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Absence of GJB6 mutations in Indian patients with non-syndromic hearing loss

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ABSTRACT

Objective: Hearing loss is the most frequent sensory defect in human being. Genetic factors account for at least half of all cases of profound congenital deafness. The 13q11–q12 region contains the GJB2 and GJB6 genes, which code connexin 26 (CX26) and connexin 30 (CX30) proteins, respectively. Mutations in the gene GJB2, encoding the gap junction protein connexin 26, are considered to be responsible for up to 50% of familial cases of autosomal recessive non-syndromic hearing loss and for up to 15–30% of the sporadic cases. It has also been reported that mutations in the GJB6 gene contribute to autosomal recessive and autosomal dominant hearing defects in many populations. The 342-kb deletion [del(GJB6-D13S1830)] of the Cx30 gene is the second most common connexin mutation after the CX26 mutations in some NSHL populations. The aim of this study was to screen GJB6 gene mutations in Asian Indian patients with autosomal non-syndromic hearing loss.
Methods: We screened 203 non-syndromic hearing loss patients, who were negative for homozygous mutations in GJB2 gene, for GJB6-D13S1830 deletion and mutations in coding regions of GJB6 using polymerase chain reaction, denaturing high performance liquid chromatography and direct sequencing.
Results: No deleterious mutation in GJB6 gene was detected in our study cohort.
Conclusion: The present data demonstrated that mutations in the GJB6 gene are unlikely to be a major cause of non-syndromic deafness in Asian Indians.

1. Introduction

Hearing impairment is the most common disorder of sensorineural functions. Approximately one child in 1000 is born with moderate to profound hearing impairment [1]. It is estimated that at least 50% of prelingual hearing loss is caused by genetic changes. Approximately 80% of cases of genetic deafness are non-syndromic and autosomal recessive forms are the most common in this group [2]. Non-syndromic hearing loss (NSHL) is genetically heterogeneous and at least 120 genetic loci for NSHL and greater than 60 loci are known for autosomal recessive NSHL (ARNSHL) (Hereditary Hearing Loss Homepage: http://webh01.ua.ac.be/hhh/). Despite large number of genetic loci that have been characterized for ARNSHL, mutations at DFNB1 locus [encompassing GJB2 and GJB6 genes] have been found to be responsible for more than half of autosomal recessive NSHL in various populations in both sporadic and familial cases [3–5]. GJB2 and GJB6 genes encode for two gap junction proteins, connexin 26 (MIM # 121011) and connexin 30 (MIM # 604418) respectively and are expressed in cochlea and encode for transmembrane protein subunit of intercellular gap junctions [6]. Mutations in GJB2 and GJB6 genes disrupt the recycling of potassium ions to the endolymph, resulting in progressive intoxication of the organ of corti [7], thereby leading to cellular dysfunction, cell death and ultimately hearing loss. Up to 50% of all patients with autosomal recessive non-syndromic prelingual deafness in different populations have mutations in the GJB2 gene. However, a large fraction (10–42%) of patients with GJB2 mutations has only one mutant allele at that locus, and some familial cases have evidence of linkage to the DFNB1 locus but have no mutation in GJB2. It was therefore hypothesized that another gene close to *GJB2* might be responsible for these cases and mutations in GJB6 gene lying close to GJB2 may contribute to these hearing loss cases. GJB6 mutations have been reported to be associated with both autosomal dominant hearing loss [8] and autosomal recessive hearing loss. Several recent studies have illustrated two large deletions, del(GJB6-D13S1830) and del(GJB6-D13S1854), involving GJB6 gene may be most common second mutations causing ARNSHL in several populations such as Spain, France, Israel, Brazil, Belgium and Australia [9,10]. Inherited hearing impairment has long been recognized in India, however, spectrum of GJB6 mutations for Indian populations is lacking. Since GJB2 and GJB6 genes are lying close to each other and digenic inheritance has been reported for these two genes, we examined the GJB6 gene in NSHL patients who were negative for GJB2 sequence variants or were carrying monoallelic GJB2 variants.

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2. Materials and methods

203 prelingual patients diagnosed with NSHL, attending out patient department of Otolaryngology Department, Post Graduate Institute of Medical Education and Research, Chandigarh, India from March 2004 to November 2007 were enrolled in the present study. The study was approved by the institute ethics committee and informed consent was obtained from all the individuals prior to enrollment. Detailed medical and family histories were obtained through a questionnaire; all individuals were evaluated by clinical examination including otoscopic exploration and pure-tone audiometry. The origin of study subjects was confirmed based on their mother language, ancestral history and marital relationships.

Patients were divided into 3 groups: Group 1 included 137 unrelated individuals with nonsyndromic hearing impairment (71 males and 66 females) without any family history of NSHL. Group 2 consisted of 63 subjects (36 males and 27 females) with family histories of hearing impairment, which appeared to be inherited in autosomal recessive mode, 23 of these patients belonged to families with two or more deaf siblings (17 males and 6 females). Group 3 comprised of 101 normal hearing unrelated individuals including 52 males and 49 females originating from same geographic region as patients. Syndromic forms and patients who had possible environmental etiology, i.e., viral infections, meningitis, acoustic trauma, or exposure to ototoxic drugs, were not included in this study. Syndromic conditions were excluded after a thorough clinical examination. Any associated middle ear or inner ear anomalies were excluded by computerized tomography scan of the temporal bone and magnetic resonance imaging. This was the standard protocol adopted in all the patients. To exclude viral etiology, IgG and IgM titres were determined in the patients.

2.1. Audiometric testing

Audiometric analysis was performed to diagnose the NSHL and to measure the severity of hearing loss. A detailed examination including otoscopy was carried out in all the subjects to rule out any evidence of syndrome. Hearing levels were measured by pure tone audiometry. Pure tone thresholds were obtained at 0.5, 1, 2, 3, 4, 6 and 8 kHz. The degree of hearing impairment was based on average threshold calculated over frequencies of 0.5, 1 and 2 kHz [pure-tone average = $PTA_{0.5–2\ kHz}$] in the better ear. Hearing impairment was then categorized following the guidelines of GENDEAF study; Group HL is defined as mild with a $PTA_{0.5–2\ kHz}$ 21–40 dB, moderate with a $PTA_{0.5–2\ kHz}$ 41–70 dB, severe with a $PTA_{0.5–2\ kHz}$ 71–95 dB, and profound with a $PTA_{0.5–2\ kHz}$ greater or equal to 95 dB. The shape of the audiogram and presence of asymmetry [defined as a 10 dB difference between ears at three frequencies, 15 dB at two frequencies, or 20 dB difference at one frequency] were also noted. In young children, behavioral audiometry was used to determine the auditory thresholds in free field conditions. All subjects underwent ABR (auditory brainstem response) assessment to objectively determine the hearing levels. Impedance audiometry including tympanometry and stapedial reflex (Madsen Orbiter 901,226 Hz probe) was done to rule out any middle ear abnormality. Type A curve was when the peak was between −100 daPa and 200 daPa and static compliance was between 0.25 ml and 1.5 ml, type B was when static compliance was lower than 0.25 ml and type C when the peak was greater than 0.25 ml but the pressure was less than −100 daPa. Otoacoustic emissions (OAE) were also done in all the patients.

2.2. Mutation detection for GJB2

Genomic DNA was extracted from whole blood following standard phenol–chloroform method. All patients and control subjects were initially screened for six known GJB2 mutations (35delG, W24X, W77X, Q124X, 167delT and 235delC) using PCR-RFLP and AS-PCR assays [11]. 51 patient samples, which were heterozygous for known GJB2 mutations and 152 patient samples, which were found to be negative for tested GJB2 mutations, were screened to identify other mutations in coding and non-coding exons of GJB2 by direct sequencing. The complete coding region of GJB2 was PCR amplified using previously described method [11].

2.3. Mutation detection for GJB6

Patients without GJB2 sequence variants ($n = 152$) as well as those carrying monoallelic GJB2 sequence variants ($n = 51$) were analyzed for del(GJB6-D13S1830) deletion [10]. Subsequently, we screened these patients for other mutations in the coding regions of the GJB6 genes.

2.4. GJB6 gene analysis

The entire coding region of GJB6 gene was amplified in four overlapping fragments using the primer pairs shown in Table 1. These amplicons were screened for the presence of variants by denaturing high-performance liquid chromatography (DHPLC) followed by direct sequencing. Mutation screening was carried out using WAVE DNA fragment analysis system equipped with autosampler (Transgenomic WAVE system D7000IF equipped with an autosampler). Prior to injection in the WAVE system, the PCR products were denatured to allow the formation of heteroduplexes for mutation detection by DHPLC. 10 μl of the PCR product was heat denatured at 95 °C for 5 min and then allowed to cool in 5 °C decrements until 25 °C was reached. Mutation detection in different amplicons was done at their respective melting temperatures (Table 1). Briefly, 10 μl of PCR product was injected into the preheated column made up of non-porous matrix consisting of polystyrene–divinylbenzene (PS–DVB) co-polymer beads that are alkylated with C-18 chains. Triethyl-ammonium acetate (TEAA) was used as a bridging molecule that helped in the adsorption of nucleic acid on the column as column is neutral in charge and not readily reacts with nucleic acid. DNA from the column was eluted out using a linear acetonitrile gradient that was

Table 1
Primer pairs and DHPLC conditions for GJB6 gene analysis.

Spanning region	Primers	Amplicon size (bp)	DHPLC T_m (°C)
Exon 2 Chr13: 19695406–19695638	F: TCAGGGATAAACCAGCGCAAT R: ACACCGGGAAAAAGTGGTCAT	233	62.3
Exon 2 Chr13: 19695192–19695479	F: GCAAGAGGACTTCGTCTGCAACA R: CGGAAAAAGATGCTGCTGGTGT	288	60.2
Exon 2 Chr13: 19694976–19695253	F: AAGCACAAGGTTCGGATAGAGG R: AGCAGCAGGTAGCACAACTCTG	278	59.6
Exon 2 Chr13: 19694767–19695045	F: CCATTTTTATGATTTCTGCGTCTG R: GTTGGTATTGCCTTCTGGAGAAGA	279	58.0