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Original Article

The Analysis of *GJB2, GJB3*, and *GJB6* Gene Mutations in Patients with Hereditary Non-Syndromic Hearing Loss Living in Sivas

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OBJECTIVES: The aim of the present study was to investigate the presence of GJB2, GJB3, and GJB6 gene mutations in non-syndromic sensorineural hearing loss (NSHL) cases living in Sivas region, to provide appropriate genetic counseling for cases who were found to have mutation, and to contribute to decrease the frequency of mutant allele in the next generation and plan treatment and rehabilitation with early diagnosis.

MATERIALS and METHODS: The study included 53 unrelated cases that were diagnosed with congenital NSHL between June 2009 and March 2010. Multiplex ligation-dependent probe amplification method was used for genotyping of GJB2, GJB3, and GJB6 gene mutations.

RESULTS: Heterozygous 35delG variant was determined in 1.9% (n=1) of cases, homozygous 35delG in 15.1% (n=8), heterozygous IVS1+1G>A mutation in 1.9% (n=1), compound heterozygous in 3.8% (n=2), and homozygous IVS1+1G>A variant in 3.8% (n=2). None of the cases had mutation in GJB3 and GJB6 genes. Mutated allele frequencies in the present study were found to be 17.9% for 35delG and 6.6% for IVS1+1G>A.

CONCLUSION: The present study showed that 35delG mutation is the most common variant in the Sivas region, and that IVS1+1G>A mutation should be investigated in hearing loss. Another result of the present study was that genetic analyzes would allow early diagnosis of hearing impairments particularly when infants whose parents have consanguinity do not pass the newborn hearing screening.

KEYWORDS: Non-syndromic hearing loss, GJB2, 35delG, IVS1+1G>A, mutation

INTRODUCTION

Hearing loss is the most common among sensory disorders in humans. There are differences between communities and the permanent bilateral sensorineural hearing loss >40 dB is seen in 1 in 500 live births. The prevalence of hearing loss increases with age; while the rate is 2.7/1000 before 5 years old, it is 3.5/1000 in the adolescence period [1].

Almost half of the hearing disorders occur due to environmental reasons, and the remaining half are caused by genetic reasons. While 70% of genetic causes are composed of non-syndromic hearing loss, 30% are syndromic [2]. Approximately 80% of cases with prelingual hearing loss have autosomal recessive, 20% have autosomal dominant, and <1% have X-linked or mitochondrial inheritance [3]. Most of the non-syndromic hearing disorders correspond to autosomal recessive inheritance [2]. In the non-syndromic type, hearing loss occurs without any systemic signs and/or symptoms [4].

Studies on the genetics of hearing loss have described many genes that may play a role in its etiology. Among these genes, the *GJB2 (Cx26)* mutations are known to be the most frequent cause of autosomal recessive non-syndromic sensorineural hearing loss (NSHL) worldwide; *GJB6 (Cx30)* and *GJB3 (Cx31)* gene mutations are less common [5, 6]. The 35delG mutation found in *GJB2 gene*

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shows distribution by geographical and ethnic origin. Among the mutations in this gene, 35delG is the most common variant in European countries, Mediterranean countries, and Turkey [5, 7-9].

Hearing impairment negatively affects the mental, cognitive, behavioral, language, and social development of the individual [10]. Genetic screening in families at risk has importance for both early diagnosis and treatment of hearing loss. Initiation of rehabilitation and treatment by early diagnosis is known to have positive effects on language and speech skills of cases with *GJB2* mutation [11, 12].

The aim of the present study was to investigate the presence of *GJB2*, *GJB3*, and *GJB6* mutations in cases with NSHL living in Sivas region, to provide appropriate genetic counseling, to contribute to decrease the frequency of mutant allele in the next generation, and to plan treatment and rehabilitation.

MATERIALS AND METHODS

Study Population

The present study included 53 unrelated cases that applied to the Sivas University Faculty of Medicine Training & Research Hospital, Otorhinolaryngology and Medical Genetics Outpatient Clinic due to a complaint of hearing loss between June 2009 and March 2010 and were diagnosed with congenital NSHL after history, physical examination, and audiological examination.

Medical history of cases; family history; history of parental consanguinity; prematurity; mode of delivery; infection during the prenatal, perinatal, and postnatal periods; trauma; use of an ototoxic drug; radiation exposure; and operation history were questioned. After obtaining a detailed medical history from all of the cases, examinations were performed by the same otorhinolaryngologist (EEA) and the same medical geneticist (HKK).

Inclusion criteria were cases with prelingual, bilateral, sensorineural, and non-syndromic hearing loss. Exclusion criteria were as follows: (1) history of ear surgery, (2) acute and chronic infection, (3) malignancy, (4) trauma, (5) ototoxic drug use, chemotherapy, or radiotherapy administration, and (6) asymmetrical, unilateral, or syndromic hearing loss.

The study was approved by the Sivas Cumhuriyet University Clinical Trials Ethics Committee (decision no.: 09/171; date: February 06, 2009). Detailed consent was obtained from all individuals to be included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Audiological Evaluation

All cases underwent audiological evaluation using an Interacoustics Clinic Audiometry brand, AC-40 model device (Interacoustics, Assen, Denmark). All audiological tests were performed by masking the other ear. Audiological examination evaluated pure tone thresholds for air conduction at 250 Hz-6 kHz and for bone conduction at 500 Hz-6 kHz and classified according to the indicator chart provided in the Katz Handbook of Clinical Audiology book [13]. Cases with a pure tone hearing threshold of 0-20 dB were classified as normal hearing, 21-40 dB as mild hearing loss, 41-60 dB as moderate hearing loss, 61-80 dB as severe hearing loss, 81-100 dB as profound hearing loss, and >100 dB as total hearing loss. In the pediatric age group who could not adapt to the hearing evaluation using pure tone audiometry, hearing loss was evaluated initially using transient otoacoustic emission (TEOAE) (ERO Scan Analyzer; Maico GmbH Salzufer, Berlin, Germany). If the TEOAE response "refer result was obtained, the test was repeated. Moreover, infants who failed the TEOAE test once again that were examined by the auditory brainstem response (ABR) (MEB-9100; Nihon Kohden Co., Tokyo, Japan) method. Diagnosis of hearing loss was confirmed with the results of ABR.

Collection of Blood Samples and DNA Extraction

Genomic DNA was isolated from collected blood by using Invisorb Spin Blood DNA Isolation Kit (Invitek, Berlin, Germany).

Multiplex Ligation-Dependent Probe Amplification (MLPA) Reaction

MLPA (P163B1 GJB-WFS1 lot 0208; MRC-Holland, Amsterdam, The Netherlands) hearing loss kit was utilized to investigate *GJB2*, *GJB3*, and *GJB6* gene mutations in patients with NSHL. The kit contains a total of 41 probes; 17 of them are for detecting mutations in the *GJB2*, *GJB3*, and *GJB6* genes. Three of the GJB2 probes are specific for point mutations (35delG, IVS1+1G>A, and 313del14).

DNA denaturation and hybridisation of MLPA probes

DNA sample was diluted to a volume of 5 µL at a range of 20-500 ng using Tris-EDTA buffer and cooled at 25°**C** following denaturation in thermal cycler (2720; Applied Biosystems, Foster City, CA, USA) at 98°**C** for 5 min. A 1.5 µL SALSA Probe mix and a 1.5 µL MLPA buffer were added in every tube and incubated at 95°**C** for 1 min and then at 60°**C** for 16 h.

Ligation reaction

For ligase reaction, every tube was incubated at 54°**C** for 15 min adding 32 µL ligase-65 mix, and then ligase inactivation was ensured by maintaining at 98°**C** for 5 min.

Polymerase Chain Reaction

Four µL PCR buffer, 26 µL water, and 10 µL MLPA ligation reaction product were added and mixed within new tubes. Then, while tubes were in the PCR device at 60°**C**, 10 µL polymerase mix containing 2 µL MLPA PCR primer, 2 µL enzyme dilution buffer, 5.5 µL water, and 0.5 µL polymerase were added, and PCR reaction was initiated immediately. PCR conditions were adjusted as follows: 35 cycles for 30 s at 95°C, 30 s at 60°C, 60 s at 72°C, and then 20 s at 72°C.

Fragment Analysis

Following PCR, 2 µL MLPA product, 0.5 µL LIZ size standard, and 20 µL formamide were mixed and loaded in the ABI Prism 310 Genetic Analyzer (ABI 310; Applied Biosystems, Foster City, CA, USA). In each experiment, four negative controls were studied together with patient samples. The resultant data were analyzed both visually and by using Coffalyser V9 program (MRC-Holland, Amsterdam, The Netherlands).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) for Windows version 14.0 was used for data analysis. Chi-square test was utilized for evaluation of data. The level of significance was accepted as 0.05. A $p<0.05$ was accepted as significant.

Table 1. Distribution of *GJB2* gene mutations with regard to gender

	Gender	
***GJB2* gene mutations**	**Male % (n)**	**Female % (n)**
No mutation	72.4 (21)	75.0 (18)
35delG/–	3.4 (1)	0.0 (0)
35delG/35delG	10.3 (3)	20.8 (5)
35delG/IVS1+1G>A	6.9 (2)	0.0 (0)
IVS1+1G>A /–	3.4 (1)	0.0 (0)
IVS1+1G>A/IVS1+1G>A	3.4 (1)	4.2 (1)
Total	100 (29)	100 (24)

Table 2. Distribution of *GJB2* gene mutations in 32 cases with profound hearing loss

***GJB2* gene mutations**	**Profound hearing loss % (n)**
35delG/–	3.1 (1)
35delG/35delG	25 (8)
35delG/ IVS1+1G>A	6.3 (2)
IVS1+1G>A /–	3.1 (1)
IVS1+1G>A/ IVS1+1G>A	6.3 (2)
Total	43.7 (14)

Table 3. Distribution of mutations with regard to consanguinity

	Parental consanguinity	
***GJB2* gene mutations**	**Yes % (n)**	**No % (n)**
No mutation	24.5 (13)	49 (26)
35delG/–	0 (0)	1.9 (1)
35delG/35delG	9.4 (5)	5.7 (3)
35delG/ IVS1+1G>A	0 (0)	3.8 (2)
IVS1+1G>A /–	0 (0)	1.9 (1)
IVS1+1G>A/ IVS1+1G>A	3.8 (2)	0 (0)
Total	37.7 (20)	62.3 (33)

Table 4. Distribution of mutations of the cases with regard to family history

	Hearing loss in relatives	
***GJB2* gene mutations**	**Yes % (n)**	**No % (n)**
No mutation	32.1 (17)	41.5 (22)
35delG/–	0 (0)	1.9 (1)
35delG/35delG	7.5 (4)	7.5 (4)
35delG/IVS1+1G>A	3.8 (2)	0 (0)
IVS1+1G>A /–	1.9 (1)	0 (0)
IVS1+1G>A/IVS1+1G>A	3.8 (2)	0 (0)
Total	49.1 (26)	50.9 (27)

RESULTS

Among 53 non-syndromic cases with NSHL, 45.3% (n=24) were female, and 54.7% (n=29) were male. The average age of the cases was 29.45±14.06 (min-max: 2-66) years.

While no mutation was detected in 73.6% (n=39) of the cases, 26.4% (n=14) had a homozygous or heterozygous mutation. Heterozygous 35delG mutation was determined in 1.9% (n=1) of cases, homozygous 35delG in 15.1% (n=8), heterozygous IVS1+1G>A mutation in 1.9% (n=1), compound heterozygous in 3.8% (n=2), and homozygous IVS1+1G>A mutation in 3.8% (n=2). None of the cases had a mutation in *GJB3* and *GJB6* genes. In the present study, mutant allele frequencies were 17.9% for 35delG and 6.6% for IVS1+1G>A.

The distribution of gene mutations examined based on genders was summarized in Table 1. On the other hand, there was no statistically significant difference between gender and the presence of *GJB2* mutation ($p>0.005$).

When the cases were classified based on the degree of hearing loss, it was determined that 60.4% (n=32) had profound hearing loss, 20.8% (n=11) had severe hearing loss, 13.2% (n=7) had moderate–severe hearing loss, and 5.7% (n=3) had moderate hearing loss. A total of 21 cases with moderate, moderate–severe, and severe hearing loss did not have a mutation; *GJB2* gene mutations were determined in 43.7% (n=14) of 32 cases with profound hearing loss. Table 2 summarizes the distribution of *GJB2* gene mutations determined in cases with profound hearing loss.

While 37.7% (n=20) of the parents were consanguineous, 62.3% (n=33) did not have a kinship. The distributions of the mutations detected in the cases according to their kinship status are shown in Table 3.

Homozygous mutation was observed in 35.0% (n=7) of 20 cases with parental kinship. In 78.79% (n=26) of cases without parental consanguinity, a mutation was not detected. Comparison of kinship and mutations were found to be significant for homozygous *GJB2* mutations in cases with parental consanguinity ($p=0.035$ and $p<0.05$).

While the most frequent type of mutation was 35delG (n=4) in cases with parental kinship, it was 35delG (n=4) in cases with no hearing loss in relatives. The difference was insignificant as the correlation between hearing loss history in the family and mutation was evaluated statistically ($p>0.05$).

When hearing loss history of the relatives was evaluated, it was found that there was a positive family history in 49.1% (n=26). Table 4 shows the distribution of mutations identified in cases by the presence or absence of family history considering hearing loss.

The cases in the study were asked about the age at which they were diagnosed with hearing loss; it was observed that 62.3% (n=33) of the cases were diagnosed within the first 12 months, 20.8% (n=11) at 1-2 years old, 5.7% (n=3) at 2-3 years old, and 11.3% (n=6) after 3 years old.

Based on the age at which hearing loss was noticed by the family, the rate of cases with mutation who were <1 year old was 22.6% (n=12), and the rate of cases without mutation was 39.6% (n=21). On the other hand, the rate was 3.8% (n=2) in cases with mutation who were >1 year old, and 34% (n=18) in cases without mutation.

The difference was found to be statistically significant with regard to the presence and absence of mutation before and after 1 year old based on the ages at which hearing loss was noticed ($p=0.0348$ and $p<0.05$).

DISCUSSION

While planning the present study, its aim was to investigate the presence and frequency of *GJB2*, *GJB3*, and *GJB6* gene mutations in cases diagnosed with NSHL in Sivas. Another goal of the present study was to contribute to the reduction of mutated allele frequency in next generations by providing appropriate genetic counseling to mutated cases and planning treatment-rehabilitations with early diagnosis.

GJB2 gene mutations are the most frequent cause of NSHL in many countries [5, 14-18]. As we mentioned previously, both prevalence of *GJB2* mutation and types of mutation vary ethnically and geographically. The most frequently observed GJB2 variant is 35delG in Caucasians and 235delC in Far Eastern [19]. The frequency of 35delG varies regionally in India. The most common variants are W24X in South India and 35delG in North India [20-23]. In a recent study by Zytzar et al. [24], the carrier frequency of 35delG was investigated in Western Siberian Russians, and the literature data from some former Soviet Union countries were also evaluated. In their study, the carrier frequencies of 35delG in the north-west and central parts of Russia were found to be high. In addition, 35delG carrier frequency was determined as 0%-3.6% in Turkic populations in Volga-Ural, Siberia, and Central Asia. The most common GJB2 variant in Turkey is 35delG. The carrier frequencies were calculated as 1.17% and 1.78% in two different studies [25, 26]. Tekin et al. [27] reported that the allele frequency of 35delG is 5%-53% in Turkish NSHL cases and is found to be the highest in Ankara. Their study included two cases from Sivas, and no variants were detected in either. In other studies performed in Turkey, the frequency of homozygous and heterozygous 35delG variants in cases with NSHL ranges between 3.9%-21.7% and 2.1%-7.8%, respectively [25, 28-32]. In our study, the most common variant in the *GJB2* is 35delG and shows similarities with the results of other studies from Turkey.

Another result determined in the present study was the presence of IVS1+1G>A, which is a splice site mutation found in exon 1 and intron one border of *GJB2* gene of patients with hearing loss. We determined heterozygous cases at a rate of 1.9% (n=1), homozygous cases at a rate of 3.8% (n=2), and compound heterozygous cases at a rate of 3.8% (n=2) for IVS1+1G>A mutation. Allele frequency was found to be 6.6% for this mutation. The presence of IVS1+1G>A mutation has been also indicated in different population studies of the literature [33-35]. The mutation, which was revealed by Denoyelle et al. [37] in 1999 for the first time, was determined to be compound heterozygous by Shahin et al. [33] and allele frequency was determined as 1%. In a series by RamShankar et al. [20], in 2003 including 215 Indian cases, it was found that IVS1+1G>A mutation was the compound heterozygous only in one case, and allele frequency was 0.2%. A study conducted in Mongolia revealed that the allele frequency of the gene was 3.5% [38]. Seeman et al. [36], in their study conducted in Czech Republic, indicated that IVS1+1G>A mutation generates 4% of pathogenic mutations in *GJB2* gene, and that it is the third most frequent pathogenic mutation. In their study, Barashkov et al. [39], discussed the molecular, audiological, and population characteristics of autosomal recessive hearing loss associated with donor attachment site of IVS1+1G>A mutation of *GJB2* gene in the Republic of Sakha (Yakut). They found that the carrier frequency of IVS1+1G>A mutation is 11.7% in cases diagnosed with neonatal hearing loss. This result is the highest ratio reported worldwide, and they highlighted that it could be resulted from Founder mutation. In the Turkish population, Sirmaci et al. [40] found IVS1+1G>A mutation in 8 of 16 cases with a known heterozygous mutation in exon 2. In addition, Subaşıoğlu et al. [41] detected c.IVS1+1G>A/35delG mutation in 1 of 21 families from central Anatolia. In a study conducted in the Çukurova region of southern Turkey, Bozdoğan et al. [28] found that IVS1+1G>A mutation is homozygous only in 1 of 77 cases, and that allele frequency is 1.4%. There are very different results in studies on the frequency of IVS1+1G>A mutation we mentioned above. We think that it would not be convenient to report a definite statement about the origin of genetic mechanisms underlying this difference. However, it can be associated with kin marriage, race, and ethnicity. Thus, Tekin et al. [38], noted in their study that this mutation can spread to the Middle East and then globally through migrations starting from Central Asia. The results of the present study revealed that the prevalence of mutation had a high rate (6.6%), and we considered that it was a result of migrations from Central Asia to Turkey throughout history.

Newborn hearing screening program has revolutionized the mean diagnostic age of hearing loss worldwide. However, it also should be remembered that cases with prelingual hearing loss can pass newborn hearing screening in certain circumstances even though they have hearing loss. In 2013, Minami et al. [42], examined the correlation between *GJB2* and hearing loss in a series of 14 cases diagnosed with hearing loss even though they passed newborn hearing screening. Their results revealed that the frequency of passing from newborn hearing screening was at least 6.9% in cases with genetic hearing loss related to *GJB2*. However, they also emphasized in their study that this rate would be higher when it was possible to screen all newborns with regard to hearing loss related to this mutation. When considering only the cases born after 2000 when the newborn hearing screening program was initiated in Japan, their study included the result that 8.9% of cases with hearing loss induced by *GJB2* mutation passed newborn hearing screening. One of the results obtained from the present study was that the age of our cases to be diagnosed with hearing loss was most frequently the first 12-month period with 62.3% (n=33), and that mutation was observed in 22.6% of these cases and only 3.8% (n=2) of the cases >1 year old had mutation, and this difference was statistically significant. In addition, when the correlation between detection of mutation and diagnostic age of hearing loss was evaluated, cases <1 year old were determined to have more hearing loss associated with mutation. This result allowed us to highlight the important contribution of the newborn hearing screening program, which was initiated in maternity hospitals by a protocol signed in 2000 in Turkey and applied in all public and university hospitals in time, for the community [43]. As it was also stated in the study by Minami et al. [42], it should be remembered that infants without hearing loss could have *GJB2* mutation. We think that through

an extensive study, it is needed to research the necessity of this genetic screening in routine by making economic cost analyzes in all newborns since we are a population where this mutation is prevalent. Therefore, it is possible to immediately start rehabilitation and treatment of infants diagnosed with hearing loss in the early period. Accordingly, it will allow eliminating a major economic burden for the community by allowing to raise individuals with less linguistic, psychological, and motor disabilities.

The present study did not reveal a statistically significant correlation between parental consanguinity and genetic mutations. However, the homozygous *GJB2* mutation was remarkable in cases born from a consanguineous marriage. This finding was another significant result of our study that consanguineous marriage is an important etiological factor for genetic hearing loss.

Studies in the literature evaluating the frequently of hearing loss in pediatric cases with *GJB2* 35delG homozygous mutation showed that there might be a correlation between severe and profound NSHL [44, 45]. In their study, Smith et al. [46], reported that 60% of children are likely to have deafness from severe to profound degree in the presence of 35delG compound heterozygosity, as well as any allele in *GJB2* gene, leading to hearing loss. Cryns et al. [47], reported that hearing loss is more severe in patients with homozygous 35delG mutation, whereas it is milder in those with compound heterozygous 35delG/IVS1+1G>A mutation. On the contrary, the literature also includes publications indicating that the degree of hearing loss varies in cases with homozygous 35delG mutation [37, 48-50]. When the cases were evaluated for degree of hearing loss also in the present study, profound hearing loss was determined to be the most frequent with 60.4% (n=32); *GJB2* gene mutations were identified in 43.7% (n=14) of cases with profound hearing loss, whereas there was no mutation in a total of 21 cases with moderate, moderate–severe, and severe hearing loss.

CONCLUSION

As can be seen from results of both the present study and the above-mentioned studies, we concluded that IVS1+1G>A gene mutation was likely to occur as founder in the Yakut population of Eastern Siberia and to spread worldwide by migrations throughout history. It will be possible to prove this hypothesis accurately only by a study designed based on ethnicity and migration maps and involving multicentered and wide patient populations.

Another important result of the present study was that genetic analyzes would allow the detection of the cause of hearing impairments particularly when infants whose parents have consanguinity do not pass newborn hearing screening. However, as in the results of similar studies in the literature, we would like to attract attention to the importance of genetically and re-audiologically evaluating newborns that are not diagnosed with hearing loss but have deafness history in their relatives and parental consanguinity.

Ethics Committee Approval: Ethics committee approval was received for this study from the Sivas Cumhuriyet University Clinical Trials Ethics Committee (decision no.: 09/171; date: February 06, 2009).

Informed Consent: Informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – H.K.K., Ö.Ö., I.S.; Design - H.K.K., Ö.Ö., I.S.; Supervision - H.K.K., E.E.A, I.S.; Resource - H.K.K., M.E.Y.; Materials - H.K.K., M.E.Y., E.E.A.; Data Collection and/or Processing - H.K.K., M.E.Y., B.B.; Analysis and/or Interpretation - H.K.K., E.E.A., M.E.Y., I.S.; Literature Search - H.K.K., E.E.A.; Writing - H.K.K., E.E.A.; Critical Reviews - E.E.A., I.S.

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