

## Molecular Pathology of 6 Novel GJB2 Allelic Variants Detected in Familial and Sporadic Iranian Non Syndromic Hearing Loss Cases

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### Abstract

**Background:** Mutations of GJB2 gene encoding connexion 26 are the most common cause of hearing loss in many populations. A very wide spectrum of GJB2 gene mutations associated with hearing loss have been detected but pathogenic role has been tested only for a part of them. In this study, we have provided genetic evidence on the pathogenicity of our previously reported novel GJB2 allelic variants.

**Methods:** The pathogenic role of GJB2 allelic variants were assessed using co segregation of each allelic variant with hearing loss in family members, absence of the allelic variants in control populations, coexistence with a second GJB2 mutation, nature of the amino acid substitution and evolutionary conservation of the appropriate amino acid.

**Results:** The GJB2 allelic variants including 363delC, 327delGGinsA, H16R and G200R have been co segregated with autosomal recessive non syndromic hearing loss in five families and are not found in control subjects. The G130V and K102Q were found in heterozygous state in two deaf individuals. G130V results in an exchange a residue highly conserved among all the connexins but was found with a rate of 1% in control subjects and K102Q results in an exchange a residue not conserved among all the connexins and not identified in control subjects.

**Conclusion:** We conclude that, 363delC, 327delGGinsA, H16R and G200R may be pathogenic. However, the pathogenicity and inheritance of K102Q and G130V can not be assessed clearly and remains to be identified.

**Keywords:** Deafness, Connexin 26, GJB2 gene, Iran

### Introduction

Hearing loss (HL) is the most frequent neurosensory disorder which is heterogenous and can also occur due to environmental causes. The majority of hearing deficiencies are of genetic origin, affecting about 60% of the HL cases in developed countries (1). The hereditary cases are mainly non-syndromic and about 80% of congenital cases are either inherited as an autosomal recessive trait or apparently sporadic (2). To date more than 100 non

syndromic hearing loss loci have been mapped and 39 genes have been identified (<http://webh01.ua.ac.be/hhh>). Despite the contribution of several different genes in causing deafness, mutations in the GJB2 gene (MIM# 121011), which encodes Connexin 26 (Cx26), have been shown to be involved in the development of syndromic and non syndromic deafness in many populations (<http://davinci.crg.es/deafness/>). Cx26 belongs to a family of transmembrane proteins which forms

gap junction channels which is expressed in a variety of tissues including cochlea. Gap junctions serve as a major communication system allowing the rapid exchange of electrolytes, second messengers and metabolites between adjacent cells. It is also thought that gap junctions play an important role in auditory transduction, by recycling endolymphatic potassium ions (3-5). A very wide spectrum of GJB2 gene mutations associated with hearing loss has been detected (<http://davinci.crg.es/deafness/>) from which a few are found at high frequencies in some populations e.g. the 35delG in Mediterranean countries (6). The majority of the GJB2 allelic variants occur at very low frequency and a pathogenic role has been tested for only a small portion. However, it is of great importance to determine the molecular bases of HL in order to provide genetic counseling in high-risk families. Here, we have provided genetic evidence and investigated the likely pathogenicity of six novel Cx26 changes identified amongst our previously reported HL patients (7).

## **Materials and Methods**

### ***Subjects and control samples***

Subjects were deaf individuals harbouring novel allelic variants 363delC, 327delGGinsA, G200R, H16R, G130V and K102Q identified in our previous project but not assessed for the pathogenic role of the novel variants (7). Moreover, we included all the available related family members to examine the co-segregation of each allelic variant with hearing loss in the family. A total of 38 subjects (five families and two individuals), aged between 4 and 88 yr (mean: 36.5 yr) were studied. Medical history and pedigree information were collected by a questionnaire. All patients were clinically characterised and checked for otoscopic and audiological examination of pure tone audiometry and were found to have severe to profound sensorineural hearing loss. The severity of the hearing loss is classified by the pure-tone average (PTA) at

500, 1000, 2000 and 4000 Hz and defined as mild (21-40 dB), moderate (41-70 dB), severe (71-95 dB) and profound (PTA >95 dB) (<http://www.gendeaf.org>). Blood samples were collected after obtaining informed consent from all family members or parents of under aged subjects. In addition, a total of 400 anonymous blood samples without known hearing loss were collected from 4 provinces (100 for each region), including Azarbaijan Sharqi in northwest, Tehran in central, Gilan in north and Chaharmahal va Bakhtiari in southwest of the country based on the original regions in there, novel variants were identified.

### ***Molecular analyses***

Genomic DNA was extracted from peripheral blood of the family members and control samples following the standard phenol chloroform procedure. The GJB2 gene consists of 2 exons in which the first exon is non-coding. The entire coding sequence of GJB2 gene was PCR-amplified in all family members using the condition and cycling strategy has been mentioned elsewhere (8, 9). Subsequently, the whole amplified samples were sequenced for the coding region (exon 2) of the gene. Sequencing of the amplified products was performed using BigDye terminator V3.1 cycle sequencing kit, (Applied Biosystems) on an ABI 3100 DNA sequencer (Applied Biosystems). The resultant sequences were compared with reference sequence M86849 using SeqScape V2.0 software.

To determine the frequency of different novel variants in the control populations, rapid screening assays including Amplification-Refractory Mutation Screening (ARMS), Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (PCR/RFLP) and nested PCR were developed for each allelic variant (Table1). (Assays conditions are available in request).

## **Results**

Altogether, six different novel variants including H16R, K102Q, 327delGGinsA, 363delC, G130V and G200R were analyzed in this study.

H16R was found to be compound heterozygous in one family originating from Azarbaijan Sharqi in northwest of Iran. This allelic variants results in an exchange of histidine at codon 16 to arginine within the intracellular 1 (IC1) domain (Fig. 1) of cx26 affecting a residue relatively conserved among all the connexins. This mutation was found in a compound heterozygote state with a previously reported missense mutation (R143W). The co-segregation of H16R with hearing loss is shown in Fig. 2.

The missence alteration of K102Q results in an exchange of lysine at codon102 to glutamine within the intracellular 2 (IC2) domain of cx26 affecting a residue is not conserved among all the connexions (Fig. 1). This variant was found heterozygous in an individual from Tehran.

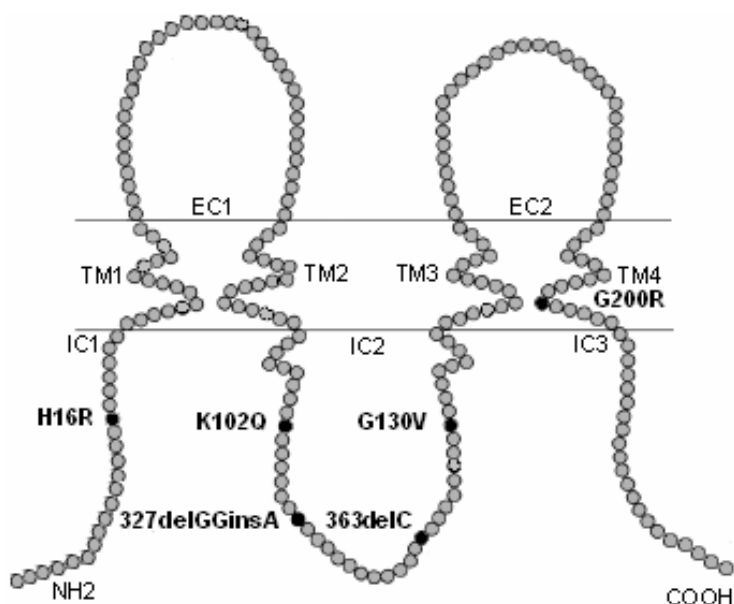
The 327delGGinsA was found to be homozygous in one family originating from Gilan in the north of the country. This frame shift mutation is predicted to lead to a substitutions of residues from codon 109 followed by premature termination at codon 111. The co-segregation of 327 delGGinsA with hearing loss is shown in Fig. 2. The 363delC was found to be homozygous in six family members of a big pedigree originating from

Chaharmahal va Bakhtiari in the southwest of the country. This frame shift mutation is predicted to lead to a substitutions of residues from codon 121 followed by premature termination at codon 167. The co-segregation of 363delC with hearing loss is shown in Fig. 2.

Another novel missence alteration G130V results in an exchange of glycine to valine within the IC2 domain of cx26 affecting a residue conserved among all the connexins (Fig. 1). This variants was found heterozygous in an individual from Tabriz.

The G200R was found to be homozygous in two families from Tehran in metropolitan. This allelic variants results in an exchange of glycine at codon 200 to arginine within the transmembrane (TM4) domain of cx26 affecting a residue not conserved among all the connexins (Fig. 1). The co-segregation of G200R with hearing loss is shown in Fig. 2.

Moreover, the presence of 6 above mentioned allelic variants in control samples from different provinces were examined using ARMS, PCR/RFLP and nested PCR. Analysis of control individuals revealed only the presence of 1/100 of G130V alteration in Tabriz (Fig. 3).

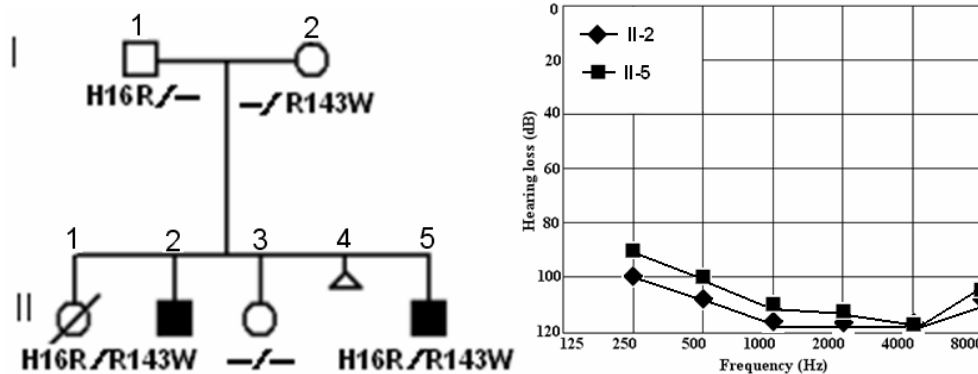


**Fig. 1:** Schematic representation of Connexin 26 illustrating the allelic variants studied; EC, extracellular domains; TM, transmembrane domains; IC, intracellular domains. The amino acid changes are represented in dark circles. H16R, K102Q, 327delGGinsA, 363delC, G130V are located in intracellular domains but G200R is located in transmembrane domain.

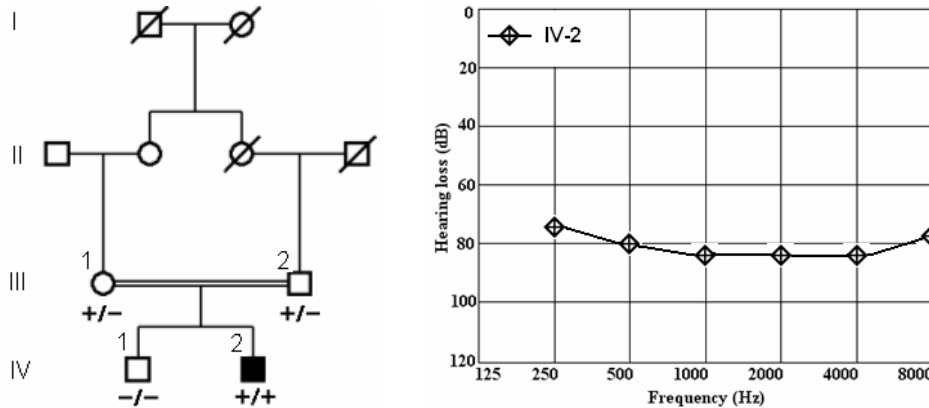
**Table 1:** Molecular assays and related conditions developed for rapid screening of 6 allelic variants

Allelic variants:	Methods of screening	TM	Primers	PCR length (bp)
H16R	ARMS	67	Common 5'-CTGTCTTCACCTGTTTTGGTGAGGTTGTGT-3' Normal 5'-TGAGCCAGATCTTTCCAATGCTGGTGGACT-3' Mutant 5'-TGAGCCAGATCTTTCCAATGCTGGTGGACC-3'	244
K102Q	ARMS	67	Common 5'-AGCCGCTGCATGGAGAAGCCGTCGTACATG-3' Normal 5'-CCATGCACGTGGCCTACCGGAGACATGAGA-3' Mutant 5'-CCATGCACGTGGCCTACCGGAGACATGAGG-3'	223
327delGGinsA	nested PCR	60 56	External F: 5' CTC CCT GTT CTG TCC TAG CT 3' External R: 5' CTC ATC CCT CTC ATG CTG TC 3' Internal F: 5' GAA GAA GAG GAA GTT CAT CA 3' Internal R: 5' CGA TGA CCT TAA ATT CAC TC 3'	807 53
363delC	PCR/RFLP: MboI	61	CX288F: 5' CTA CCG GAG ACA TGA GAA GA 3' CX436R: 5' AGA TGA CCC GGA AGA AGA TG 3'	149
G130V	PCR/RFLP: NlaIV	59	CX123F: 5' CTC CCT GTT CTG TCC TAG CT 3' CX929R: 5' CTC ATC CCT CTC ATG CTG TC 3'	807
G200R	PCR/RFLP: XbaI	59	CX123F: 5' CTC CCT GTT CTG TCC TAG CT 3' CX929R: 5' CTC ATC CCT CTC ATG CTG TC 3'	807

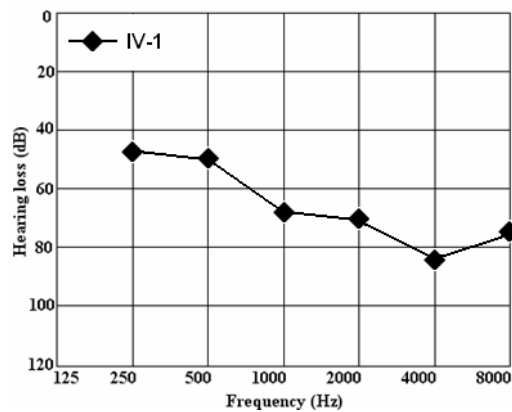
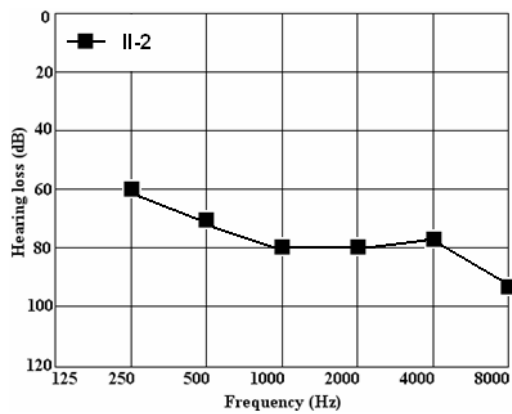
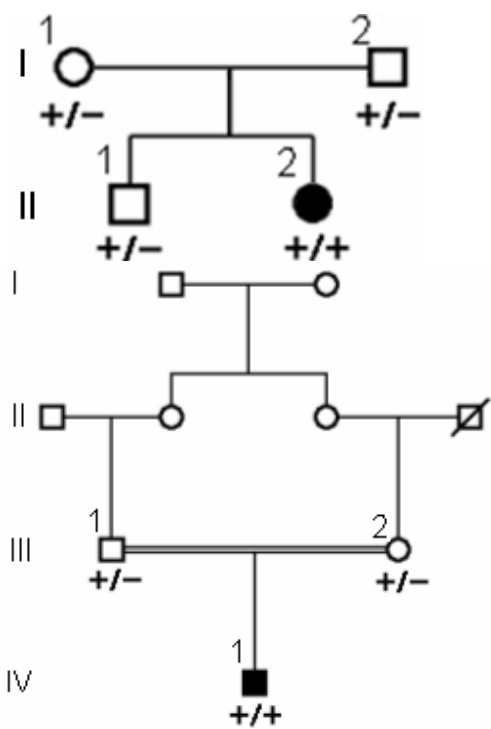
a  
H16R



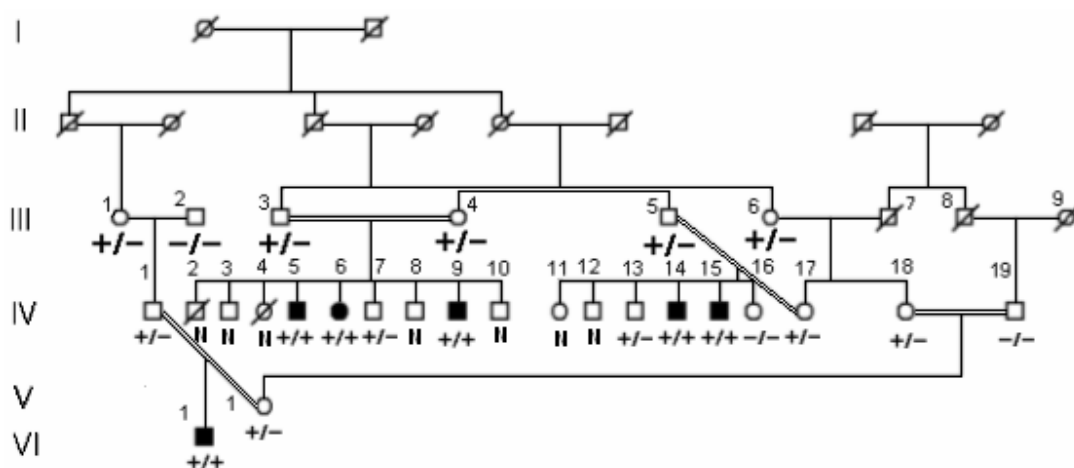
b  
327delGGinsA

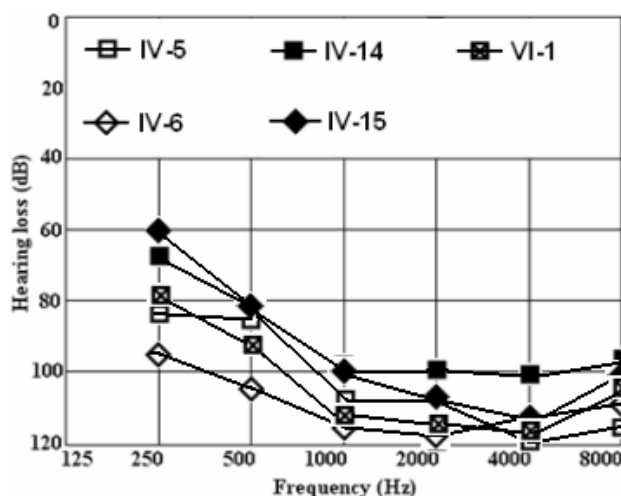


c  
G200R



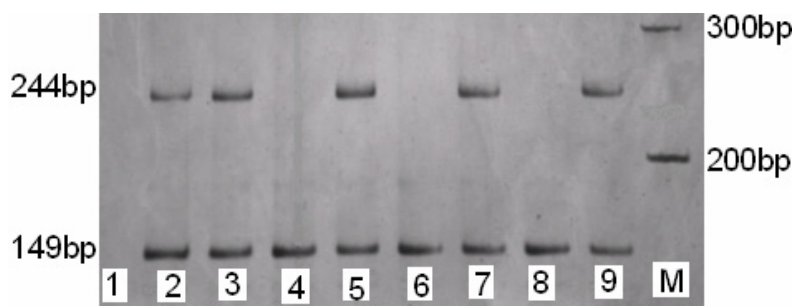
d  
363delC



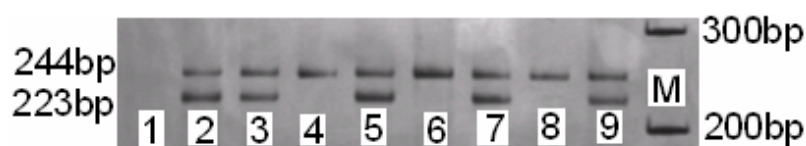


**Fig. 2:** Pedigrees and audiograms of five families segregating GJB2 gene allelic variants. An average levels of hearing losses of both ears have been shown at 250-8000 Hz. '+' represent the mutant chromosome, '-' represent the wild type and 'N' indicates DNA unavailable. (a) Co-segregation of H16R and the average level of hearing loss in a family from Azarbaijan Sharqi (b) Co-segregation of 327delGGinsA and the average level of hearing loss in a family from Gilan. (c) Co-segregation of G200R and the average level of hearing loss in two families from Tehran. (d) Co-segregation of 363delC and the average level of hearing loss in a family from Chaharmahal va Bakhtiari.

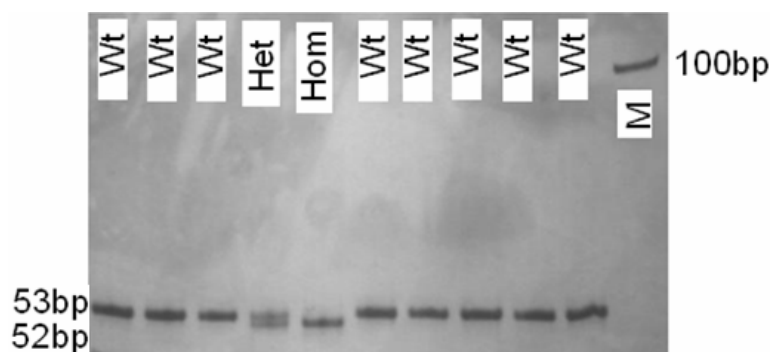
**a**

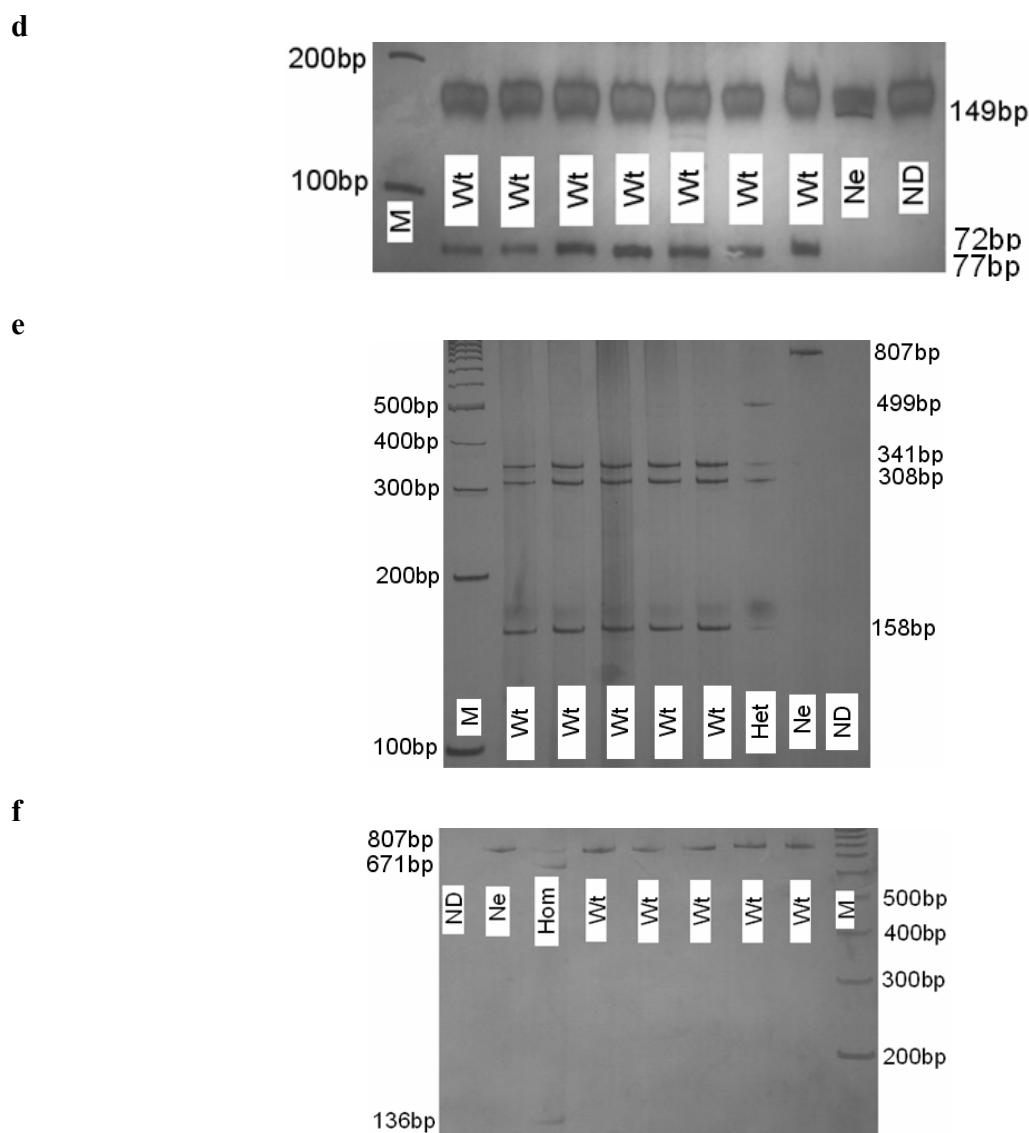


**b**



**c**





**Fig. 3:** ARMS, PCR/RFLP and nested PCR assays were developed for rapid screening of 6 allelic variants. ‘Wt’ represents the wild type, ‘Hom’ represents the homozygote, ‘Het’ represents the heterozygote, ‘M’ represents the marker (ladder), ‘Ne’ and ‘ND’ indicate no restriction enzyme and no DNA respectively. (a and b) ARMS screening of H16R and K102Q allelic variant respectively. Lanes 1 show negative controls (no DNA), Lanes 2 and 3 represent control heterozygotes amplified by mutant and normal primers respectively. Lanes 4 and 5 represent control normal DNAs amplified by mutant and normal primers respectively. ARMS results of DNA samples using mutant and normal primers are shown in lanes 6, 8 and 7, 9 respectively. Lanes 10 show ladder size strands. The lower bands and upper bands in fig a and b represent the 149bp and 244bp control products respectively. (c) nested PCR screening of 327del GG insA allelic variant. wild type, Homozygote and heterozygote alleles were detected as one or two bands of 52 and 53bp. (d) Screening of 363delC allelic variant by PCR/RFLP. The normal 149bp PCR product is digested with MboI to give products of 72 and 77bp. This mutation destroys the MboI resctriction site. (e) PCR/RFLP for screening of G130V allelic variant. The N1aIV digests the normal 807bp PCR product in two sites giving three fragments of 341, 308 and 158bp. This mutation destroys the N1aIV restriction site in one of two positions giving two strands of 499 and 308bp. (f) Screening of G200R allelic variant using PCR/RFLP assay. This mutation creates a XbaI restriction site. Therefore, the mutant 807bp PCR product is digested with XbaI to give products of 671 and 136bp.

## Discussion

Our previous review on 3214 chromosomes of autosomal recessive non syndromic hearing loss families revealed a specific combination of GJB2 mutations types and frequencies in different regions of Iran. The average of 16.1% of GJB2 mutations in familial and sporadic deaf chromosomes (with a gradual decrease of its frequency from north and northwest to the south and southeast) were detected. We determined 35delG as the most prevalent GJB2 mutation, accounting for 71.6% of the GJB2 mutations chromosomes with a carrier frequency of 1.25%. The R127H, W24X, delE120, -3170G>A and V27I+E114G were the following common mutations in different population of Iran (7, 10). While the prevalence of GJB2-related deafness in Iran is 2-3 times less than that reported from European populations, mutations of this gene are the major cause of deafness reported so far. Moreover, six novel GJB2 allelic variants H16R, K102Q, 327delGGinsA, 363delC, G130V and G200R were detected in our previous study which has been the subject of this study regarding their pathogenicity (7). A novel variant with autosomal recessive non syndromic hearing loss inheritance supposed to be a pathogenic allele if the following factors would be considered: co segregation with hearing loss in different families, absence of the mutation in 100 non-deaf control subjects, coexistence with a second GJB2 mutation, nature of the amino acid substitution and evolutionary conservation of the amino acid residues over several species and across different connexins (11-13). Co-segregation of novel allelic variants with deafness in several different family members could clarify the inheritance pattern and pathogenic role of each variant. We found four of six allelic variants H16R, 327delGGinsA, 363delC, and G200R homozygous or compound heterozygous co-segregating with congenital bilateral severe to profound hearing loss in several family members. The remaining two allelic variants K102Q and G130V were heterozygote found as the sole variants in two deaf individuals which co-segrega-

tion studies could not be performed for them. The failure of our previous study to detect the second allele of these heterozygote GJB2-related patients might be due to mutation in non-coding region of the gene that we did not screen. It could be also due to another connexin gene such as GJB6 or interaction with other gene product. However, more than 500 deaf subjects have been tested for a large deletion of the GJB6 gene namely del (GJB6-D13S1830) in Iran, but no mutation has been detected (14-16). These heterozygote GJB2-related patients might be a simple carrier and their deafness may have another cause (17-19). In summary; four of six allelic variants H16R, 327delGGinsA, 363delC, and G200R have been co segregated with autosomal recessive non syndromic hearing loss in five families and are not found in control subjects. Moreover, two frame shift alterations 327delGGinsA and 363delC predicted to lead to truncated proteins and H16R was found coexisted in trans with a previously reported missense mutation allele R143W causing profound hearing impairment. We conclude that, H16R, 327delGGinsA, 363delC and G200R are responsible for the deafness phenotype and may be pathogenic. However, the pathogenicity and inheritance of K102Q and G130V can not be suggested clearly by our data and remains to be identified. The data presented here will improve the genetic counselling of hearing impaired patients.

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