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Prevalence of the IVS1(+1)G→A and 35delG mutations in the *GJB2* gene of Turkish patients with nonsyndromic hearing loss

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Abstract: *GJB2* encodes connexin 26, a gap junction protein that is assumed to be a component of the potassium recycling pathway in the inner ear. Loss or malfunction of these gap junctions, as might be reflected by mutations in *GJB2*, may disrupt potassium movement from the hair cells through the supporting cell network to the endolymph, leading to hearing impairment.

One mutation, the deletion of 1 guanosine residue from a stretch of 6 between nucleotide positions 30 and 35 (35delG) at codon 10, is the most common deafness-causing allelic variant of *GJB2* in sporadic patients and autosomal recessive families. Mutations in the *GJB2* gene represent the most common cause of autosomal recessive, sensorineural hearing loss. The 35delG mutation accounts for more than two-thirds of identified mutations. In this study, mutations, especially in the connexin 26 (*GJB2*), connexin 30 (*GJB6*), and 12srRNA genes, among 173 unrelated patients with prelingual nonsyndromic autosomal recessive deafness were screened and investigated by using the polymerase chain reaction-based restriction fragment length polymorphism (PCR-based RFLP), single-strand conformation polymorphism (SSCP), and sequence analysis methods. In patients with severe to profound hearing loss, 2 different mutations and 1 polymorphism (35delG and IVS1(+1)G→A mutations and V153I polymorphism) were found. The 35delG mutation was detected as the most common pathogenic allele among the Turkish patients and accounted for 50% of all mutant *GJB2* alleles. The 35delG and IVS1+1G→A mutations in the Cx26 gene were detected with total allele frequencies of 16.47% and 4.33%, respectively, and the V153 polymorphism was found in a heterozygous state at an allele frequency of 3.47%. However, the 342-kb deletion in the Cx30 gene and mitochondrial (mt)1555A→G in the 12srRNA gene mutations could not be detected among the studied patients.

Key words: *GJB2*, nonsyndromic hearing loss, IVS1(+1)G→A, 35delG, V153I

Sendromik olmayan işitme kayıplı Türk hastaların GJB2 genindeki IVS1(+1)G→A ve 35delG mutasyonlarının yaygınlığı

Özet: *GJB2*, iç kulaktaki potasyum geri dönüşüm yolunun bir bileşeni olarak varsayılan koneksin 26, ara bağlantı proteinini, kodlar. *GJB2* genindeki mutasyonlar tarafından ortaya çıkan bu ara bağlantıların kayıp veya hasarı, tüy hücrelerinden endolimfteki destek hücre bağlantısına doğru olan potasyum hareketini bozarak işitme kaybına yol açar. Kodon 10'da 30-35. nükleotit pozisyonları arasındaki 6 guanin nükleotit dizisinden bir guanine nükleotidinin delesyonu olan bir mutasyon, sporadik hastalar ve otozomal resesif ailelerde *GJB2*'nin alelik varyantlarına neden olan en yaygın sağırlık sebebidir. *GJB2* genindeki mutasyonlar otozomal resesif sensörinöral işitme kaybının en yaygın nedenlerinden biridir. 35 del G mutasyonu, tanımlanan mutasyonların 2/3 oranından daha fazla oranda yer alır. Bu çalışma kapsamında otozomal resesif, prelingual sendromik olmayan işitme kayıplı, birbirinden bağımsız 173 Türk hasta arasında özellikle connexin 26 (*GJB2*) connexin 30 (*GJB6*) ve 12srRNA genlerindeki yaygın mutasyonlar, Polimeraz Zincir Reaksiyonu Restriksiyon Parça Uzunluk Polimorfizmi (PZR-RFLP), SSCP ve dizi analiz yöntemleri ile araştırılmıştır. İki farklı mutasyon ve bir polimorfizm, (35delG, IVS1+(1)G→A mutasyonları ve V153I polimorfizmi) şiddetli ve ağır tipte

işitme kaybına sahip hastalarda bulunmuştur. 35delG mutasyonu Türk hastalarımız arasında en yaygın patojenik allel olarak tespit edilmiştir ve tüm mutant *GJB2* allellerin yaklaşık % 50'sinde yer almaktadır. Cx26 geninde 35delG ve IVS1+1G→A mutasyonları sırasıyla % 16,47 ve % 4,33 toplam allel frekansıyla tespit edilmiştir. Hastalarımız arasında V153 polimorfizmi % 3,47 allel frekansı ile heterozigot halde bulunmuştur. Diğer taraftan Cx30 genindeki 342-kb delesyon ve 12 srRNA genindeki mitokondriyal (mt)1555A→G mutasyonları çalışılan hastalar arasında bulunamamıştır.

Anahtar sözcükler: *GJB2*, sendromik olmayan işitme kaybı, IVS1(+1)G→A, 35delG, V153I

Introduction

Congenital and early childhood hearing loss of over 35 dB HL occurs in 1 out of every 1000 live births, and more than half of these cases are of hereditary origin (1). Approximately 70%-80% of genetic hearing loss is described as "nonsyndromic," as there are no other accompanying signs or symptoms to suggest a syndrome. To date, around 100 nonsyndromic hearing loss (NSHL) loci have been identified. Although the highest number of these loci and the corresponding genes are responsible for a dominant mode of inheritance, they constitute only 15%-20% of NSHL; the great majority of the NSHL cases (75%-80%) are inherited in an autosomal recessive fashion (1,2). Among the 37 known loci for autosomal recessive (AR) NSHL, DFNB1 mapping to chromosome 13q12 is by far the most prevalent. This locus contains 2 gap junction genes, *GJB2* and *GJB6*, and encoding gap junction proteins, connexin 26 and connexin 30, respectively (2). Mutations in *GJB2* are estimated to be responsible for more than 50% of ARNSHL. More than 100 different mutations of *GJB2* have been identified (Hereditary Hearing Loss Homepage at http://hereditaryhearingloss.org/). One particular mutation, 35delG, accounts for up to 70% of the pathological alleles in the European and American Caucasian populations, as well as in some other ethnic groups, with a carrier frequency ranging from 1.2% to 3.5% (1,3). It is associated with dominant and syndromic hearing loss, as well (4). Previous studies confirmed that 35delG is also a fairly common mutation among the Turkish hearing-impaired population (3,5-7).

In the present study, the *GJB2* gene of 111 adult Turkish patients between the ages of 19 and 28, with congenital or early childhood deafness, and 62 deaf children between the ages of 5 and 15 years from 2 schools for the deaf in Ankara, Turkey, were examined in order to determine the molecular pathology underlying deafness in these patients.

Materials and methods

Subjects

Our subjects consisted of 2 groups of hearing-impaired patients. The first group was selected from among the male military conscripts, or candidate conscripts, who were referred to the Mevki Military Hospital ENT Department (Ankara) for evaluation of fitness for obligatory military service. Of these, 111 consecutive patients between the ages of 19 and 28 with a bilateral prelingual hearing loss were included in the study. A routine otoscopic examination and tympanometry was carried out prior to pure tone audiometry in all patients in order to detect any otopathologies that might have caused or contributed to the existing hearing loss. Hearing levels were classified as mild (25-41 dB HL), moderate (41-56 dB HL), moderately severe (56-71 dB HL), severe (71-91 dB HL), and profound (over 91 dB HL) (8). The patients also met the criteria of having no symptoms or past medical history suggestive of intrauterine infection, no history of neonatal and early childhood infection or diseases that might have caused the hearing impairment, and no history of head trauma or cranial operation and no accompanying signs and symptoms which indicate a syndromic hearing loss.

The second group was made up of 62 hearing-impaired children, ranging in age from 5 to 15 years, from 2 schools for the deaf in Ankara, again in accordance with the above criteria. The hearing impairment level of the children was either severe or profound (over 71 dB HL).

For both groups, a thorough family history was taken and the patients or parents were questioned regarding the origin of the family, affected siblings or any other kin, any consanguineous marriage in the family, and any other inherited disease among the family members. Each of the 173 subjects was representative of 173 independent families.

DNA studies

Having acquired informed consent for the study from either the parents or the patients, DNA was extracted from peripheral blood according to standard procedures (9). Analysis of the common 35delG mutation was performed in all probands by polymerase chain reaction-based restriction fragment length polymorphism (PCR-based RFLP) using *Mva*I (or *Bst*NI) restriction endonuclease. Samples heterozygous or negative for the 35delG mutation were further screened for IVS1(+1)G→A, 167delT, and mitochondrial 1555A→G mutations by PCR-based RFLP using *Bsp*MI (or *Bfu*AI), *Pst*I, and *Alw*26I restriction endonucleases, respectively. The 342-kb deletion in the Cx30 gene was also screened in all subjects by a PCR-based assay as previously described (5). The 35delG and IVS1(+1)G→A mutations, determined by enzyme digestion, were also confirmed by the dideoxy chain-termination sequence reaction (10). The entire coding region of patients that were heterozygous or did not have any of the above mutations was analyzed by sequencing or single-strand conformation polymorphism (SSCP) to detect whether there was any other Cx26 gene mutation. For sequencing, the entire coding region of exon 2 was amplified with the primers Cx2F: 5'-GTGCATTCGTCTTTTCCAGAGCA-3' and Cx2R: 5'-TTGACA GCTGAGCACGGGTTG-3'. The resulting 794-bp PCR fragments were sequenced using appropriate overlapping primers to cover the entire exon 2 of the *GJB2* gene. For SSCP analysis (11), the coding exon was amplified using primers, giving 4 overlapping segments of about 250 bp in size. The resulting fragments were analyzed with 8% nondenaturing polyacrylamide gel electrophoresis (PAGE). The samples with a shifted band in the SSCP analysis were sequenced and a G→A alteration at nucleotide 457 (codon 153 Val→Ile) was found. This region was analyzed in all of the samples by SSCP. Since this mutation creates a cleavage site of the enzyme *Bsp*14071, all subjects with the same shifted band in SSCP were also confirmed by RFLP analysis using the *Bsp*1407I enzyme.

Results and discussion

A total of 34 cases in Group I and 18 cases in Group II were found to be mutant for 1 or 2 of the 3 Cx26 mutations tested, which were 35delG, IVS1(+1) G→A, and V153I. The sequence results of the 35delG, IVS1(+1)G→A, and V153I mutations are shown in Figures 1, 2, and 3, respectively, and the frequencies of these mutations among Turkish NSHL patients are given in Table 1.

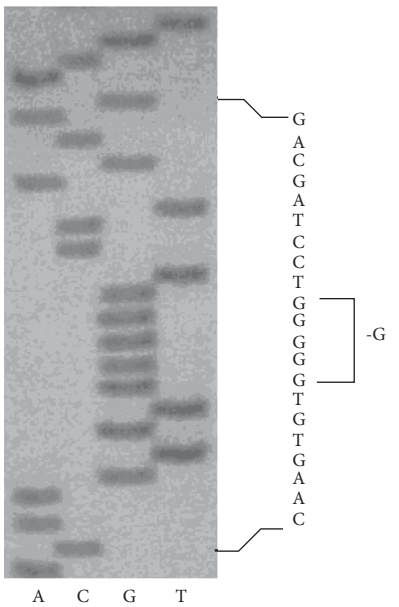

Figure 1. DNA sequence results of 35delG.

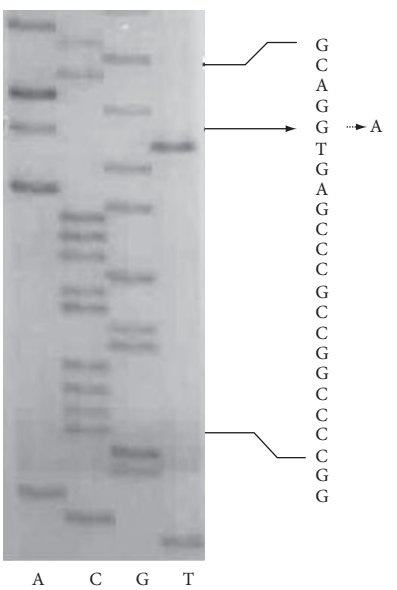

Figure 2. DNA sequence results of IVS1(+1)G→A.

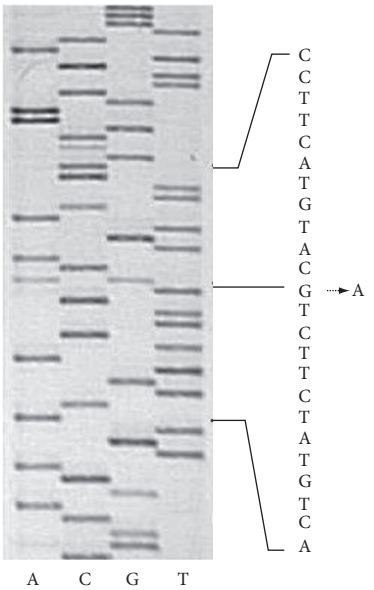

Figure 3. DNA sequence results of V153I mutation (nt 457 G→A).

The frequencies of these mutations in the study group were 15.76% in Group I and 17.74% in Group II for 35delG, 5.40% in Group I and 2.42% in Group II for IVS1(+1)G→A, and 2.25% in Group I and 0.81% in Group II for V153I. The allele frequencies of 35delG, IVS1(+1)G→A, and V153I in the *GJB2* gene were 47.90%, 12.60%, and 5.04%, respectively.

As shown in Table 2, 11 patients in Group I (9.91%) and 6 in Group II (9.68%) were found to be homozygous for the 35delG mutation. Only 3 patients (2.70%), all from Group I, were homozygous for IVS1(+1)G→A, and 2 of these 3 patients had severe to profound hearing loss. On the other hand, 4 patients in Group I (3.60%) were compound heterozygous for 35delG and IVS1(+1)G→A, and all but 1 of these 4 had milder hearing loss. In Group II, 2 patients (3.22%) were found to be compound heterozygous for 35delG and IVS1(+1)G→A.

Table 1. Allele frequencies of mutations and polymorphisms among Turkish patients with NSHL.

Group \ Mutant alleles	35delG	IVS1+1G>A	Val153I	342-kb del	mt1555 A>G	167delT
Group I	35/222 (15.76%)	12/222 (5.40%)	5/222 (2.25%)	0/222	0/222	0/222
Group II	22/124 (17.74 %)	3/124 (2.41%)	1/124 (0.80%)	0/124	0/124	0/124
Total	57/346 (16.47 %)	15/346 (4.33 %)	6/346 (1.73%)	0/346	0/346	0/346

Table 2. Genotype frequencies of patients.

Group \ Genotype	35delG/35delG	35del/IVS1(+1)G®A	35delG/?	IVS1(+1)G®A/IVS1(+1)G®A	IVS1(+1)G®/?	V153I/?
Group I	11/111 (9.91%)	4/111 (3.60%)	9/111 (8.11%)	3/111 (2.70%)	2/111 (1.80%)	5/111 (4.50%)
Group II	6/62 (9.68%)	2/62 (3.22%)	8/62 (12.90%)	0/62 (0.00%)	1/62 (1.61%)	1/62 (1.61%)
Total	17/173 (9.83%)	6/173 (3.47%)	17/173 (9.83%)	3/173 (1.73%)	3/173 (1.73%)	6/173 (3.47%)

Sequence and/or SSCP analysis of 11 cases (8 from Group I and 3 from Group II) that were negative for the above mutations and 19 cases (13 from Group I and 6 from Group II) that were heterozygote for 1 of these 3 mutations revealed that there were other mutations in exon 2 of the *GJB2* gene.

Although the complete coding region of the *GJB2* gene was sequenced in cases with a heterozygous 35delG, IVS1(+1)G→A, or V153I mutation, a mutation in the second allele could not be found. Therefore, these patients and the patients who did not carry any mutations in the *GJB2* gene were tested for the presence of the 342-kb deletion in the Cx30 gene and for the 1555A>G mutation in 12srRNA. These mutations were not found in any of these patients.

Since the first report by Kelsell et al. in 1997 (2), there has been a plethora of reports implicating mutations in the Cx26 gene as the major cause of hereditary nonsyndromic sensorineural deafness. The most prevalent of these mutations, 35delG, has also been identified at an allele frequency of 20%-30% in populations with ARNSHL in general, and 52%-90% in subjects with Cx26-related prelingual deafness in different studies conducted on European and American Caucasian populations (12-14). This percentage drops drastically in Middle Eastern, Asian, and African populations (15-18).

There have been several studies investigating the prevalence of *GJB2*- and 35delG-related deafness among Turkish hearing-impaired subjects, in which the allele frequency of 35delG was reported to account for 73% (3), 90% (5), and, in a smaller cohort, 64% (7) of mutations in the *GJB2* locus. Bayazıt et al. (2003), however, found 35delG in only 40% of the alleles of *GJB2*-related deafness in their Turkish subjects (6). In another study by Tekin et al., the distribution of 35delG was found at such a high heterogeneity throughout the country that the prevalence varied between 5% and 50% from region to region (19). We found that the 35delG mutation accounted for 47.1% of the mutations in the *GJB2* gene, and the frequency of 35delG was 16.47% among our study group as a whole. In previous studies, the homozygosity of the 35delG mutation was reported as 19% in Turkish patients with ARNSHL (20). This figure was 9.83% in our study groups. This difference may be due to the large clinical heterogeneity in our selected patients. Our subject families mostly originated from central, southern, southeastern, and northwestern Turkey. Although it was not statistically analyzed, we could not note any significant variability between regions in terms of 35delG prevalence, contrary to the report of Tekin et al. (19).

One interesting finding, as an important outcome of this study, is the existence of IVS1(+1)G→A, a splice-site mutation in the noncoding exon 1 of Cx26, among Turkish hearing-impaired subjects at a significant frequency. There are very few studies worldwide reporting the identification of this particular mutation, in which the allele frequency was found to be between 1% and 2.5% among Cx26 mutations (17). This mutation was not reported in Turkish patients with ARNSHL in previous studies. The frequency of 4.33%, when both of our groups are combined, is also the highest for IVS1(+1)G→A in the literature.

The V153I mutation has been alternatively reported as either a polymorphism (6,21) or a recessive pathogenic allele (22,23). Meşe et al., in 2004, demonstrated that the V153I mutation was unable to form functional channels in the paired *Xenopus* oocyte expression system; their study thus supported the view that V153I could be pathogenic (24). This mutation was found in 5 patients in a heterozygous state at a frequency of 4.91% in our study group. When we screened this mutation in 90 individuals who had no hearing impairment, we found that 6 individuals (6.6%) were carrying this mutation in a heterozygous state. These results support previous reports that this alteration is a polymorphism rather than a mutation.

In summary, 35delG is the most common cause of nonsyndromic genetic hearing loss among Turkish deaf people. The phenotypes of Cx26-related deafness are highly variable; however, according to hearing threshold levels, 35delG mostly leads to moderately severe or profound hearing loss, not only in monogenic but also in digenic forms. Our results also revealed that the IVS1(+1)G→A mutation is found with a considerably high prevalence among Turkish patients who are hearing impaired. Sequence and SSCP analysis of the *GJB2* gene in a total of 30 cases revealed that 34.45% of the *GJB2* alleles did not carry any mutation in the *GJB2* gene. Therefore, there must be another gene responsible for deafness in the Turkish population.

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