6* and *GJB3*) and in the 5' non-coding region of *GJB2* including the splice sites. Eleven out of 47 *GJB2*-heterozygous patients analyzed carried the splice site mutation -3170G>A in the 5'UTR region of *GJB2*. One out of these 11 patients showed homozygous -3170G>A genotype in combination with p.R127H. Next to the *GJB2* mutations we noted 2 cases of deletion in *GJB6* [ $\Delta$ (*GJB6*-D13S1830)] and 3 (2 new and 1 described) base substitutions in *GJB3* [c.357C>T, c.798C>T and c.94C>T (p.R32W)] which are unlikely disease-causing. Our results suggest the importance of routine screening for the rather frequent -3170G>A mutation (in addition to c.35delG) in patients with hearing impairment.

## Introduction

Hearing impairment (HI) represents the most frequent neuro-sensory disorder worldwide. Contrary to former assumptions ascribing the basis of this highly heterogenous disease mainly to the ageing process and external factors, in recent years it has been demonstrated that at least 2/3 of all hearing deficiencies are of genetic origin. Approximately 70% of all hereditary hearing impairment cases occur as non-syndromic forms without additional clinical symptoms. *GJB2* is the gene responsible for a large proportion of these diseases (OMIM 121011). The *GJB2* gene encodes the gap junction protein connexin 26 (CX26) which is expressed in the cochlea and may play a role in K<sup>+</sup> circulation between different partitions in the cochlea (1,2). Gap junctions comprise the major system of intercellular communication for electrolytes, second messengers and metabolites. Therefore, it is not surprising that alterations in several members of the connexin protein family contribute to the development of hearing impairment, e.g. connexin 26, 30 and 31 (3-7).

Currently, more than 100 different mutations have been described within *GJB2* making it the most frequently examined gene in patients with hearing impairment (8). Although major progress has been made over the last several years in understanding *GJB2*-based hearing impairment, various patients belonging to multiple ethnic groups were described with only one single heterozygous *GJB2* mutation. The pathogenic mutations in this gene are generally inherited recessively and therefore induce hearing impairment only if occurring in both alleles; assessment and genetic counseling of single mutation carriers are difficult.

Several studies have demonstrated that most cells generally express more than one connexin isoform and assemble homomeric or heteromeric connexins leading to the formation of homotypic or heterotypic gap junctions (9-11). Four connexins (CX26, CX30, CX31 and CX43) were identified as prominently expressed in the cochlea. Moreover, digenic inheritance of mutations in two distinct connexin genes was shown to lead to hearing impairment (12). Therefore, we screened the patients with a heterozygous *GJB2* mutation for alterations in further connexin genes (*GJB3* and

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*GJB6*) and for mutations in the basal *GJB2* promoter as well as in the non-coding *GJB2* region.

## Materials and methods

**Subjects and selection criteria.** In this study 410 patients were involved. Patients showed congenital, bilateral non-syndromic sensorineural hearing impairment and were recruited from the outpatient service of the Department of Otolaryngology, University of Debrecen. The control group was composed of 156 unrelated individuals. Information on the medical history and pedigree structure was obtained in personal interviews with the affected individuals or with their unaffected relatives. Written informed consent was obtained from all participants and from parents of patients younger than 18 years. Control cases were collected comprising 156 individuals with normal hearing also originating from Hungary. The study was approved by the Ethics Committee of the University of Debrecen.

Case histories were obtained using a questionnaire regarding the following aspects, with special attention to disease: age at onset of SNHI, hearing aids, symmetry of the hearing impairment, middle ear infections, medical treatment, noise damage, trauma, meningitis, ototoxic agents, tinnitus, vertigo and other clinical manifestations (neurologic, ophthalmologic, gynecologic, pediatric, dermatologic, and orthopedic) to exclude other syndromic forms of hearing impairment.

**Audiological methods.** All patients involved in the study underwent otoscopic and audiometric examinations by using age-appropriate methods. We used air- and bone-conduction at 125, 250, 500, 1000, 2000, 4000, and 8000 Hz for all affected participants. The audiometric configuration, assessment of age at onset, severity and pattern of hearing loss by pure-tone audiometry, tympanometry and auditory brainstem response were performed according to the recommendations of the European Workgroup on Genetics of Hearing Impairment (13).

**Genetic analysis.** Anticoagulated venous blood (6 ml) was obtained from all tested individuals, and DNA was extracted according to the manufacturer's recommendations (Puregene kit, GENTRA System, Minneapolis, MN, USA).

***GJB2* analysis.** The coding exon of *GJB2* was sequenced using a capillary sequencer model CEQ 8000 (Beckman Coulter) (14). Sequences were compared with the reference

sequence (GenBank no. AF281280) using the DNAsis software (MWG).

Mutation c.380G>A (p.R127H) was screened in control individuals applying a restriction fragment length polymorphism (RFLP) assay. Using primers *GJB2*,3F (gtggcctac cggagacatgag) and *GJB2*,5R (gggcaatgcgttaaactggc) a 409-bp PCR product was amplified. The mutation abolishes a restriction site for *Sfa*NI resulting in three bands (143, 189 and 77 bp) for the mutant and four bands (107, 36, 189 and 77 bp) for wild-type alleles after *Sfa*NI digestion and polyacrylamide gel electrophoresis.

The 5' non-coding region of *GJB2*, promoter region -3458 to -3331 relative to the A of the ATG translation initiation codon located in exon 2 (15), and the non-coding first exon including both splice sites were sequenced using primers *GJB2*P,H2.1F (cagggcgctgggggcacttgggg) and *GJB2*P,H2.1R (caaccgctctgggtctcgcgttc). Sequences were aligned with the reference sequence (Gen Bank no. U43932.1) utilizing the CEQ software (Beckman Coulter). The splice site mutation -3170G>A (IVS1(+1)G-->A) was screened by an RFLP assay. Applying primers FOR (ggtgtgggtgcgggttaaaggcg) and MUT\_REV (cagtcggggccggcgggggtca) a 221-bp PCR fragment was generated. Since the mutation destroys a restriction site for *Eco*91I, wild-type alleles show two bands (199 and 22 bp) and mutated alleles only one band (221 bp) after *Eco*91I digestion. Samples revealing a mutated allele were sequenced on both strands.

***GJB3* analysis.** The coding exon of *GJB3* was sequenced using primers described elsewhere (16,17). Sequences were compared with the reference sequence (GenBank no. NT\_079620) using the CEQ software (Beckman Coulter).

The c.94C>T (p.R32W) sequence variant was screened applying primers 1f (acctattcatcatcacgatgg) and 1r (gagtgtgca gcagtagagg) (16). The variant has just one restriction site for *Bcn*I while the wild-type allele has two. After digestion and electrophoresis, two DNA fragments (240 and 282 bp) for the mutant and three fragments (79, 161 and 282 bp) for the wild-type allele were observed.

***GJB6* analysis.** Applying a multiple PCR strategy the 342-kb deletion [ $\Delta$ (*GJB6*-D13S1830)] was examined. Using primers for1 (gccatgcattgtggcctacta), rev1 (actatctgaatcagctcattc) and for2, del (cattgttgtaactaacctcca, GenBank no. AL590096.16) a 441-bp PCR product was generated for wild-type alleles and a 480-bp fragment was produced for an allele representing the deletion.

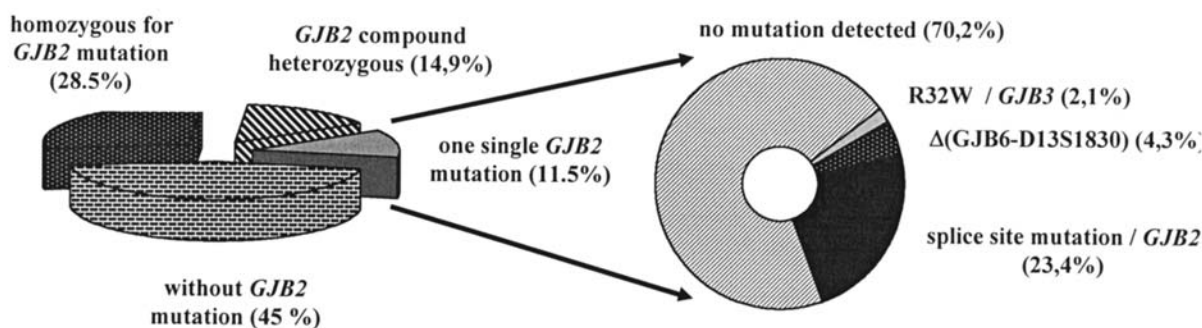


Figure 1. Distribution of *GJB2* mutations detected in Hungarian hearing impaired patients and the frequency of further connexin mutations.



**Audiologic.** *GJB2* mutations were detected in 225/410 patients (55%). All individuals were diagnosed with prelingual, sensorineural, bilateral, and moderate to profound hearing impairment. In most of the cases both ears presented a similar degree of hearing impairment. The auditory deficit involved all frequencies, and the morphology of the audiometric curves was flat in 153/225 (68%) of the cases and slightly decreased towards the high frequencies in 72 (32%) of the cases. No other configurations were observed.

***GJB2* mutations.** The most common *GJB2* mutation in the Hungarian population is the c.35delG (18). The mutation frequency of c.35delG in this cohort was 39% (319/820 alleles analyzed) which is in accordance with our previous studies (19). One hundred and seventeen patients showed the c.35delG mutation in *GJB2* in homozygous form (28.5%) and 61 were c.35delG compound heterozygous (14.8%). Forty-seven patients (11.4%) with sporadic hearing impairment showed only one *GJB2* mutation (c.35delG, p.V27I, p.G59V, p.R127H or p.E129K) (Fig. 1 and Table I). Most of these patients (24/47, ~51%) revealed the c.35delG mutation, whereas 3/47 (6.4%) showed the c.79G>A (p.V27I) mutation, 2/47 (4.3%) patients the c.385G>A (p.E129K) and only one the c.177G>T (p.G59V). A high number of the c.380G>A (p.R127H) mutations were found (17/47, ~36.2%) in our patients. Subsequently the c.380G>A (p.R127H) DNA change revealed 4/156 (allele frequency 1.3%) heterozygous control individuals.

***GJB2*-non-coding region and promoter analysis.** The first non-coding exon including the splice sites was analyzed for DNA changes. Ten heterozygous and one homozygous patient (11/47) showed the already described splice site mutation -3170G>A in the non-coding *GJB2* region (23.4%). No -3170G>A splice site mutation was found in 156 control individuals (<0.32% allele frequency).

***GJB6* mutations.** All 47 *GJB2*-heterozygous patients were analyzed for the 342-kb deletion [ $\Delta$ (*GJB6*-D13S1830)] using a multiple PCR strategy. Only two patients out of 47 *GJB2*-heterozygous patients showed the deletion in heterozygous form, accounting for ~4.3%. This deletion [ $\Delta$ (*GJB6*-D13S1830)] was analyzed in 46/185 hearing impaired patients without *GJB2* mutations and the corresponding control samples as well. In contrast to other studies, none of the 46 patients negative for *GJB2* mutations carried this deletion. Furthermore, 156 controls exhibited only wild-type alleles.

***GJB3* mutations.** By sequencing the coding region of *GJB3*, 3 common *GJB3* (2 new and 1 described) base substitutions (c.357C>T, c.798C>T, and p.R32W) were detected in our patients and the corresponding control samples, respectively. The new c.357C>T, c.798C>T base changes were not the result of a change of amino acid and they were likely polymorphisms since they also occurred in the control population. The c.357C>T variant occurred with an incidence of ~6% (controls) to 15% (patients), a frequency comparable

to other studies (17,20). The SNP c.798C>T occurred in 6% (patients) to 11% (controls) of all analyzed individuals, which also concurs with other research (20). In addition the c.94C>T (p.R32W) variant in *GJB3* was detected in one out of 47 heterozygous patients (2.1%). This DNA variant was the only *GJB3* mutation resulting in a change of amino acid. Nine out of 156 control individuals (5.7%) carried this DNA change in the heterozygous state.

## Discussion

Mutations in *GJB2*, the gene encoding the gap-junction protein connexin 26, are the most common cause of recessively inherited prelingual hearing impairment in Europe. Since several studies have demonstrated that single mutations in different connexin genes lead to digenic hearing impairment, we investigated a patient cohort consisting of Hungarian patients with one heterozygous *GJB2* mutation for mutations in further connexin genes and the regulatory region of *GJB2*.

***GJB2* promoter and the non-coding region.** The basal *GJB2* promoter was characterized by Tu and Kiang in 1998. Using reporter gene assays they localized the promoter region to position -3458 to -3331 relative to the translation initiation codon. Furthermore, a critical regulatory region extending from -3427 to -3399 was determined (15). Analyzing this DNA region via direct sequencing in *GJB2*-heterozygous Hungarian patients revealed no DNA change suggesting an infrequent involvement of promoter mutations in the development of hearing impairment in our patients.

In contrast, mutation -3170G>A which is located within the splice site, and therefore may have a pathogenic impact, was detected in 23.4% of *GJB2*-heterozygous patients (Table I). It is likely that the -3170G>A allele is either not transcribed or the mRNA is unstable, thereby leading to hearing impairment. This recessive mutation occurs most frequently in combination with c.35delG resulting in severe to profound hearing impairment (21,22). Less common is the occurrence in conjunction with *GJB2* mutations p.R184P and c.167delT (23-25). Our new observation for the Hungarian population is similar. Ten heterozygous -3170G>A carriers also had the c.35delG mutation, whereas only one individual had the p.R127H mutation in combination with homozygous splice site mutations. The severity of HI varies from moderate to profound in the cases with [*GJB2*: c.35delG] + [5'UTR *GJB2*: -3170G>A] genotype. The binaural mean pure tone average (PTA) threshold for air conduction at 0.5, 1 and 2 kHz (PTA<sub>0.5,1,2 kHz</sub>) was moderate in two patients, severe in three and profound in five. The patient with the homozygous splice site mutation was a 3-year-old girl. Auditory brainstem response (ABR) was performed and showed moderate HI.

***GJB6* and  $\Delta$ (*GJB6*-D13S1830) deletion.** It is known that CX26 can form heteromeric and heterotypic channels with other inner ear connexins in mammals (26). Co-immunostaining showed expression of CX26 (*GJB2*) and CX30 (*GJB6*) in the same gap junction plaques (27,28). Interaction of both proteins was demonstrated pointing to their ability to form heteromeric CX26/CX30 connexins *in vivo* (29). The

Table I. Sequencing results for the three analyzed connexin genes in Hungarian patients.

Patient	<i>GJB2</i> (Cx26) coding sequence	<i>GJB2</i> (Cx26) -3170G>A	<i>GJB6</i> (Cx30) $\Delta$ ( <i>GJB6</i> -D13S1830)	<i>GJB3</i> (Cx31) coding sequence	Genotype
1	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
2	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[IV] + [IV]	[ <i>GJB2</i> : c.35delG] + [?]
3	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
4	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
5	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
6	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
7	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
8	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
9	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [c.798C>T]	[ <i>GJB2</i> : c.35delG] + [?]
10	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [c.798C>T]	[ <i>GJB2</i> : c.35delG] + [?]
11	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [c.798C>T]	[ <i>GJB2</i> : c.35delG] + [?]
12	[c.35delG] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
13	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.35delG] + [?]
14	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
15	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
16	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
17	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
18	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
19	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
20	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
21	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
22	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [c.357C>T]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
23	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.798C>T] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
24	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
25	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
26	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
27	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
28	[c.380G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[ <i>GJB2</i> : c.380G>A, p.R127H] + [?]
29	[c.385G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.385G>A, p.E129K] + [?]
30	[c.385G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[ <i>GJB2</i> : c.385G>A, p.E129K] + [?]

Table I. Continued.

Patient	GJB2 (Cx26) coding sequence	GJB2 (Cx26) -3170G>A	GJB6 (Cx30) $\Delta$ (GJB6-D13S1830)	GJB3 (Cx31) coding sequence	Genotype
31	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
32	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[c.798C>T] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
33	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
34	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
35	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
36	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[IV]+ [IV]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
37	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[c.798C>T]+ [IV]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
38	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
39	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]
40	[c.35delG] + [WT]	[-3170G>A] + [WT]	[WT] + [WT]	[c.94C>T] + [WT]	[GJB2: c.35delG] + [5'UTR GJB2: -3170G>A]/ [GJB3: c.94C>T, p.R32W]
41	[c.380G>A] + [WT]	5'UTR [-3170G>A] + [-3170G>A]	[WT] + [WT]	[WT] + [WT]	[-3170G>A] + [3170G>A]/ [p.R127H]
42	[c.35delG] + [WT]	[WT] + [WT]	[ $\Delta$ (GJB6-D13S1830)] + [WT]	[WT] + [WT]	[GJB2: c.35delG] + [ $\Delta$ (GJB6-D13S1830)]
43	[c.35delG] + [WT]	[WT] + [WT]	[ $\Delta$ (GJB6-D13S1830)] + [WT]	[WT] + [WT]	[GJB2: c.35delG] + [ $\Delta$ (GJB6-D13S1830)]
44	[c.79G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[c.357C>T] + [WT]	[GJB2: c.79G>A, p.V27I] + [?]
45	[c.79G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.79G>A, p.V27I] + [?]
46	[c.79G>A] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.79G>A, p.V27I] + [?]
47	[c.177G>T] + [WT]	[WT] + [WT]	[WT] + [WT]	[WT] + [WT]	[GJB2: c.177G>T, p.G59V] + [?]

WT, wild-type.



*GJB6* situation is particular because of its chromosomal localization within 50 kb to *GJB2*. Mutations in *GJB6* have been shown to result in non-syndromic autosomal dominant and recessive hearing impairment (6,30). Recently, the impact of *GJB6* in the development of hearing impairment was confirmed by the generation of a knock-out mouse model (31). These mice exhibited severe hearing impairment. The cochlear sensory epithelium started to degenerate by cell apoptosis after P18 resulting in the deterioration of hearing in adult animals (31). A common 342-kb spanning deletion [ $\Delta$ (*GJB6*-D13S1830)] was identified in patients who carried one heterozygous pathogenic *GJB2* mutation (c.35delG) (30,32,33) (Table I). These patients are thought to develop hearing impairment with variable severity due to a digenic pattern of inheritance (34).  $\Delta$ (*GJB6*-D13S1830) leaves the *GJB2* coding region intact but deletes a large region close to *GJB2* and truncates *GJB6*. Thus, the deletion may eliminate an upstream regulatory element for *GJB2* that is essential for the normal expression of this gene in the inner ear. This deletion is mainly found in combination with a *GJB2* mutation, and the associated HI is assumed to be caused either by the deletion of a putative *GJB2* regulatory element or by digenic inheritance (12,33). Our two patients with the c.35delG mutation combined with the  $\Delta$ (*GJB6*-D13S1830) mutation showed more severe hearing impairment compared to the other patients, whereas the group of patients combining the c.35delG with the splice site mutation -3170G>A showed significantly less severe hearing loss. This result is consistent with the multicenter study of Snoeckx and coworkers (35). The frequency of the 342-kb deletion [ $\Delta$ (*GJB6*-D13S1830)] affecting *GJB6* varies tremendously between different populations. In our study, as well as in other studies of Polish and Austrian patients, the deletion was observed infrequently (36,37). In contrast, a multicenter study in nine countries revealed high mutation frequencies with the highest ones (32-72%) observed in France, Spain, Israel, and the UK (33). Furthermore, the frequency of a digenic cause for hearing impairment based on *GJB2*/[ $\Delta$ (*GJB6*-D13S1830)] mutations in North American patients varied between 16 and 20% (38-40). According to our results, mutations within the coding region of *GJB6* and the 342-kb deletion encompassing parts of *GJB6* seem not to be a common cause for hearing impairment in Hungarian patients.

***GJB3* sequence variants.** In several studies mutations within *GJB3* were described which resulted in HI (5,17,20,41). In a family with palmoplantar keratoderma and various forms of HI, Kelsell and coworkers were the first to detect the p.R32W mutation which occurred in combination with two missense mutations in *GJB2* (p.M34T and p.D66H) segregating with the skin disease (41). Since p.R32W affects a highly conserved residue, the authors supposed that the high frequency HI observed in their patients resulted from defective CX31/CX26 channel formation caused by additive mutations in both genes. In contrast, Lopes-Bigas and coworkers demonstrated that p.R32W in *GJB3* did not segregate with HI and/or skin disease in their Spanish patient cohort (42). They presented a mother of a patient who carried c.35delG (*GJB2*) in combination with p.R32W (*GJB3*) and showed normal hearing and absence of skin disease. The authors

presumed that p.R32W (*GJB3*) most likely represents a polymorphism with a frequency of 7.5% in the Spanish population. Rouan and coworkers subsequently showed that the intracellular coupling of HeLa cells transfected with R32W-CX31 was comparable to wild-type CX31, which is consistent with the view that R32W is a polymorphism of CX31 (43). In our study p.R32W was the only mutation that caused an amino acid exchange found in our *GJB2*-heterozygous patients (Table I). Only one patient, who was compound heterozygous for c.35delG and the splice site mutation -3170G>A in *GJB2*, displayed this mutation. Since 5.7% of the Hungarian control individuals analyzed also showed this DNA change, it most likely represents a common polymorphism.

In summary, approximately one fourth of all patients with one heterozygous *GJB2* mutation carried another mutation in the 5'UTR region of *GJB2* (splice site mutation -3170G>A). RFLP analysis as presented in this study provides an efficient tool to analyze patients with one heterozygous *GJB2* mutation at a relatively low cost. Therefore, we suggest the importance of the additional routine screening for the -3170G>A mutation in *GJB2*-heterozygous hearing impaired patients. Further mutations in the *GJB3* and *GJB6* genes are less frequently responsible for the hereditary hearing impairment in our patients.

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