Original Article

Investigating Seven Recently Identified Genes in 100 Iranian Families with Autosomal Recessive Non-syndromic Hearing Loss

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Objectives: Hearing loss (HL) is the most common sensory disorder, and affects 1 in 1000 newborns. About 50% of HL is due to genetics and 70% of them are non-syndromic with a recessive pattern of inheritance. Up to now, more than 50 genes have been detected which are responsible for autosomal recessive non-syndromic hearing loss, (ARNSHL). In Iran, HL is one of the most common disabilities due to consanguineous marriages. The aim was to investigate the prevalence of three new ARHL genes (GJB4, GJC3, and SLITRK6) reported in neighboring countries among Iranian families with ARNSHL.

Methods: One hundred unrelated families with at least two affected siblings in consanguineous marriage, who were negative for GJB2 gene mutations, were selected. By using three STR markers for each gene, homozygosity mapping was performed.

Results: Two families showed linkage to GJB4, six families were linked to GJC3 and only one family linked to SLITRK6. The samples of these families who showed linkage were sent for Sanger sequencing to detect the causative mutations. However, after analyzing the sequencing results, no mutation could be detected in either of the families. Molecular analysis for these nine families is underway in order to determine the pathogenic mutations using whole exome sequencing.

Discussion: These data demonstrate a very low prevalence of mutation in these three genes (GJB4, GJC3, and SLITRK6) in the Iranian population, since no mutation was detected in our study group of 100 families.

Keywords: Autosomal recessive non-syndromic hearing loss, homozygosity mapping, linkage analysis, Iran

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Introduction

Hearing loss (HL) is a common sensorineural defect that affects a person's life by disrupting their ability to communicate. In industrialized countries, 1 in 1000 infants is either born with profound HL or is affected during the pre-lingual period of their lives. In Iran, HL is the second most common disorder after intellectual disability [1]. HL can be classified by different systems. The first classification method is based on the time of HL appearance. If HL is manifested before a child speaks, it is called pre-lingual, while after a child speaks it is called post-lingual. The second method for classification of HL is whether the HL occurs along with other symptoms or disorders (syndromic), which accounts for 20-30% of HL, or whether the disorder is only HL (non-syndromic), which accounts for 70-80% of HL. Deafness is also classified based on the degree of HL. For example if the severity of HL is more than 91 dB (decibels), it is considered as profound. The severity of HL ranges between 71-90 dB in severe forms, 56-70 dB in moderately severe, 41-55 dB in moderate, 26-40 dB in mild, 16-25 dB in slight, and finally 10-15 dB classified as normal hearing. The pattern of inheritance in HL can be different, which means that another classification can be its pattern of

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inheritance. The most common pattern, which accounts for 80% of HL, is autosomal recessive. HL can also be dominant, which accounts for 20%. X-linked and mitochondrial pattern types only account for less than 1% of HL [2-4]. Autosomal recessive non-syndromic HL (ARNSHL) is usually pre-lingual. In Iran, due to its high percentage of consanguineous marriages, autosomal recessive disorders are more prevalent compared to other populations [5].

ARNSHL is a genetically heterogeneous disorder in which 95 loci have been mapped and 55 genes have been identified to date (http://hereditaryhearingloss.org/). Our previous study showed that mutations of the GJB2 gene do not contribute to the major genetic load of deafness in the Iranian population (~16%). [6,7] Recently, many countries are paying more attention to HL, and focusing their studies on the genes causing this disorder. In the past couple of years, some countries around Iran have found mutations in three new HL genes (GJB4, GJC3, and SLITRK6).

The GJB4 gene (OMIM# 605425) located on chromosome 1p34.3, has 2 exons compromising 3984 base pairs. The protein encoded by GJB4 is connexin protein 30.3 that is a component of a gap junction, and is composed of 266 amino acids and is about 30.3 kDa. Gap junctions are specialized in cell-cell contacts between almost all eukaryotic cells which provide direct intracellular communication. Gap junctions allow the passive diffusion of molecules up to 1 kDa, which includes nutrients, small metabolites, and ions. Structurally, gap channels are composed of two hemichannels called connexons, which themselves are formed from six connexin molecules.[8] Gap junctions are regulated through post-translational modifications of the C-terminal cytoplasmic tail and phosphorylation modulates assembly and their physiological properties. They are continuously synthesized and degraded, allowing tissues to rapidly adapt to changing environmental conditions. Connexins play a key role in many physiological processes including cardiac and smooth muscle contraction, regulation of neuronal excitability, epithelial electrolyte transport and keratinocyte differentiation. Mutations in connexin genes are associated with human diseases including sensorineural deafness, a variety of skin disorders, peripheral neuropathy and cardiovascular disease. Lopez et al. detected a deletion (c.154del4) in GJB4 that causes a frameshift in 2002.[9] In 2007, Yang et al. studied 380 Taiwanese. Among them, 260 had non-syndromic hearing loss. They reported seven mutations in connexin 30.3.[10] In 2012, Kooshavar et al. studied Iranian patients who were heterozygote for GJB2 mutation. They reported five variants and one of the variants, c.542C>T, was not detected in the normal population [11].

The gene GJC3 (OMIM#611925) located on chromosome 7q22.1 comprises two exons. The protein encoded by this gene is a member of the gap junction proteins called connexin 31.3, and it is composed of 279 amino acids.[12] All cells in solid tissues are coupled by gap junctions, thus it is not surprising that mutations in connexin genes have been linked to a variety of human diseases, including cardiovascular anomalies, peripheral neuropathy, skin disorders, cataracts, and deafness. Yang et al. reported a heterozygote transversion (c.807A>T) in two Taiwanese patients with ARNSHL in 2007.[10] In 2010, Hong et al. reported a novel missense mutation in HeLa cells (p.E269D) in the GJC3 gene.[13] Afterwards, in 2013, Su et al. reported a novel missense mutation (p.L23H) in HeLa cells that affects protein connexin 31.3. [14]

SLITRK6 gene (OMIM# 609681) is located on chromosome 13q31.1 with two exons comprising 6561 base pairs. The proteins encoded by SLITRK6 are integral membrane proteins with two N-terminal leucine rich repeat (LPR) domains. Mostly, these proteins are expressed predominantly in neural tissues and have neurite-modulating activity. SLITRK6 protein is composed of 841 amino acids. Mutations in this gene are associated with deafness and myopia [15]. In 2013, Tekin et al. studied three consanguineous Amish families that had sensorineural HL. They reported a nonsense mutation (c.1240 C>T) in exon 2 of SLITRK6. They also reported a mutation c.890 C>T in a consanguineous Turkish family.[16] Morlet et al. studied closely related Amish families in 2014. They reported a homozygote nonsense mutation (c.11240 C>T) which causes defects in cochlea of the inner ear and results in HL.[17] Table (1) shows these genes and their locus, if available.

Table 1. The candidate genes, their locations, the reported linked families in different countries.

Gene	Chromosome	Locus	Country	Mutation	References
GJB4	1p34.3	Unknown	Taiwan	p. R22C (c. 64 C>T) p. V137M (c. 109 G>A) p. V74M (c. 220 G>A) p. R98C (c. 292 C>T) p. R10H (c. 302 G>A) p. A124W (c. 370 C>T) p. C169W (c. 507 C>G)	Yang et al. 2007
			Iran	p. T181M (c. 542 C>T)	Kooshavar et al. 2013
GJC3	7q22.1	Unknown	Taiwan	p. E269D (c.807 A>T)	Yang et al. 2007
			Turkey	p.S297X (c.890C>A)	Tekin et al. 2013
SLITRK6	13q31.1	Unknown	Greece	p.R181X (c.541C>T)	
			Amish	p. Q414X (c.1240C>T)	Morlet et al. 2014

These genes have either not been worked on Iranian families, or they have been studied in small populations. Our goal was to study these genes in a large sample size from different ethnic regions of Iran, determining and comparing their prevalence with neighboring countries.

Methods

Patients-After providing a family history, all hearing-impaired family members underwent a clinical investigation, including a physical examination, and a pure-tone audiometry at 2500-8000 Hz was performed for all individuals. One hundred unrelated patients with ARNSHL who were negative for the GJB2 mutation were selected for this study. These patients were from consanguineous families with at least two affected and one normal sibling. No clinical features, including mental retardation, that would indicate that the HL was part of a syndrome, were observed. In addition, no gross vestibular involvement was noted. The HL phenotype was severe to profound, and was not known to be caused by inflammatory middle ear disease or specific environmental factors. Participating subjects were clinically evaluated by their medical history and physical examination. The consent form, as approved by the University of Social Welfare and Rehabilitation Sciences; Tehran, Iran, was signed by each family.

Homozygosity Mapping - In this project, families were analyzed for allele segregation of three genes using three or four short tandem repeats (STR) microsatellite markers flanking these genes. Since the allelic frequency for these STR markers were not available for the Iranian population, we determined the heterogeneity for these markers in ten unrelated normal individuals from diverse ethnic groups. A minimum of two microsatellite markers per locus were genotyped in the parents, with at least two affected children and one healthy sibling in each family. The STR markers that were either within the gene or closer to the gene, and the repeats which were tri or tetra rather than di repeats, were given priority over the rest of the markers. STR amplification was carried out using the PCR method, and then the PCR products were analyzed on 8% polyacrylamide gel, followed by silver nitrate staining. The linked families were further investigated using Sanger sequencing to confirm the mutation. Primers for sequencing were selected using bioinformatics software, Genome Browser, primer 3, and oligoanalyzer.

Mutation analysis - Complete DNA sequencing was performed for each linked family using intronic primers flanking the exons and exon–intron boundaries by Big Dye Terminators (Applied Biosystems 3130 Genetic Analyzer, Foster City, CA).

Results

In the present study, among the 100 investigated families with ARNSHL, nine were linked to one of the three genes, GJB4, GJC3, or SLITRK6, after homozygosity mapping. Unfortunately, no mutation was detected after Sanger Sequencing was performed. Table (2) shows the STR markers selected for these genes from the Genome Browser. (http://genome.ucsc.edu/)

Table 2. The panel of microsatellite markers for the homozygosity mapping of each of the candidate regions and the number of linked families to each gene.

No.	Locus	Gene	No. of linked families	Clinical features	STR Markers
1	Unknown	GJB4	2	Severe to profound HL	D1S1570 D1S496 D1S195
2	Unknown	GJC3	6	Mild to moderate HL	D7S477 D7S2498 D7S2480 D7S2432
3	DFNMYP	SLITRK6	1	Moderate to profound HL, myopia	D13S251 D13S253 D13S282

Discussion

Hearing loss is very heterogeneous in different populations. Here we investigated the prevalence of three different genes (GJB4, GJC3, and SLITRK6) in ARNSHL in Iran among 100 families who were negative for GJB2 (DFNB1) mutations. Nine families showed linkage to one of these genes. Lopez et al. reported a deletion (154del4) in GJB4 in 2002 that causes a frameshift. [9] In 2007, Yang et al. reported seven other mutations in GJB4 in a Taiwanese population. [10] Recently, five variants were reported by Kooshavar et al. in an Iranian population, from which c.542C>T was not detected in the normal population. They studied patients that were heterozygote for a GJB2 mutation, since about 18.29% of ARNSHL is due to a mutation in this gene in Iran. [11] The families in our cohort were selected from different parts of Iran, but no mutation was detected among them. Yang et al. in 2007, reported two Taiwanese with c.807A>T transversion in the GJC3 gene. [10] In 2010, Hong et al. reported a novel missense in HeLa cells that stops the function of connexin. [13] Tekin et al. studied three Amish families with consanguineous marriages who were affected with sensorineural HL along with high myopia in 2013. They reported a mutation p.Q414X (c.1240C>T) in exon two of the SLITRK6 gene. They also reported a mutation, p.S297X (c.890C>A), in a consanguineous Turkish family. [16] In 2014, Morlet et al. studied nine Amish families. They reported a novel nonsense missense variant p.Q414X (c.1240C>T). All the patients were homozygote for this mutation. [17] The linked family in our study group was only affected with HL without displaying any symptoms of myopia. The explanations for not detecting any mutation while the families were linked could be that the STR markers are close to other genes that can cause HL, or it could be due to SNPs. They could be linked to the SNP but not to the gene itself. Finally, this method, homozygosity mapping, is an indirect method and the results are not very precise.

The next step for detecting mutations for the families that have been linked to one of the three mentioned genes is whole exome sequencing (WES). The human genome comprises about 3 × 109 bases, with coding and noncoding sequences. About 3 × 107 base pairs (1%) of the genome are the coding sequences. It is estimated that 85% of the disease-causing mutations are located in the coding regions of the genome. Since sequencing determines every nucleotide in a DNA sequence, exome sequencing offers a look into the genome that large-scale studies of common variations, such as the genome-wide association study (GWAS), cannot provide. GWAS can only identify variations in DNA that are common in the population, in at least one percent of people, but WES detects not only the ones known to vary, but it can also reveal rare mutations, mostly monogenic disorders. WES has been used for variant detection among both common and rare diseases, as well as for SNP associations and pharmacogenetics. Another advantage of using this technique is that WES is fast and it is not as costly as whole genome sequencing.[18] If no mutation is detected after WES, this could mean that these three genes, GJB4, GJC3, and SLITRK6, have a very low prevalence in the Iranian population.

Conclusion

Based on our findings, these three genes, GJB4, GJC3, and SLITRK6, have a very low prevalence in the Iranian population. However, further studies are recommended on these genes, with a more precise and specific mutation detection system such as WES.

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