Genetic linkage analysis of DFNB1 (GJB6) and DFNB4 (SLC26A4) loci with autosomal recessive non-Syndromic hearing loss (ARNSHL) in Kermanshah, Iran

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Received: 19 August, 2017; Accepted: 12 June, 2017

Abstract

Background: Hearing Loss (HL) is the most common sensory disorder in human with an incidence of about one in 650 alive neonates. It is estimated that at least 50% of pre-lingual HL has a genetic basis. Almost 70% of genetic HL are non-syndromic (NSHL) and of NSHL cases, the autosomal recessive form (ARNSHL) comprises about 80%. Iranian population especially the Kurdish ethnicity with high consanguinity rate offers suitable opportunity for the study of ARNSHL. The aim of this study was to clarify the role of DFNB1 (GJB6) and DFNB4 (SLC26A4) loci in ARNSHL in Kermanshah, Iran.

Materials and Methods: DFNB1 (GJB6) and DFNB4 (SLC26A4) loci were analysed in a cohort study on 28 ARNSHL families (GJB2-negative) from Kermanshah province in Iran. Genetic linkage analysis was applied on 140 samples from 140 individuals by polymerase chain reaction – polyacrylamide gel electrophoresis (PCR-PAGE) technique. Silver staining was used for visualizing the bands. At least, two informative screening markers were analyzed for each locus. Haplotypes were analyzed to determine linkage.

Results: None of the families studied showed linkage to DFNB1 and DFNB4 loci.

Conclusion: Our experiment, similar to previous studies, imply the absence of GJB6 mutations in Iran. None of the families showed linkage to DFNB4 locus. As it normally ranks second after DFNB1 in Iran and other parts of the world, more studies are warranted on more families to elucidate the role of this locus as well as other loci in etiology of ARNSHL.

Keywords: autosomal recessive non-syndromic hearing loss (ARNSHL); DFNB1; SLC26A4; Iran

Introduction

Hearing loss (HL) is the most common sensory deficit in human in developing countries with incidence of about one in 650 alive neonates (1). Two third of the hearing impaired people in the world live in developing countries (1). One study showed that in 2013 at least 360 million people (more than 5% of the global population) have disabling HL (2). One in every 500 children has severe to profound congenital bilateral HL. This number rises to 2.7 per 1000 children for up to 5 year old children and 3.5 per 1000 individuals during adolescence (3-5).

In early childhood, severe HL prevents from suitable speech acquisition and subsequent literacy (6) and delays the cognitive and psychological development.
HL can be classified by different criteria including severity (mild: 20 to 39 dB, moderate: 40 to 69 dB, severe: 70 to 89 dB, or profound: ≥90 dB), origin (conductive, sensorineural or mixed), age of onset (pre-lingual or post-lingual), and presence or absence of associated features (syndromic or non-syndromic) (7). It is estimated that at least 50% of pre-lingual HL has genetic basis. Some believe that 25% of the causes of unknown HL is genetic. Almost 70% of genetic HL are non-syndromic (NSHL) that they have no symptom except HL (8).

NSHL has different types according to the inheritance pattern; autosomal dominant (DFNA, 20%), autosomal recessive (DFNB, 80%), X-linked (DFNX, <1%), Y-linked (DFNY, <1%), and mitochondrial (less than 1%) (9-12).

HL is one of the most heterogeneous human genetic traits (8-12), of which over 70 DFNB loci have been detected for ARNSHL (13) and over 134 loci are associated with NSHL (13). Main genes related to ARNSHL are the GJB2, SLC26A4, OTOF and TMC1 genes. Alterations in the SLC26A4 gene make it the second most frequent cause of ARNSHL after GJB2 (8-12). Recessive mutations of the SLC26A4 (PDS) gene on chromosome 7q can cause sensorineural HL with goiter (14) (Pendred syndrome) or NSHL without goiter (Li et al., 1998) (at the DFNB4 locus). The mutations disrupt in vitro transmembrane anion/base exchange activity of the SLC26A4 polypeptide, pendrin (15). The epidemiology of SLC26A4 deafness may vary among Asian and Western populations, as it has already been reported for recessive GJB2 mutations at the DFNB1 locus (16, 17).

Iran is a multiethnic, large country with a population of at least 77 million with the average consanguineous marriage rate of 38.6% (18). However, the heterogeneous genetic linkage analysis was applied for GJB2-negative families by PCR-PAGE.

Population due to different ethnicities (19) makes generalizations about the population difficult. Thus, focus on special ethnicities such as Kurdish ethnicity, with a high consanguinity rate, offers suitable opportunity for studying of ARNSHL.

In Iran, most studies on ARNSHL have been limited to GJB2 as the most common cause of ARNSHL. Experiments on other loci would provide information about the role of other loci in pathogenesis of ARNSHL in this population and could be used for more comprehensive genetic screening of the disease and more efficient genetic counseling (20).

In the present study, a cohort of 28 ARNSHL families from Kermanshah, a Western province of Iran, was investigated to determine the contribution of DFNB1 (GJB6) and DFNB4 (SLC26A4) to the etiology.

**Methods**

**Subjects and clinical evaluation.** In this descriptive study, 28 GJB2-negative families with Iranian origin (Kurdish ethnicity) were collected from Kermanshah province of Iran.

An informational questionnaire was filled out for each family and the pedigrees were drawn. Inclusion criteria included autosomal recessive mode of inheritance that involves consanguineous marriage of healthy parents and presence of at least two patients in each family. Families with the possibility of exposure to environmental risk factors (infections, the use of ototoxic drugs, head trauma, etc.) and Skeletal disorders, vision, thyroid and kidney disorders were excluded from the study. Pure tone audiometric test was performed for air and bone conduction at different frequencies ranging from 250 to 8000 Hz. Participants signed an informed consent form before the experiment. Blood Samples of participants were collected in EDTA-containing tubes. Genomic DNA was extracted using a salting out procedure. DNA qualities were checked on 1.2% agarose gel. A NanoDrop ND-1000 spectrophotometer (Saveen Werner, USA.) was used to determine DNA concentration and purity.

The study was approved by the Review Board of Kermanshah University of Medical Sciences.

**GJB2 mutation screening.** GJB2 mutation screening was performed according to a protocol described before (20). Totally, 28 ARNSHL families negative for GJB2 mutations families were selected for genetic linkage study.

**Selection of DFNB loci.** Two loci including DFNB1 and DFNB4 were selected for screening based on the reviews of most frequent loci. Screening STR markers were selected based on their physical distance.
Genetic linkage analysis of DFNB1 (GJB6) and DFNB4 (SLC26A4) loci … Shahmohammadi et al.

found at NCBI UniSTS. STR markers of each locus and their primer sequences are listed in Table 1.

**Table 1**: The list of two DFNB loci screened in this study. The corresponding genes and details of screening markers are shown. Categories and functions of their encoded proteins are mentioned.

<table>
<thead>
<tr>
<th>Locus (gene), Physical location (bp), Category &amp; function</th>
<th>Marker</th>
<th>Physical position (bp)</th>
<th>PCR product range</th>
<th>Forward primer (5 → 3)</th>
<th>Reverse primer (5 → 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJB6: 20796101..20806534) Ion homeostasis Protein: Connexins</td>
<td>D13S1275</td>
<td>-</td>
<td>180-214</td>
<td>ATCACTTGAATA AGAAGCCATTTG</td>
<td>CCAGCATGACCTT TACCAG</td>
</tr>
<tr>
<td></td>
<td>D13S175</td>
<td>20848506 20848618</td>
<td>101-113</td>
<td>TATTGGATACTT GAATCTGCTG</td>
<td>TGCATCACCTCAC ATAGGTTA</td>
</tr>
<tr>
<td></td>
<td>D7S2420</td>
<td>10688928-106890211</td>
<td>240-290</td>
<td>CCTGTATGGAGG GCAAACTA</td>
<td>AAATAATGACTG AGGCTCAAAAAC</td>
</tr>
<tr>
<td></td>
<td>D7S496</td>
<td>107154713-107154849</td>
<td>129-141</td>
<td>ACAAACAGTCAA CCCACAAT</td>
<td>GCTATAACCTCAT AANAAACCAAAA</td>
</tr>
<tr>
<td></td>
<td>D7S2456</td>
<td>107331501-107331642</td>
<td>140-152</td>
<td>AAGAAGTGCATT GAGACTCC</td>
<td>CCGCCTTAGTAAA ACCC</td>
</tr>
<tr>
<td></td>
<td>D7S2459</td>
<td>107683218-107683460</td>
<td>238-252</td>
<td>CTGAAATTGAC CTGAAACCTT</td>
<td>ACAGGGGTCTCTC ACACATTTA</td>
</tr>
<tr>
<td></td>
<td>D7S2425</td>
<td>108347079-108347322</td>
<td>234-246</td>
<td>CTAAGTCTTGAGA AGACATTACC</td>
<td>CCTTTTCAGATG TTTATCCA</td>
</tr>
</tbody>
</table>
Linkage Analysis. PCR of STR markers was conducted according to a standard procedure. The master mix content and the related concentrations are shown in Table 2. The thermal profile of PCR is shown in Table 3.

Table 2: The content and concentrations of the PCR master mix in 1 ml volume.

<table>
<thead>
<tr>
<th>Material</th>
<th>PCR mix β (1.5 mM) (µl)</th>
<th>PCR mix β (2 mM) (µl)</th>
</tr>
</thead>
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<tr>
<td>ddH20</td>
<td>862</td>
<td>852</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MgCl2 50 (mM)</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>dNTP mix (25 mM of each)</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3: The thermal profile of PCR for different STR markers used in this study.

<table>
<thead>
<tr>
<th>Stage</th>
<th>D7S2459</th>
<th>D7S2420</th>
<th>D7S496</th>
<th>D7S2425</th>
<th>D7S2456</th>
<th>D13S1275</th>
<th>D13S175</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
</tr>
<tr>
<td>5 Min</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
</tr>
<tr>
<td>Denaturation temperature</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
</tr>
<tr>
<td>1 Min</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
<td>95 C</td>
</tr>
<tr>
<td>Annealing temperature</td>
<td>56 C</td>
<td>59 C</td>
<td>50.5 C</td>
<td>57.5 C</td>
<td>56.2 C</td>
<td>52.6 C</td>
<td>51 C</td>
</tr>
<tr>
<td>1 Min</td>
<td>56 C</td>
<td>59 C</td>
<td>50.5 C</td>
<td>57.5 C</td>
<td>56.2 C</td>
<td>52.6 C</td>
<td>51 C</td>
</tr>
<tr>
<td>Extension temperature</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
</tr>
<tr>
<td>1 Min</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
</tr>
<tr>
<td>10 Min</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
<td>72 C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>40</td>
<td>40</td>
<td>35</td>
<td>32</td>
<td>32</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

Genotyping was carried out by PCR-polyacrylamide gel electrophoresis (PAGE). Silver staining was used for visualizing the bands. At least, two informative screening markers were analyzed for each locus. Haplotypes were analyzed to determine linkage.

In haplotypes, numbers are referred to the band size, with the smallest band size being specified as allele number one, etc.
Results

In total, 28 families (all negative for GJB2 homozygous mutations) were included in our study. About 74% of families involved first-cousin marriage.

One sample image of PCR-PAGE is shown in Figure 1. The haplotypes of four families are shown in Figure 2. In each haplotype, two informative screening markers (D7S2459, D7S2420) for DFNB4 are considered.

None of the families showed linkage to DFNB1 (GJB6) and DFNB4 (SLC26A4) loci. Genotyping was confirmed by including positive and negative samples for DFNB4.

Discussion

In the present study, 28 families with ARNSHL and negative for GJB2-mutations were studied using genetic linkage analysis. No linkage to either DFNB1 (GJB6) or DFNB4 (SLC26A4) was found. The GJB2 gene plays a major role in the pathology of ARNSHL all over the world. In a previous study, the average rate of mutations in GJB2 was found to be 18.56% in Iran(19). This emphasizes the fact that the contribution of other loci to ARNSHL should be evaluated.

In the present study, we aimed to evaluate DFNB1 linkage on samples negative for GJB2
mutations to investigate exon 1 (non-coding) of GJB2 as well as GJB6 mutations, also at the same locus. Common deletions in the GJB6 gene (the del (GJB6-D13S1830) and del (GJB6-D13S1854)) are relatively common in some populations (21-25), but sounds to be very rare in others (26-30) and at least should be considered in those in whom only a single mutation has been detected on GJB2 screening. Gene Clinics advises that very few individuals (0.5%) will be homozygous for two GJB6 deletion alleles (31). In the Republic of Macedonia, molecular characterization of deafness was performed in 130 NSHL profoundly deaf children from different ethnic origins and no del (GJB6-D13S1830) mutation was detected(32). In an Iranian study, mutational analysis in GJB2 and GJB6 genes was done. However, no mutation was found in the GJB6 gene (33).

In this study, no linkage to DFNB4 (SLC26A4) was found. In a study on Chinese ARNSHL population, the SLC26A4 mutation was found in 9.52% (4/42) of Yunnan minority patients and 9.84% (19/193) of Han Chinese patients. No significant difference was found in deafness associated gene mutational spectrum and frequency between the Yunnan minority and Han patients (34). In another Chinese study, in Zhejiang province, 13.6% (24/176) of patients were shown to carry at least one mutant SLC26A4 allele (35).

In a study on 303 unrelated Czech patients with early hearing loss (298 with NSHL and 5 with PS), all GJB2-negative, were tested for SLC26A4 mutations. This study showed that SLC26A4 mutations are present mostly in patients with EVA/MD and/or progressive HL and those with affected siblings (36).

In the Handan Center Hospital (Handan, China), among the 1,000 newborn babies that underwent the first hearing screening, 61 cases were found to have hearing impairment. Upon genetic screening of SLC26A4, four cases showed the heterozygous IVS7-2A>G mutation and one case exhibited heterozygous 1226G>A mutations (37). This shows that the gene plays a significant role in congenital deafness.

In another study, the prevalence of mutations of SLC26A4 among Tibetan, Tu nationality, and Mongolian subjects in the northwest of China was found to be high; mutant allele frequency of SLC26A4 was 4.54%, 6.12%, and 15.79%, respectively(38).

In South Asia, about 5% of ARNSHL cases have SLC26A4 mutations (39, 40). In a survey in Iran, 12 families out of 80 (15%) Iranian families with 2 or more HL patients showed linkage to DFNB4 locus with clues for 5 families to be syndromic (41). In another Iranian research, three out of 36 (8.3%) ARNSHL families were linked to DFNB4 locus. One additional family, diagnosed as Pendred syndrome was also found to be linked to the locus (20). In another study, out of 34 families negative for GJB2, 3 families (8.8%) showed linkage to DFNB4. Therefore, DFNB4 is ranked second after DFNB1 and contributes significantly to HL in Iran (42). In Japanese joint study, SLC26A4 mutations were identified in 82 of the 100 (82%) patients with deafness associated with enlarged vestibular aqueduct (EVA) (43). Therefore, phenotype (EVA)-directed genotyping can be applied in molecular diagnosis of this type of deafness.

In our survey, none of the families showed linkage to the DFNB4 locus. To ensure our data, we included positive and negative control samples. It seems that the locus might not play a major role in this Kurdish population from Kermanshah province. Because of its second rank after DFNB1 in Iran and other parts of the world, more studies should be done on more families to obtain a more precise account of its role in this population. The role of other loci in deafness should also be evaluated. In case, DFNB4 is found to be rare in this Kurdish population, it is recommended to include it in the genetic screening of deafness associated with EVA. Therefore, CT scan or MRI of the inner ear might be useful phenotyping strategy after GJB2 mutations are ruled out as the cause of deafness in family.

**Conclusion**

In conclusion, our experiments like previous ones, imply the absence of GJB6 mutations in Iran. Therefore, the screening of the mutations is not recommended in molecular diagnostics of deafness in Iran. Furthermore, it is possible that DFNB4 (SLC26A4) is not playing a major role in the studied population. Further studies on larger sample size is warranted to clarify the issue.
Conflicts of Interest

There is no conflict of interest among authors.

Acknowledgment

This project was funded by a grant from Kermanshah University of Medical Sciences (Iran).

References


