



Genetic Linkage Analysis of DFNB3, DFNB9 and DFNB21 Loci in GJB2 Negative Families with Autosomal Recessive Non-syndromic Hearing Loss

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Abstract

Background: Autosomal recessive non-syndromic hearing loss (ARNSHL) is the most common hereditary form of deafness, and exhibits a great deal of genetic heterogeneity. So far, more than seventy various DFNB loci have been mapped for ARNSHL by linkage analysis. The contribution of three common DFNB loci including DFNB3, DFNB9, DFNB21 and gap junction beta-2 (GJB2) gene mutations in ARNSHL was investigated in south of Iran for the first time.

Methods: In this descriptive study, we investigated sixteen large families with at least two affected individuals. After DNA extraction, GJB2 gene mutations were analyzed using direct sequencing method. Negative samples for GJB2 gene mutations were analyzed for the linkage to DFNB3, DFNB9 and DFNB21 loci by genotyping the corresponding short tandem repeat (STR) markers using polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis (PAGE) methods.

Results: GJB2 mutations (283G>A and 29delT) were causes of hearing loss in 12.5% of families with ARNSHL and no evidence of linkage were found for any of DFNB3, DFNB9 and DFNB21 loci.

Conclusion: GJB2 mutations are associated with ARNSHL. We failed to find linkage of the DFNB3, DFNB9 and DFNB21 loci among GJB2 negative families. Therefore, further studies on large-scale population and other loci will be needed to find conclusively linkage of DFNB loci and ARNSHL in the future.

Keywords: Autosomal recessive non-syndromic hearing loss, DFNB loci, Genetic linkage analysis

Introduction

Hearing loss is regarded as a common neurosensory disorder, and affects approximately 1-2 in 1000 live births (1). Hearing loss is a multifactorial defect caused by genetic, environmental factors or a combination of both (2). The role of genetic factors in the pathogenesis of hearing loss far outweighs the environmental parameters, and about 60% of the hearing loss is caused by genetic changes (3). The inheritance patterns of genetic

hearing loss are autosomal recessive (77%), autosomal dominant (22%), X-linked (1%), and mitochondrial (<1%) (4). Based upon the presence or absence of distinctive clinical features, hearing loss can be classified into syndromic (20-30%) and non-syndromic (70-80%) forms (5). Autosomal recessive non-syndromic hearing loss (ARNSHL) is the most frequent hereditary form of hearing loss (HL), and displays a very allelic and locus het-

erogeneity (6). To date, more than seventy loci have been mapped for ARNSHL by linkage analysis, and they are designated as DFNB followed by an identification number (7). Totally 40 genes have been identified in these loci (8).

In attempt to find candidate locus for ARNSHL, DFNB1 (MIM 220290) was initially identified by Guilford *et al.* which accounts for large percentage of ARNSHL (9, 10). In the DFNB1 locus, mutations in GJB2 gene, encoding the gap junction beta-2 protein connexin 26 are estimated to be responsible for 10-50% of ARNSHL in many populations (11). Therefore, the GJB2 gene mutations only explain a fraction of ARNSHL. Other common loci are associated with ARNSHL including DFNB3 (MYO15A gene, MIM 602666), DFNB9 (OTOF gene, MIM 603681) and DFNB21 (TECTA gene, MIM 603629) (8, 12). The role of DFNB3 locus in ARNSHL pathogenesis was initially identified in a population of villagers of Bengkala, Bali (13). The MYO15A gene within the DFNB3 locus encodes myosin XVA, an unconventional myosin, is a motor protein in hair cells of cochlea that deliver Whirlin protein to the tips of stereocilia, and play fundamental role in formation of stereocilia structure (9). Another gene, OTOF (DFNB9), encodes otoferlin, a large trans membrane protein, play an important role in exocytosis of synaptic vesicles at the synapse between inner hair cell and auditory nerve fibers (14). It has been previously reported that OTOF mutations lead to a unique form of ARNSHL called auditory neuropathy (15). The TECTA (DFNB21), a highly polymorphic gene, encodes alpha-tectorin, a glycoprotein that interacts with beta-tectorin to form the noncollagenous matrix of the tectorial membrane (16). The TECTA gene mutation may be disrupt the structure of this matrix that leads to insufficient transmission and amplification of sound in the inner ear (16).

Due to large number of consanguineous marriage in Middle East compared to European and American population, ARNSHL is 2-3 times more common in the Middle East (12). Iran, with a heterogeneous population due to various ethnicities and with high consanguineous marriage rate (38.6%),

represents a worthy opportunity for genetic linkage analysis of ARNSHL (17).

Therefore, we aimed to address the question of “whether DFNB3, DFNB9 and DFNB21 loci can be contributed to ARNSHL or not,” by genetic linkage analysis these loci in GJB2 negative families.

Materials and method

Families

In this descriptive study, 32 families with at least two affected children were identified from Hormozgan province of Iran, during 2013-2014. HL screening for members of the families was performed by pure tone audiometric test and clinical examination. HL informational questionnaires were obtained from all families and the pedigrees were drawn based on the filled-out questionnaires and interview with the members of the families by genetic counselors. Families with the following criteria were excluded from the study: syndromic hearing loss, exposure to known environmental risk factors such as trauma ototoxic drugs, rubella during pregnancy and excessive noise. Based upon pedigree of families, sixteen families with 2-4 affected children were finally screened as ARNSHL. This study was approved by the Ethics and Human Rights Committee of Hormozgan University of Medical Sciences, Bandar Abbas, Iran and the informed consents and demographic characteristics were filled out by all participants before enrolment.

DNA extraction and GJB2 gene mutation screening

Peripheral blood samples (5 ml from each) were taken from all members of the families and genomic DNA was extracted from the whole blood using the salting-out method as previously described (18). All affected individuals were screened for mutations in exon 2 of the GJB2 gene using direct sequencing technique. To amplify the exon 2 of the GJB2 gene, the primers F (5'-CTCCCTGTTCTGTCTAGCT-3') and R (5'-CTCATCCCTCTCATGCTGTC-3') were used in the PCR procedure, which yield a 809 bp frag-

ment. The PCR solutions contained 100 ng genomic DNA, 10× PCR buffer, 10 pmol of each primers, 10 nmol of each deoxyribonucleotide triphosphates, 1.5 mmol MgCl₂ and 1 U Taq polymerase in a final volume of 25 µl. Initial denaturation at 95 °C for 2 min was followed by 35 cycles of denaturation at 94 °C for 30 sec and annealing at 59 °C for 30 sec, an extension at 72 °C for 30 sec and a final extension at 72 °C for 5 min. The PCR products were separated on 1% agarose gel and visualized with ethidium bromide staining. The amplified products were analyzed using direct sequencing method (Macrogen, South Korea). We used Chromas software to analyze the chromatograms and then the sequencing results were compared with the Human Genome Database and GenBank.

STR markers genotyping and linkage analysis

Negative samples for GJB2 gene mutations were tested for linkage analysis of DFNB3, DFNB9 and DFNB21 loci using STR markers shown in Table 1. Based upon STRs physical distance in NCBI Map Viewer and NCBI UniSTS, we select-

ed the STRs and their primers. A panel of 11 different STR markers was genotyped for DFNB3, DFNB9 and DFNB21 loci, according to the selection criteria including greater heterozygosity values, shorter amplicon and locating near the known locus. The PCR solutions contained 100 ng genomic DNA, 10×PCR buffer, 10 pmol of each primers, 10 nmol of each deoxyribonucleotide triphosphates, 1.5 mmolMgCl₂ and 1U Taq polymerase in a final volume of 25 µl. A two-step touchdown thermal cycling was designed for each STR markers based on the T_m of both primers. PCR was started with an initial denaturation step 94 °C for 5 min was followed by 7 cycles of denaturation (94 °C, 15 sec), annealing (60 °C, 40 sec) and extension annealing (72 °C, 40 sec) followed by a final 25 cycles of denaturation (96 °C, 40 sec), annealing (58 °C, 40 sec), and extension (72 °C, 40 sec) and final extension at 72 °C for 10 min. Finally, the PCR products were separated by electrophoresis procedure on 10% polyacrylamide gels stained by silver nitrate.

Table 1: STR markers of each locus and their primer sequences

Reverse primer (5'→3')	Forward primer (5'→3')	PCR product range	Physical position (bp)	Marker	Locus (gene), Physical location(bp)
GGCACCATAATCATGTC AGACAAT	CTTGACTCCTACAAATCCTGGCA	169-185	14260705-14260882	D17S921	DFNB3 (<i>MYO15A</i>) 18012020..18083116
AAGGGCTTGCTTTGAC	ACTATCCGCCAATACA	119-131	16102497-16102619	D17S953	
ATATTTCAATATTGTAACCAGTCCC	CCAACATCTAGAATT AATCAGAATC	139-163	17264482-17264618	D17S2196	
TCAATGGAGGAAT CCTACTT	ATGATTTGTGTACCTTA TGTATGTT	164-204	28606342-28606533	D2S365	DFNB9 (<i>OTOF</i>) 26680071..26781566
CCGTGCTCTATGCCAG	TCCATCTTTGCGTGC	130-160	27303911-27304064	D2S2247	
TTAGAGCACACATGGTCACTCC	AGGCTGAATCCACCTCC	203-221	26839873-26840075	D2S174	
GGCGATTATGAATAATCCTGC	CACTGCGCCTAGCCTC	182-200	26559144-26559325	D2S2223	
GCTTGATCATGGTGTATTATCTT	TCATTCTACAAGACTAG CATTACC	172-212	121049124-121049321	D11S4107	DFNB21 (<i>TECTA</i>) 120973375..
TTAGACCATTATGGGGGCAA	AGAACCAAGGTCGTAA GTCCTG	172-199	120828264-120828438	D11S925	121061515
TTTTGTCTAGCCATG ATTGC	TCGTGAGANTACTGC TTTGG	101-123	128624097-128624205	D11S912	
AATGGGCACCTCCACCC TATTAGT	GTCITCCCACCTGGAT ATGGGTA	145-155	126292160-126292309	D11S4151	

Results

Families and clinical data

From sixteen families (85 people) who enrolled in this study, 41 individuals had ARNSHL (48.23%). Nine out of sixteen studied families have consanguineous marriage (56.25%). The subjects divided into moderate (41-60 dB), severe (61-80 dB) and profound (≥ 80 dB) hearing impairment according to World Health Organization (WHO) classification of hearing loss. The frequencies of the moderate, severe and profound hearing loss among affected subjects were 9.75% (n=4), 63.25% (n=26), and 26.8% (n=11) respectively.

Screening of GJB2

We analyzed mutations in exon 2 of the GJB2 gene using direct sequencing technique. We found two homozygous pathogenic variants in exon 2 of the GJB2 gene including 283G>A and 29delT

mutations in two studied families (12.50%). These families were excluded from further linkage analysis. Fig. 1 shows 283G>A mutation.

Linkage analysis of DFNB3, DFNB9 and DFNB21 loci

We failed to find linkage of the DFNB3, DFNB9 and DFNB21 loci among fourteen GJB2 negative families. If at least three STR markers (fully informative) of a DFNB locus did not show homozygosity among the affected individuals of a family, then the locus would be determined unlinked. All members of GJB2 negative families, including parents, deaf and healthy siblings were heterozygous for all studied STR markers. As a sample, Fig. 2 shows the PCR products for D2S2223 marker of DFNB9 locus on 10% polyacrylamide gel. The pedigree of one of the unlinked family is shown in Fig. 3.

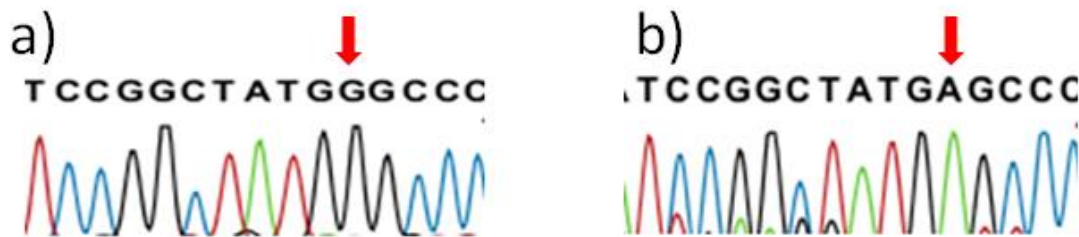


Fig. 1: Results of GJB2 gene sequencing
a) chromatogram of a wild type of GJB2 gene, b) chromatogram of 283G>A mutation of GJB2 gene

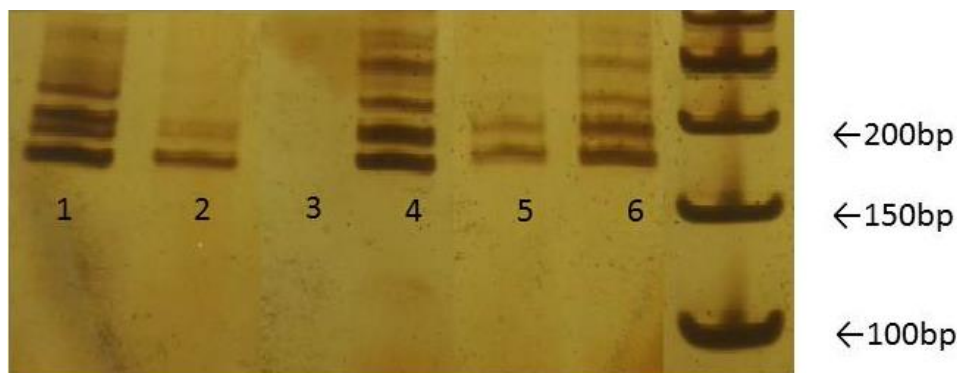


Fig. 2: RA families PCR products for D2S2223 marker of DFNB9 locus on 10% polyacrylamide gel, DNA Ladder 50bp were used. Left to right: 1 father, 2 mother, 3 negative control, 4 and 5 deaf children and 6 a healthy child; the result showed that all bands are heterozygous.

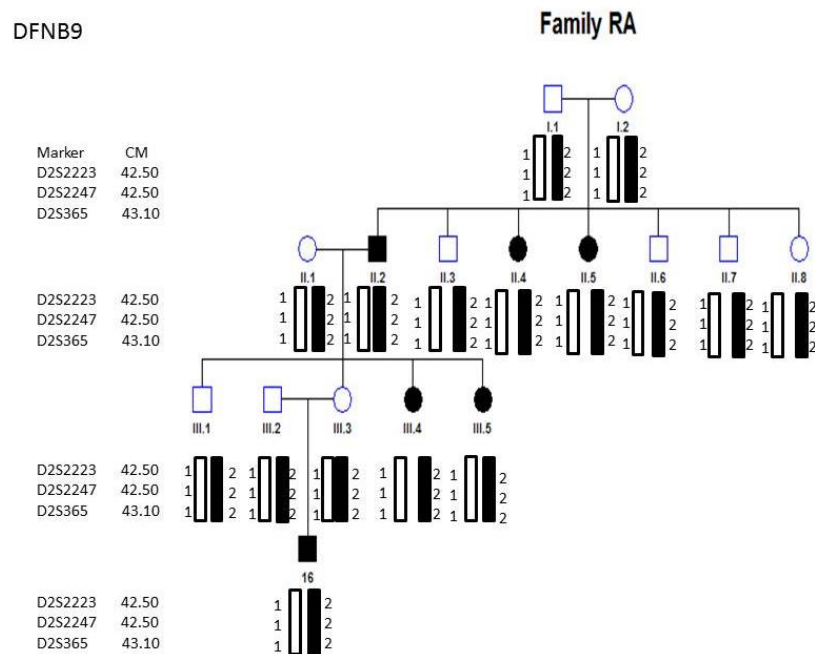


Fig.3: Pedigree of RA family and genotyping of STR markers (locus DFNB9) (LDB map)

Discussion

Our results showed that GJB2 mutations (283G>A and 29delT) were causes of hearing loss in 12.5% of families with ARNSHL and no evidence of linkage were found for any of DFNB3, DFNB9 and DFNB21 loci. Due to the large number of DNFB loci, direct identification of ARNSHL genetic background is laborious. However, previous literatures have suggested that DFNB1 (GJB2), DFNB3 (MYO15A), DFNB4 (SLC26A4), DFNB7/11 (TMC1), DFNB9 (OTOF) and DFNB21 (TECTA) loci are the most common cause of ARNSHL in various populations (8, 12). Therefore, we investigated the mutations of GJB2 gene and linkage of DFNB3, DFNB9 and DFNB21 loci in GJB2 negative families with ARNSHL in south of Iran for the first time. We found that GJB2 gene mutations (283G>A and 29delT) were associated with deafness in 2 out of 16 families (12.50%). Our results are in accordance with the findings of previous studies published in this field (19). The rates of GJB2 gene mutations in Iranian patients with ARNSHL were 11%-18.3% (19, 20). The

GJB2gene mutations are ethnic-specific and the 35delG mutation prevalent in Caucasians, 167delT in Ashkenazi Jews, and W24X in Pakistan and Indian population (10, 21, 22). By contrast, the 35delG and R127H mutations in GJB2 gene are prevalent in Iranian population (19). Though, the population they had studied was ethnically different from the south Iranian population.

We failed to find linkage of the DFNB3, DFNB9 and DFNB21 loci among GJB2 negative families. Similar to our findings, two studies reported that MYO15A gene mutations are not associated with ARNSHL (17, 23). However, several reports revealed the association between MYO15A gene mutations and hearing loss (24-27). In a study 8 out of 140 families (5.71%) linked to the DFNB3 locus (9). In a recent study on 40 Iranian ARNSHL families from Qom and Markazi provinces, 2 families were linked to DFNB3 (5.8%) (28). In addition, previous studies have reported that the MYO15A gene mutations accounts for 5% and 5.5% of ARNSHL in Pakistani and Iranian population respectively (29, 30). Therefore, the prevalence of the DFNB3 locus varies among various ethnicities in Iran. DFNB9 (OTOF) is

mapped to 2p23.3 and No family was linked to this locus in our study. Similarly, in a study on 36 large ARNSHL families no linkage was found to DFNB9 (31). Conversely, the frequency of OTOF gene mutations have reported 2.3% (13/557) in Pakistani families (32), 5.0% in Turkish (24), 1.4% (1/73) in Chinese (33) and 2.7% in Iran (17).

In accordance to a study (28) we were not found evidence of linkage for DFNB21 (TECTA) in our population. Previous several studies on Iranian families have reported different frequencies of DFNB21 mutations in various ethnics 2.7-6.6% (17, 34).

Conclusion

GJB2 gene mutations affected 12.50% of families with ARNSHL in our population. In contrast to several previous studies, we failed to find linkage of the DFNB3, DFNB9 and DFNB21 loci among GJB2 negative families. This may be a consequence of racial differences populations or using low number of subjects in the present study. In spite of the no linkage reported in this study, possible linkage of DFNB3, DFNB9 and DFNB21 loci, and ARNSHL could not be completely ruled out. Therefore, it seems that further studies on large-scale population will be needed to conclusively find linkage of DFNB3, DFNB9 and DFNB21 loci, and ARNSHL.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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