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*GJB*2 Gene Related Nonsyndromic Hearing Loss in Mazandaran Province, North of Iran

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Abstract

Introduction: Congenital hearing loss is the most common sensory deficit in the world and mutations in *GJB*2 gene are the most common cause of deafness in many populations. Frequency of *GJB*2 mutations is estimated about 16% in Iran and varies among different provinces with a decreasing trend from north to south. The aim of this study was to investigate the frequency of *GJB*2 mutations in Mazandaran province, north of Iran, among non-syndromic hearing loss patients. **Methods:** 262 patients from 204 families participated in this study. After genomic DNA extraction, *GJB*2 gene analysis was carried out using DNA sequencing of both coding and non-coding regions by ABI 3130XL genetic analyzer. **Results:** 30.15% of all subjects showed mutations in *GJB*2 gene. Four mutations, including c.35delG (Gly12Valfs*), IVSI-1 + 1G > A, c.95G > A (Arg32His) and c.224 G > A (Arg75Gln) comprises 69.89% of all mutations in this study c.35delG and IVSI-1 were the most common mutations among patients respectively. Codon 75 mutation (c.224G > A. p: Arg75Gln) with autosomal dominant inheritance was seen in 7 cases from 3 families. 22 patients showed only one mutation in *GJB*2 gene and in 126 (48.09%) individuals, parents had a consanguineous marriage. **Discussion:** Frequency of *GJB*2 gene related hearing loss among patients was higher than average (16%) in this province. This study also showed a dominant inheritance pattern of *GJB*2 gene in this area. Consanguineous marriage also showed highly frequent among parents. More investigation needs to clarify cause of hearing loss in those 22 patients with one mutation in *GJB*2 *gene*, either two gene inheritance or another gene may be responsible for hearing loss.

Keywords

Hearing Loss, *GJB*2, Mazandaran, Hereditary Deafness, Nonsyndromic

1. Introduction

Deafness is one of the most common diseases in the world which is caused by hereditary or environmental factors or a combination of both [1] [2]. Congenital deafness has a prevalence of 1.3 per 1000 population and in 70% of cases is not associated with other clinical symptoms [3] [4] [5] [6]. Between 75% - 80% cases of non-syndromic deafness are inherited in an autosomal recessive pattern. Generally the frequency of hearing loss is reported 1 in 1000 live births in US and developed countries. The prevalence of deafness in Iran is higher due to the high rate of consanguineous marriages, approximately 3 per 1000 [7] [8]. This disorder is extremely heterogeneous and more than 150 genes are identified causing deafness to date. So far, 121 genes responsible for non-syndromic deafness have been identified, of wich76 genes have autosomal recessive inheritance, others have autosomal dominant (54 genes) and X-linked (5 genes) inheritance (http://hereditaryhearingloss.org).

*GJB*2 gene, located at 13q12 and encodes Gap Junction beta2 protein, also known as connexin 26 protein and is necessary for cells to communicate with each other. This protein is localized in the cochlea and plays a role in k+ recycling pathway during auditory transduction [9] [10]. Different types of mutation in *GJB*2 gene such as Missense, Nonsense and frameshift are the most common cause of non-syndromic deafness with autosomal recessive inheritance pattern. Although some mutations in this gene have been reported to be associated with autosomal dominant inheritance pattern [11]. So far, more than 300 pathogenic mutations have been reported for *GJB*2 gene that is associated with autosomal recessive non-syndromic deafness (http://databases.lovd.nl/shared/genes/GJB2). The most frequent mutations in this gene is c.35delG that occurs due to a G deletion in a sequence of 6 guanines from position 30 - 35 coding are and cause a frameshift and premature stop codon [11] [12] [13] [14].

The type and prevalence of mutations vary in different populations. The frequency of *GJB*2 mutations is reported from different provinces in Iran. It is generally as high as 22% to 38% in West Azarbaijan and East Azarbaijan province, Gilan and Golestan in the north and northwest provinces, to the average level of 16% in central provinces like Qom, Kerman, Isfahan, Yazd, Lorestan and as low as 4% to 0% in South Provinces like Sistan and Baluchestan [14] [15]. In general, it is estimated about 16% prevalence for *GJB*2 related hearing loss in Iran [14] [15]. Consanguineous marriage increases the frequency of homozygousity. It is estimated that 38% of the marriage is consanguineous and approximately 70% of cases are first cousins [16]. Also about 30,000 to 40,000 children are born every year with congenital disorders such as deafness [17] [18].

Deaf persons often require different kinds of medical care, specialized education and social services, reduced work productivity which leads extra cost to the society. Although *GJB*2 mutations are highly frequent in Northern provinces in Iran (Golestan, Mazandaran and Gilan), there are very few previous reports about *GJB*2 gene mutation frequency from Mazandaran province. The aim of this study was to investigate the frequency of *GJB*2 mutations in Mazandaran province, which could be useful for genetic counseling and prenatal diagnosis.

2. Materials and Methods

2.1. Subject

This prospective study was recruited 262 patients from 204 families with hearing loss from Mazandaran province, north of Iran who referred to Novin genetic diagnostic laboratory and counseling center, during the 11-year period, 2009-2020). After genetic counseling, family who was suspected to the hereditary form of hearing loss was included in this study. Any hearing loss with environmental cause was excluded from this study. All ethical issues, including confidentiality and informed consent were included in this study. All subjects were informed and were approved by the ethics committee of Mazandaran University of Medical Sciences.

2.2. DNA Extraction and PCR Amplification

A total of 5 to 10 ml of peripheral blood was collected in a EDTA container tube from each person, then genomic DNA was extracted according to established protocols [19] [20]. The quality and quantity of DNA samples were measured by Nanodrop spectrophotometer (Thermo Sci., Newington, NH). Two appropriate pairs of primers (**Table 1**) were applied (Macrogene, South chorea) to amplify exon 1 and exon 2 of *GJB*2 gene using polymerase chain reaction (PCR) followed by DNA sequencing method. A 850 bp and a 950 bp fragment was achieved by PCR amplification in a total volume of 25 µl respectively. Briefly, 2 µM of each primer (0.7 µl), 0.1 mM each dNTP (0.5 µl), 1× PCR buffer (2.5 µl), 1.5 mM $MgCl_2$ (0.7 µl), 0.5 unit of Taq polymerase (0.2 µl) and 19.3 µl distillated water (Cinnagen, Iran) were used in each PCR tube. The following amplification conditions: initially denatured at 95˚C for 5 minute, followed by 35 cycles of denaturation at 95˚C for 1 minute, annealing at 60˚C for1minute and extension at 72˚C for 1 minute and a final extension at 72˚C for 5 min. Quality of PCR product was checked by electrophoresis, 5-µl of each PCR product on was run on 1% agarose gel.

Table 1. Specific primers to amplify exon 1 and exon 2 of the *GJB*2 gene.

Exon 1: Forward	5'-GTGCGGTTAAAAGGCGCCAC-3'
Exon 1: Reverse	5'-GGTGCCATCGCGTCCACTT-3'
Exon 2: Forward	5'-TGCTTGCTTACCCAGACTCA-3'
Exon 2: Reverse	5'-TTGTGGCATCTGGAGTTTCA-3'

2.3. DNA Sequencing and Genotype Analysis

Samples were subjected to further investigations using Sanger sequencing 3130XL Genetic Analyzers (ABI applied Biosystem, USA) for all individuals as a part of the diagnostic genetic laboratory services. In brief, specific primers were used to amplify entire exonic as well as 5' and 3' flanking intronic regions of the exon1 and exon2 of *GJB*2 gene, followed by DNA sequencing to find any variations or short deletion/insertions as described previously. Sequence analysis was performed using reference sequences from GenBank database. Gene Runner software (http://www.generunner.com) was used to align patient's gene sequence, and Ref. sequence along with manual check of chromatogram from patient's DNA sequence using *FinchTV* chromatogram viewer software (Geospiza) also was applied [19] [20].

2.4. Statistical Analysis

Statistical analysis was performed using descriptive statistics. Allelic and genotype frequencies of the study variants were reported using basic descriptive statistics.

3. Results

262 patients from 204 families with hearing loss, including 154 severe were screened for *GJB*2 gene. 79 individuals (30.15%) showed mutations in *GJB*2 gene, which 72 individuals (27.48%) had two mutations with autosomal recessive inheritance and 7 individuals (2.67%) showed one mutation with autosomal dominant inheritance in *GJB*2 gene (**Figure 1**). Also 161 (61.45%) individuals showed no mutations in *GJB*2 gene. From 262 patients, 22 (8.39%) individuals showed only one recessive mutation in *GJB*2 gene and were carrier (**Table 2** and **Figure 1**).

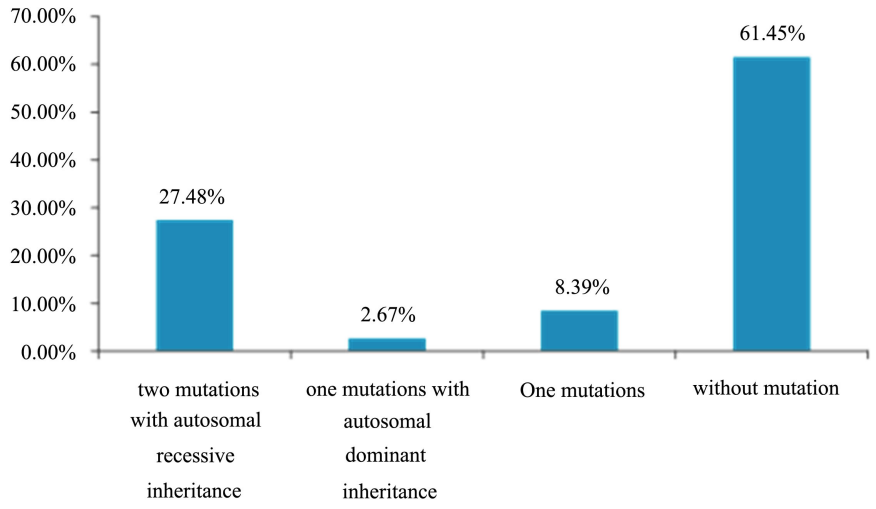

Figure 1. Frequency of autosomal recessive, autosomal dominance and individuals with one mutation in *GJB*2. 61.45% showed no mutation in *GJB*2 gene (n = 262).

Table 2. Carrier frequency for *GJB2* allele variant in 183 deaf individuals in whom *GJB2* related hearing loss could not be identified (n = 22).

Genotype	Codon change	Total case	Frequency (%)
IVSI + 1G > A/_		8	36
c.35delG/_	Gly12Val fs/_	7	31.8
c.380G > A/_	Arg127His/_	3	13.6
c.95G > A/_	Arg32His/_	2	9
c.478G > A/_	Gly160Ser/_	1	4.5
c.138T > G/_	Asp46Glu/_	1	4.5
Total		22	

35delG and IVSI-1 + 1G > A were the most common mutations among patients in this study, respectively (Table 1). Also 4 mutations, including c.35delG (Gly12Valfs), IVSI-1 + 1G > A, c.95G > A (Arg32His) and c.224 G > A (Arg75Gln) comprises 91.27% of all mutations among 173 mutant alleles in this study (Table 3). Codon 75 mutation (Arg75Gln) with autosomal dominant inheritance was seen in 7 cases from 3 families. Two polymorphisms (c.457G > A and c.79G > A) also were seen in this study with total frequency of 2.28%.

Genotype composition and genotype frequency of the patients are shown in Table 4. As it is expected, homozygous patients for three most frequent recessive mutations are more common among patients (Table 3). In 126 (48.09%) individuals, parents had consanguineous marriage. Three family pedigrees, one with autosomal dominant and two with autosomal recessive inheritance are shown in Figure 2.

4. Discussion

The *GJB2* gene related hearing loss in Iran varies between 35% to zero, shows a decreasing trend from north to south of the country with average about 16% according to the previous studies [21] [22] [23] [24]. *GJB2* gene-related hearing loss is generally higher in the North and is reported as high as 38% in some area in Gilan and Golestan provinces [15]. A previous study performed by Najmabadi *et al.* in 664 autosomal recessive non-syndromic hearing loss families from different geographic areas including 47 probands from North of Iran reported 38.3% frequency of *GJB2* gene related hearing loss [25]. Also in another study by Bazazzadegan *et al.*, which was conducted on 2322 families from 31 provinces of Iran during twelve years, the highest prevalence of *GJB2* gene mutations was belongs to the north of Iran, with 33% in Mazani and Gilaki ethnic groups [26]. In this study that 262 deaf individuals from Mazandaran province were recruited, the results showed that the frequency of *GJB2* mutations related non-syndromic hearing loss was 30.15% respectively. 4 mutations, including c.35delG, IVSI-1G > A, c.95G > A and c.224G > A accounted for about 70% of all detected mutations.

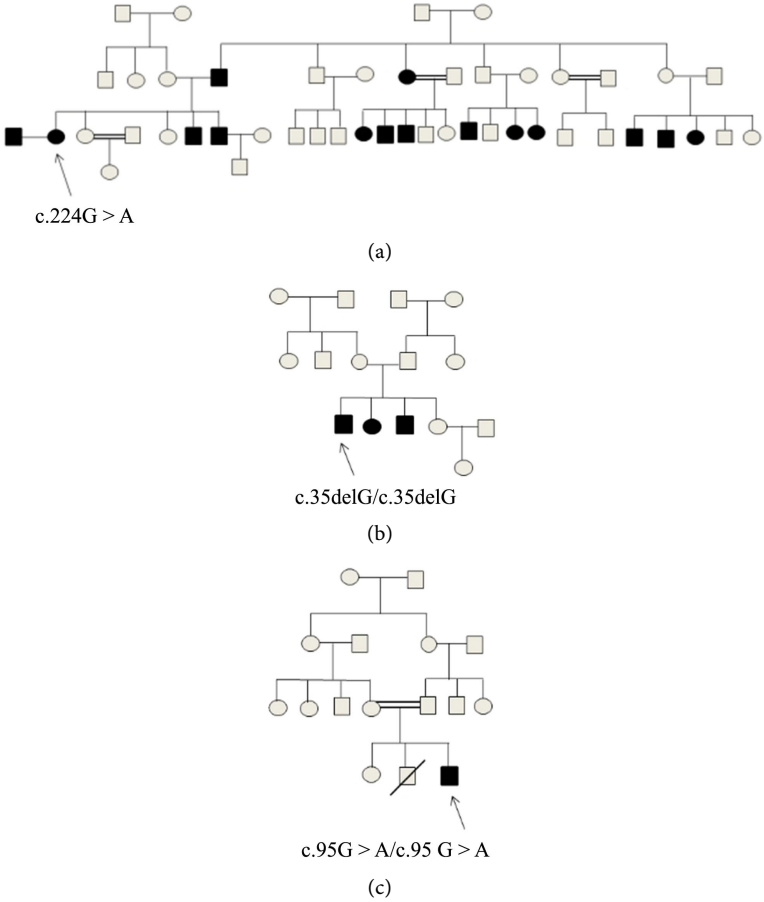

Figure 2. A: example of autosomal dominant pedigree in one family with c.224A > G (Arg75Gln) mutation. A relatively big family with 15 affected members. B & C: Two family pedigree that shows c.35delG and c.95G > A (Arg32His) mutation with autosomal recessive inheritance.

Table 3. Number and prevalence of different mutations found in *GJB2* gene in affected individuals with hearing loss (n = 262).

Mutation	Codon change	Number of alleles	Frequency (%)
c.35delG	Gly12Val fs	108	62.42
IVS1 + 1G > A		25	14.45
c.95G > A	Arg32His	18	10.4
c.224G > A	Arg75Gln	7	4
c.551G > C	Arg184Pro	5	2.8
c.380G > A	Arg127His	3	1.73
c.478G > A	Gly160Ser	2	1.15
c.138T > G	Asp46Glu	1	0.57
c.274G > T	Ala92Ser	1	0.57
c.229T > C	Trp77Arg	1	0.57
c.428G > A	Arg143Gln	1	0.57
c.71G > A	Trp24Ter	1	0.57
c.408C > A	Tyr136Ser	1	0.57
Total		173	

Table 4. List of *GJB2* genotype with biallelic recessive pathogenic mutation in 72 deaf individuals.

Genotype	Codon change	Total case	Frequency (%)
c.35delG/c.35delG	Gly12Val fs/Gly12Val fs	43	16.41
c.95G > A/c.95G > A	Arg32His/Arg32His	7	2.67
IVSI + 1G > A/IVSI + 1G > A		2	0.76
c.551G > C/c.551G > C	Arg184Pro/Arg184Pro	1	0.38
c.35delG/ IVSI + 1G > A	Gly12Val fs/IVSI + 1G > A	9	3.43
c.35delG/c.551G > C	Gly12Val fs/Arg184Pro	2	0.76
c.35delG/c.229T > C	Gly12Val fs/Trp77Arg	1	0.38
c.35delG/c.95G > A	Gly12Val fs/Arg32His	1	0.38
c.478G > A/IVSI + 1G > A	Gly160Ser/IVSI + 1G > A	1	0.38
IVSI + 1G > A/c.408C > A	IVSI + 1G > A/Tyr136Ser	1	0.38
c.95G > A/IVSI + 1G > A	Arg32His/IVSI + 1G > A	1	0.38
c.551G > C/IVSI + 1G > A	Arg184Pro/IVSI + 1G > A	1	0.38
c.35delG/c.428G > A	Gly12Val fs/Arg143Gln	1	0.38
c.35delG/c.71G > A	Gly12Val fs/Trp24Ter	1	0.38
Total		72	

c.35delG was the most common *GJB2* mutation accounted for 108 out of 226 (47.78%) mutated alleles in this study. c.35delG first was reported by Zlante *et al*. 1997 [27]. In Caucasians, this mutation is responsible for 20% of all hereditary hearing loss [28] [29] [30]. Frequency of c.35delG carrier is reported 1.26 % in Northern Europe and 1.96% in Southern Europe [31] [32]. Also its' frequency is reported 1.52%, 0.64%, 1% and 0.64% among American, Asian, Oceanic, and African populations respectively [4] [32]. In Azarbayjan population, Iran neighbor, frequency of c.35delG mutation has been estimated 18% [33] and in Turkish population, it is reported 5% - 53% respectively [34]. In Iran, c.35delG also is the most frequent mutation among deaf individuals [22] [35] [36]. The c.35delG frequency is more in North and Northwest comparing to south of the country [23] [37]. In one study on 298 familial and 592 sporadic subjects from ten provinces, c.35delG was the most prevalent mutation accounted for 193 out of 259 (74.5%) of the *GJB2* mutated alleles [35]. Also, c.35delG frequency was reported 27%, 9.1% and 18% for Gilan, Golestan and East Azarbayjan provinces in the north and 0% in Sistan and Baluchestan province in the south respectively [35]. In this study, from 524 alleles tested, 108 (20.64%) had c.35delG mutation, which is similar to the finding of previous studies.

IVSI + 1G > A is a splice site mutation in non-coding exon 1 of the *GJB2* gene that was reported by Denoyelle *et al*. [38]. It has been found in several populations and frequent among Czech, Hungarian, Yakut and Turkish [39] [40]. This mutation is the third most common *GJB2* mutation in Czech [39] and also it is reported 4.33% with total allele frequency in Turkish population [41] [42]. In

Iran, the IVS1 + 1G > A is one of five most prevalent *GJB*2 mutations with frequency about 4.5% [23], with as high as 9.1%in Kurdish ethnic group [14] [43]. Results from this study also showed IVS1 + 1G > A as a second most common mutation in Mazandaran with the frequency of 14.45% (25 of 226) of the *GJB*2 mutated chromosomes.

c.95G > A (Arg32His) is a third most frequent mutation accounted for 10.4% (18 out of 173) mutated chromosomes in this study. Previous studies reported in one out of 70 consanguineous families from Isfahan, estimated 2.5% frequency in central area of Iran [44], 5 out of 1328 total allele tested (0.37%) in other study [25] and also 2 out of 131 (1.52%) in an unrelated nonsyndromic hearing loss samples from central part of Iran [1]. Results achieved from this study revealed slightly higher frequency of 3.43% (18 out of 524 alleles) from total chromosome studied.

c.224G > A (Arg75Gln) cause autosomal dominant hearing loss [26]. In this study, 7 individuals from 3 families (7 out of 173 affected alleles) (4%) were seen with this mutation, with non-syndromic hearing loss (**Table 1** and **Figure 1**). To date, 54 dominant mutations in *GJB*2 gene have been identified worldwide (http://hereditaryhearingloss.org), of which about two-third can cause syndromic hearing loss and remaining causes autosomal dominant non-syndromic hearing loss [26] [45].

For those 22 patients who showed only one mutation in *GJB*2 gene, a digenic inheritance could be involved or another gene rather than *GJB*2 may be responsible for hearing loss. Because hearing loss is heterogeneous, so it is a good candidate for digenic inheritance [46]. Several examples of digenic inheritance have been reported for hearing loss, such as PCDH15 and CDH23, SLC26A4 and FOX1, SLC26A4 and KCNJ10, MYO7A and PCDH15, PCDH and USH1G [47]. For *GJB*2 gene, different digenic inheritance including *GJB*2 and *GJB*6, *GJB*2 and *GJB*3, *GJB*2 and MIFT has been reported before [48] [49] [50]. More investigation is needed to find other mechanisms that are involved.

c.457G > A, (Val153Ile) substitution was seen 12 out of 262 (4.58%) deaf individuals. This change has been reported as a pathogenic allele with recessive inheritance [51] [52] as well as a polymorphism [53] [54]. In one study, Mes *et al*. demonstrate that the c.457G > A change could be pathogenic as they showed that mutated protein was unable to form functional channels in Xenopus oocyte expression system [55]. In another review study, Mahdieh *et al*. revealed c.457G > A with a frequency of 93 out of 148 (62.84%) among polymorphic alleles and 2.4% in total reported alleles and concluded it as a polymorphism [22]. Similarly, Bazzazzadegan *et al*. found 64% (89 out of 4644 alleles) in 2322 nonsyndromic hearing loss probands from different ethnics in Iran and conclude this change is a polymorphism [23]. The pathogenic effect of this amino acid substitution is controversial and remains to be confirmed.

Finally, our finding showed that 79 individuals (30.15%) showed mutations in *GJB*2 gene in this study, also in 161 individuals (61.45%), *GJB*2 gene was not involved in their hearing problem. This study also showed dominant inheritance

of *GJB*2 gene in this area. Along with mutation detection, also we found that about 48% of patients resulted from consanguineous marriage. There was also some limitation in this study. The audiometry test result from all patients was not available, so the exact phenotype-genotype correlation study was not possible in this investigation. As deafness is a heterogeneous disease, in cases that *GJB*2 gene is not responsible for the disease, another gene may involve, so analysis of all other known genes for hearing loss needs to be analyzed using next generation sequencing system.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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