RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1011501
İst Tıp Fak Derg 2022 / J Ist Faculty Med 2022

GJB2-RELATED NON-SYNDROMIC HEARING LOSS VARIANTS' SPECTRUM AND THEIR FREQUENCY IN TURKISH POPULATION

GJB2 İLİŞKİLİ NON-SENDROMİK İŞİTME KAYBI VARYANTLARININ SPEKTRUMU VE TÜRK TOPLUMUNDAKİ SIKLIKLARI

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Cite this article as: Gulec C, Aslanger AD, Karaman V, Wollnik B, Tepgec F, Kayserili H, et al. *GJB2*-related non-syndromic Hearing loss variants' spectrum and their frequency in Turkish population. J Ist Faculty Med 2022;85(2):162-9. doi: 10.26650/IUITFD.1011501

ABSTRACT

Objective: Hearing loss (HL) is one of the most prevalent chronic conditions in children and has consequences in speech, language, education, and social functioning which impede the quality of life. Due to the major involvement of the genetic factors in HL, especially non-syndromic HL (NSHL), genetic diagnosis and genetic counseling have a major impact on early management of the affected individuals and their families. Herein, we report the *GJB2* gene variants and their frequencies in NSHL cohort at a tertiary health center between 2002-2021 to contribute for the future genetic counseling of Turkish NSHL patients.

Materials and Methods: Two exons of the *GJB2* gene were amplified in 402 NSHL patients by two separate PCR reactions and sequenced using the Sanger technique.

Results: We found 13 different *GJB2* variants in 35% (141/402) of the patients with NSHL. 53.9% were homozygous and 33.3% were compound heterozygous for the most common (59.21%) variant, c.35delG. Approximately 13% of the patients were found to carry the variants in the heterozygous state. The most frequent *GJB2* variant c.35delG was followed by c.71G>A (6.38%), c.-23+1G>A (3.54%) and c.233delG (2.48%). We found heterozygous p.Asp50Glu (c.150C>A) alteration in four of eight patients with keratitis, ichthyosis, deafness (KID) and palmoplantar keratoderma (PPK) syndrome.

Conclusion: Our results further emphasize the well-known prevalance of the *GJB2* c.35delG alteration being the most pre-

ÖZET

Amaç: İşitme kaybı, çocukluk çağındaki en önemli kronik sağlık sorunlarından biridir ve yaşam kalitesini konuşma, eğitim ve sosyal ilişki sorunlarına yol açarak azaltır. Özellikle non-sendromik işitme kaybında genetik faktörlerin rolü etkilenmiş kişi ve ailelerinin genetik tanı ve genetik danışma aşamalarında doğru yönlendirilmesi açısından kilit bir rol oynar. Bu nedenle, non-sendromik işitme kaybı olan hasta ve ailelerinin önümüzdeki yıllarda genetik tanı ve danışmasına katkıda bulunmak amacıyla, bu çalışmada, 2002-2021 yılları arasında sinirsel tip işitme kaybı tanısı alan hastalardaki *GJB2* gen varyantlarını ve sıklıklarını sunmaya çalıştık.

Gereç ve Yöntem: *GJB2* geninin iki ekzonu, 402 hasta DNA'sında iki ayrı PCR ile çoğaltıldı ve Sanger yöntemi ile dizilendi.

Bulgular: Non-sendromik işitme kaybı olan olguların %35'inde (141/402) *GJB2* geninde 13 farklı değişim saptadık. Hastaların %53,9'u en yaygın (%59,21) varyant olan c.35delG değişimini homozigot taşırken, %33,3'ü birleşik heterozigot olarak taşıyordu. Yaklaşık %13'ünde ise değişim heterozigot olarak belirlendi. Çalışma grubumuzda en yaygın *GJB2* varyantı olan c.35delG değişimini sırasıyla c.71G>A (%6,38), c.-23+1G>A (%3,54) ve c.233delG (%2,48) değişimleri izlemiştir. Keratit-ihtiyoz-sağırlık (KID) ve palmoplantar keratoderma (PPK) sendromu tanılı sekiz hastanın dördünde heterozigot p.Asp50Glu (c.150C>A) değişimi saptandı.

Sonuç: Sonuçlarımız, Türkiye'deki non-sendromik işitme kaybı hastalarındaki c.35delG varyantının uzun zamandır bilinen baskınlığını bir kez daha göstermektedir. Ayrıca, tek mutant alel

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Submitted/Başvuru: 19.10.2021 • **Revision Requested/Revizyon Talebi:** 15.11.2021 •
Last Revision Received/Son Revizyon: 25.11.2021 • **Accepted/Kabul:** 13.01.2022 • **Published Online/Online Yayın:** 16.02.2022

dominant variant in the Turkish NSHL patients. The high rate of mono-allelic state could be considered as coincidental due to high allelic heterogeneity of NSHL, or possibly suggestive for digenic inheritance.

Keywords: Sensorineural hearing loss, *GJB2* gene, c.35delG alteration, mutation frequency

saptanan hastaların oranı, non-sendromik işitme kaybının alelik heterojenitesi nedeniyle rastlantısal olarak değerlendirilebileceği gibi, digenik kalıtımı da düşündürebilir.

Anahtar Kelimeler: Sinirsel tip işitme kaybı, *GJB2* geni, c.35delG değişimi, mutasyon sıklığı

INTRODUCTION

Hearing loss (HL) is one of the important health problems with social and psychological outcomes. Though both environmental and genetic factors are involved in the etiology of HL, hereditary factors are responsible for more than 70% of the cases. HL is classified as syndromic and non-syndromic, depending on the presence or the absence of accompanying findings, respectively. Non-syndromic HL (NSHL), which constitutes most of the cases (70-80%), is further subdivided by mode of inheritance as autosomal recessive, autosomal dominant, X-linked, and mitochondrial HL. Autosomal recessive NSHL (ARNSHL) constitutes 80% of NSHL (1). Bi-allelic variants in any one of the identified over 100 genes are known to cause ARNSHL. This considerable number of genes responsible for hearing loss shows the hearing mechanism's complex structure which involves channel proteins, integral membrane protein, adhesion molecules, enzymes and extracellular matrix components (2). However, the most frequent mutations causative for ARNSHL are found in the *GJB2* gene.

The *GJB2* (*CX26*, GenBank M86849, OMIM: *121011) gene encodes connexin protein providing a chemical connection of the cell with neighboring cells or extracellular space. The main task of GJB2 in the hearing system is to modulate the potassium ion recycling which is required for the action potentials of hair cells in the organ of Corti (3-6). Generally, loss-of-function mutations of the *GJB2* gene cause autosomal recessive non-syndromic hearing loss (ARNSHL). In contrast, gain-of-function mutations of the *GJB2* gene are responsible for the autosomal dominant keratitis, ichthyosis, deafness (KID) or palmoplantar keratoderma (PPK) syndrome, characterized by extensive hyperkeratotic lesions in the skin, keratitis leading to a loss of visual acuity and profound progressive deafness (7).

Diversity in the clinical manifestations of *GJB2* variants can mainly explain the genotype-phenotype correlation. The nonsense mutations causing early truncation and missense mutations preventing the formation of gap junctions may be responsible for profound hearing loss. Some other particular alterations do not impair the formation of functional gap junctions but reduce conductance levels and alter gating properties, causing mild or moderate hearing loss (8-10). On the other hand, *GJB2* mutations like p.Asp50Glu (c.150C>A), p.Gly12Arg (c.34G>C), p.Ala40Val (c.119C>T), and p.Gly45Glu (c.134G>A) which are associated with KID/PPK do not seem to impair the gap junction formation, but they mildly affect gap junction channel properties (11, 12).

ARNSHL's prevalence depends on the frequency of carriers in the population. Knowing the frequency and types of mutation in a population allows early therapeutic intervention. As a result of recent developments in genetic technology, the diagnostic approach to patients with hearing loss now includes Whole Exome Sequencing (WES) after scanning of the *GJB2* gene. Thus, for the appropriate implementation of the *GJB2* gene scanning in a population, the variant frequency data may need to be updated. In this regard, we aimed to investigate the frequency and spectrum of *GJB2* gene variants in our NSHL patients diagnosed with *GJB2*-related disease (ARNSHL).

MATERIALS AND METHODS

The bilateral hearing-loss patients with clinical findings that suggest a possible traumatic or infectious causative, and unilateral hearing-loss patients were excluded from the study. The patients with a syndromic form of hearing loss, except for those with KID or PPK, were also excluded.

Peripheral blood samples of 2 ml were collected upon approval of the patients and families for genetic testing. DNA isolations were performed by using commercial kits according to the instructions (Mammalian Blood and Cells and Tissue DNA Isolation Kit, Roche). Two exons of the *GJB2* gene were amplified by three separate PCR reactions using specific primers (Table 1). To perform an efficient sequencing, the second exon of the *GJB2* gene, which has a relatively large size (681 bp), was amplified and sequenced with two separate PCRs. All PCR reactions were carried out with 2.5 mM $MgCl_2$, 0.2 µM of each primers, 0.2 µM of each dNTP, 1U *Taq* DNA polymerase (Thermo Fisher Scientific) and 100 ng genomic DNA. Purification of the PCR products was performed with Exonuclease and Alkaline Phosphatase enzymes (Thermo Fisher Scientific). Sanger sequencing reactions were carried out with an automated sequencer (ABI 3500). An analysis of sequencing data was performed with the SeqScape software (SeqScape v3.0) using the *GJB2* reference requence (NM_004004.6) fetched from the USCS Genome Browser (https://genome-euro.ucsc.edu/). The study was approved by the Ethics Committee of Istanbul University, Istanbul Faculty of Medicine (Date: 22.01.2021, No: 108).

Table 1: Primers used to amplify non-coding and coding two exons of *GJB2* gene

Primer	Exon	Sequence	Lenghth (bp)	Tm (°C)	Expected PCR product size (bp)
GJB2_F1	1	5′-GTGCGGTTAAAAGGCGCCA-3′	19	66.4	265
GJB2_R1		5′-GGCAACCGCTCTGGGTCT-3′	18	63.8	
GJB2_F2-I	2	5′-CTCCCTGTTCTGTCCTAGCT-3′	20	56.2	840
GJB2_R2_I		5′-GACTGAGCCTTGACAGCTGA-3′	20	59.3	
GJB2_F2-II	2	5′-CTCCCTGTTCTGTCCTAGCT-3′	20	56.2	804
GJB2_R2-II		5′-CCCTCTCATGCTGTCTATTTC-3′	21	56.5	

bp: Base pair, Tm: Melting temparature

RESULTS

Among 402 families with at least one member clinically diagnosed with ARNSHL based on pedigree, 35% (141/402) were found to carry the pathogenic variant in the *GJB2* gene. Of these, 53.9% were homozygous, and 33.3% were compound heterozygous for the identified variants. Approximately 13% of the patients were found to carry a single (mono-allelic) *GJB2* variant (Table 2).

We identified 13 different *GJB2* gene variants in our cohort (Table 2). The *GJB2* gene variants most frequently observed were c.35delG (59.2%), c.71G>A (6.38%), c.-23+1G>A (3.54%), c.233delC (2.48%) and c.358_360delGAG (1.77%). Five variants (c.35delG, c.71G>A, c.-23+1G>A, c.358_360delGAG and c.233delC) were found in the homozygous state, seven were found to be compound heterozygotes, c.35delG being the most frequent one. One patient was found to be compound heterozygous, involving different alleles other than c.35delG. Eighteen (12.7%) of the patients were found to carry a single *GJB2* variant, most frequently (55%) being c.35delG.

Allele (Figure 1) and genotype frequencies (Table 3) consistently showed that c.35delG variant predominates the cohort either in homozygous or in compound heterozygous state. The c.71G>A variant was the second most frequent one.

In eight patients clinically diagnosed with KID syndrome four were found to carry c.150C>A (p. Asp50Glu).

Table 2: Patient allele counts of *GJB2* variants revealed in the NSHL cohort

Mutation	Patient counts			Allele count	Allele frequency	Reference
	Homozygous	Compound heterozygous	Mono-allelic			
c.35delG (p.G12Vfs*2)	67	23	10	167	76.82	13
c.71G>A (p.W24*)	3	12	0	18	8.19	14
c.-23+1G>A	2	3	3	10	4.56	15
c.233delC (p.L79Cfs*3)	2	3	0	7	3.18	16
c.358_360delGAG (p.E120del)	2	0	1	5	2.73	17
c.167delT (p.L56Rfs*26)	0	2	0	2	0.91	18
c.269T>C (p.L90P)	0	1	1	2	0.91	13
c.327_328delGG (p.E110Dfs*4)	0	1	0	1	0.45	19
c.439G>T (p.E147*)	0	1	0	1	0.45	20
c.94C>T (p.R32C)	0	1	0	1	0.45	21
c.487A>G (p.M163V)	0	0	1	1	0.45	22
c.551G>C (p.R184P)	0	0	1	1	0.45	18
c.239A>G (p.Q80R)	0	0	1	1	0.45	33
Total	76 (53.91%)	50 (33.33%)	18 (12.76%)	220		

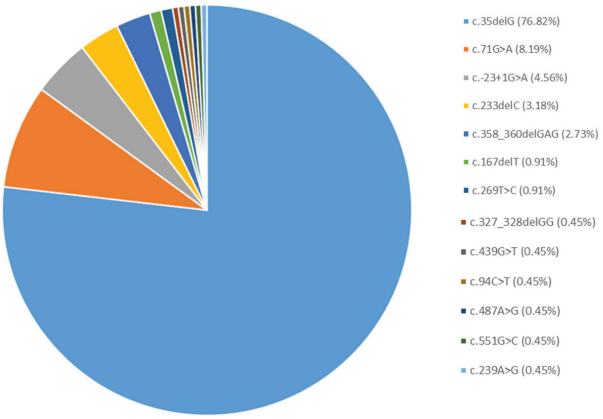

Figure 1: Allele frequency of *GJB2* variants identified in the cohort.

neighboring countries. The most frequent variant was c.35delG, which was identified in more than half of our patients. This mutation is the most common pathogenic variant found among Caucasians, Mediterraneans, and different European populations (12, 23-27). Though c.35delG was reported as the most frequent alteration by different studies in various countries, its frequency was not the same among populations. The variable frequency of c.35delG among different populations can be explained by the founder effect. Many studies suggest that the c.35delG allele originated from South Europe, Italy (28). Interestingly, the frequency of this variant displays a geographic gradient as observed by its frequency decrease from Southern to Northern Europe and from Western to Eastern Asia (29). This geographic gradient can be

Table 3: Patients with different *GJB2* allele genotypes in the cohort

Genotype (HGVS*)	Zygosity	Patient count (%)
c.[35delG];[35delG]	Homozygous	67 (56.78)
c.[35delG];[71G>A]	Compound Heterozygous	12 (10.17)
c.[35delG];[35=]	Heterozygous	10 (8.47)
c.[35delG];[233delC]	Compound Heterozygous	3 (2.54)
c.[71G>A];[71G>A]	Homozygous	3 (2.54)
c.[35delG];[-23+1G>A]	Compound Heterozygous	3 (2.54)
c.[-23+1G>A];[-23=]	Heterozygous	3 (2.54)
c.[358_360delGAG];[358_360delGAG]	Homozygous	2 (1.69)
c.[35delG];[167delT]	Compound Heterozygous	2 (1.69)
c.[233delC];[233delC]	Homozygous	2 (1.69)
c.[-23+1G>A];[-23+1G>A]	Homozygous	2 (1.69)
c.[358_360delGAG];[358_360=]	Heterozygous	1 (0.85)
c.[35delG];[327_328delGG]	Compound Heterozygous	1 (0.85)
c.[35delG];[439G>T]	Compound Heterozygous	1 (0.85)
c.[35delG];[94C>T]	Compound Heterozygous	1 (0.85)
c.[269T>C];[358_360 delGAG]	Compound Heterozygous	1 (0.85)
c.[269T>C];[269=]	Heterozygous	1 (0.85)
c.[487A>G];[487=]	Heterozygous	1 (0.85)
c.[551G>C];[551=]	Heterozygous	1 (0.85)
c.[239A>G];[239=]	Heterozygous	1 (0.85)

*: Human Genome Variation Society (http://varnomen.hgvs.org/recommendations/DNA/)

DISCUSSION

Here we report, the variant spectrum and frequency of the *GJB2* gene in NSHL patients with *GJB2*-related entities (ARNSHL or KID/PPK). Our results were consistent with the previous studies conducted in Turkiye or in observed even in some individual countries. In Iran, for instance, the frequency of the c.35delG is highest in the North-West and lowest in the South-East populations, consistent with the findings of neighboring countries like Turkiye and Pakistan (30). A similar frequency range for

c.35delG variant has been observed in different cities of Turkiye ranging from 5% to 53% (31).

HL is a health problem, although being nonlethal it poses life quality issues. Though the recent developments in HL treatment partly begin to overcome these limitations, the psychological outcomes of the disease can not be ignored. The current solutions to this problem include early diagnosis, management, follow-up, educational and social support for the families, genetic counseling, and possible cochlear implantation. Due to the high rate of heredity in HL, genetic counseling has a major impact on new cases before genetic testing. On the other hand, the high ratio of autosomal recessive inheritance calls attention to consanguineous marriages. Many studies suggest that the variability in the frequency of some *GJB2* variants in different populations resulted from the founder effect for the frequent variants and consanguineous marriages for the rare variants (15, 29, 31). Within our cohort, five alterations in a homozygous state were consistent with their high frequency of consanguineous marriages in the population from Turkiye. As a result of high allele frequency, c.35delG was found in 87% and 46% of the patients at homozygous and compound heterozygous state, respectively. These ratios may clinically have importance because c.35delG mutation has been shown to have significantly severe hearing impairment in homozygous patients, compared with 35delG/non-35delG compound heterozygotes (8). Interestingly, one of our patients who was in a compound heterozygous state had two different mutations (c.[269T>C;358_360delGAG]) other than the most frequent one (c.35delG). Taken together with the high ratio of compound heterozygosity, this finding suggests that ARNSHL is a health problem not restricted to consanguineous marriages.

Some *GJB2* mutations reported previously from Turkiye were not found in our patient cohort (32). These variants include c.360_362delGAT (p.delE120), c.310_323del14, c.299_300delAT, c.517C>T (p.P173S) and c.238C>A (p.Q80K). This discrepancy supports the idea that the *GJB2* variant spectrum and frequencies vary by geographic origins and the size of the cohorts. Even in the same cohort, the spectrum of variants can fluctuate by the method used and by the size of the cohort. For instance, compared to our previous report, we observed in this present study an additional ten rare variants and one frequent variant (c.-23+1G>A) in the *GJB2* gene due to the inclusion of the non-coding exon of *GJB2* and the analysis of a higher number of patients (33).

The finding that 12.7% of our patients had a single mutant allele of the *GJB2* gene suggests some possibilities, like the presence of a second mutation that might be located in a non-coding or regulatory regions of the *GJB2* gene. Another possibility is that the bi-allelic mutations responsible for the disease might be located in a different gene other than the *GJB2*. In the latter case, the mutation we found in the *GJB2* gene might be just a coincidental variant carried by the patient. However, digenic inheritance involving the *GJB2* gene should also be considered to explain mono-allelic *GJB2* gene variants in the patients. Digenic inheritance in ARNSHL was reported by previous studies (34, 35). Mono-allelic variant carriers of the *GJB2* gene were shown to cause ARNSHL with the presence of other mono-allelic variants in another gene like *GJB6*, *GJB3*, *MITF*, *TMPRSS3*, *GJB4*, *GJA1*, and *GJC3* (36-40). However, there are conflicting data regarding *TMPRSS3/GJB2* digenic inheritance (41). Despite all the supporting publications, the molecular mechanism underlying the digenic ARNSHL remains to be elucidated. Considering all these data, we can at least say that, to elucidate the molecular etiology of ARNSHL in the patients with mono-allelic *GJB2* mutation, digenic inheritance should be kept in mind. For such patients, exome sequencing is presently recommended for genetic diagnosis. However, to confirm the digenic inheritance, a segregation analysis of the candidate variants in the patient's family is also needed. Besides, digenic inheritance carries a possibility of a lead to an incorrect exclusion of the variant in segregation analysis.

In addition to the patients with ARNSHL, we analyzed the *GJB2* gene in the patients with KID/PPK syndrome. Of eight patients clinically diagnosed with KID/PPK, four were found to carry p.D50N (c.150C>A) variant in heterozygous state. Though this study covered only the *GJB2* gene, variants in other connexin genes like *GJB6* (CX30), *GJB4* (CX30.3), *GJB3* (CX31), and *GJA1* (CX43) that are expressed in epidermis and appendages, are known to cause skin disorders (42). However, only *GJB6* variants are known to cause KID/PPK or an overlapping Clouston syndrome (43). Like *GJB2*, *GJB6* also has allelic heterogeneity, and pathogenic *GJB6* variants can cause autosomal recessive and dominant deafness or type 2 ectodermal dysplasia 2 (Clouston type). Therefore, sequencing of the *GJB6* gene should be included in the diagnostic algorithm of the HL patients with skin lesions.

The *GJB2* gene has two exons, and most pathogenic *GJB2* variants, including c.35delG, are located in the second exon of the gene. Therefore, sequencing of this exon is expected to detect most of the causative variants. Consistent with previous studies, sequencing of the second exon was sufficient for the diagnosis of most patients. However, our patients' third most frequent mutation (c.-23+1G>A) is located in the splicing site of the non-coding first exon. Sequencing of this non-coding exon is also required to increase the diagnostic yield of the patients with ARNSHL.

In summary, sequencing only the coding exons of the *GJB2* gene would lead to molecular diagnosis in approximately 33% of the patients. Further, including the non-coding exon would yield an additional 2% diagnostic rate. To identify pathogenic variants in the remaining 65% of the cases, it seems necessary to implement NGS techniques, due to the possible digenic inheritance and multigenic etiology of NSHL. Increasing the availability and decreasing the cost of the NGS have made it the most favorable technique for the diagnosis of genetic diseases in the past years. Depending on the *GJB2* mutation spectrum and frequency in a population, NGS seems to be a recommendable method after excluding the most frequent mutations by Sanger sequencing. It should be noted that the NGS technique may reveal some coincidental variants that could be confusing for the clinician.

The efficiency of NGS in the diagnosis of HL depends both on the cohort and the covered genes. Depending on the patient selection criteria (like ethnicity, with or without positive family history), a positive diagnostic rate was reported to range from 10 to 83% (44). Similarly, in a large multiethnic cohort including 1119 unrelated patients who were tested with NGS gene panel (targeted genomic enrichment and massively parallel sequencing), it was shown that screening of 89 genes increased the diagnostic rate by 2% compared to the screening of 66 genes. This difference was stated to account for 4% of all positive diagnoses (45). Considering the remarkable contribution rate (18%) of gross copy number changes in hearing loss, we can deduce that the MLPA technique may also need to be implemented for the diagnostic algorithm of NSHL (35).

In conclusion, our update on NSHL cohort for *GJB2*-related entities has supported the previous knowledge about the most frequent *GJB2* pathogenic variants and revealed the possibility of compound heterozygosity of the rare variants and potential digenic inheritance.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: This study was approved by the Ethics Committee of Istanbul University, Istanbul Faculty of Medicine (Date: 22.01.2021, No: 108).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Z.O.U., B.W.; Data Acquisition- H.K., A.D.A.; Data Analysis/Interpretation- Z.O.U., V.K., F.T., Ç.G.; Drafting Manuscript- Ç.G., Z.O.U.; Critical Revision of Manuscript- Z.O.U., H.K., Ç.G.; Approval and Accountability- Z.O.U., H.K., B.W., Ç.G., A.D.A., V.K., F.T.

Conflict of Interest: Authors declared no conflict of interest

Financial Disclosure: This study was partially funded by Scientific Research Projects Coordination Unit of Istanbul University. Project number: 37697.

Acknowledgement: We especially thank to Psychologist Melike Ulubil for her support in the ascertainment of the families and for meticulously drawn pedigrees. We remember the esteemed Nermin Baserer, Professor of the Ear, Nose and Throat Department of Istanbul Faculty of Medicine, with appreciation, and respect for her valuable support on the genetics of hearing loss. We always remember Abdullah Üzümcü, PhD with love, respect, and vivid memories.

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