RESEARCH ARTICLE

GJB2 and *GJB6* gene mutations found in Indian probands with congenital hearing impairment

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Abstract

Genetically caused deafness is a common trait affecting one in 1000 children and is predominantly inherited in an autosomal-recessive fashion. Several mutations in the *GJB2* gene and a deletion of 342 kb in *GJB6* gene (del*GJB6*-D13S1830) have been identified worldwide in patients with hearing impairment. In the present study, 303 nonsyndromic hearing-impaired patients (140 familial; 163 sporadic) were examined clinically and screened for mutations in *GJB2* and *GJB6* genes. Mutations in *GJB2* gene were found in 33 (10.9%) patients of whom six (18.2%) were carriers for the mutant allele. The most frequent mutation was p.W24X accounting for 87% of the mutant alleles. In addition, six other sequence variations were identified in the *GJB2* gene viz., c.IVS1+1G>A, c.167delT, c.235delC, p.W77X, p.R127H (polymorphism), p.M163V. None of the samples showed del(*GJB6*-D13S1830) or any point mutations in *GJB6* gene.

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Introduction

Hearing impairment (HI) is the most frequent sensory disorder involving a multitude of factors, and at least 50% of the cases are due to genetic etiology (Morton and Nance 2006). A disruption of different classes of proteins involved in ion homeostasis, cytoskeletal and extracellular matrix components, transcription factors, receptors, cellular trafficking proteins and molecules belonging to the cadherin superfamily may all cause HI, with or without associated syndromic features (Bitner-Glindzicz 2002). Syndromic forms constitute about one third of congenital HI, and the remaining two-thirds are nonsyndromic. Among the nonsyndromic forms, the large majority (70%–80%) are autosomal recessive, often characterized by being profound and prelingual (Cryns *et al*. 2004).

To date, about 80 loci for autosomal recessive nonsyndromic deafness have been identified (http://dnalab-www.uia.ac.be/dnalab/hhh/), exhibiting extreme heterogeneity. *DFNB1* was the first locus incriminated in autosomal recessive deafness, and two genes have been associated with this locus: *GJB2* and *GJB6*, encoding gap-junction proteins connexin 26 and connexin 30, respectively. Despite this heterogeneity, up to 50% of prelingual recessive nonsyndromic deafness can be attributed to mutations in *GJB2* (Denoyelle *et al*. 1997; Green *et al*. 1999). More than 90 different mutations of the *GJB2* gene have been reported. Many are 'private' mutations, having been observed in only one or few pedigrees, although very common alleles have also been identified in several populations including the c.35delG allele in Caucasians, c.167delT allele in Ashkenazi Jews, c.235delC allele in east Asian population, and p.R143W mutation in Ghana (Nance 2003). Also, a functional study conducted by Mani *et al*. (2008) on Cx26 mutations provided evidence that Cx26 mutations affect gap-junction activity by misregulation at multiple levels.

The second gap-junction protein, connexin 30 is expressed in the same inner-ear structures as connexin 26 and both the connexins are functionally related (Lautermann *et al*. 1999). The importance of *GJB6* to normal hearing

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has been confirmed by the identification of a large deletion del(*GJB6*-D13S1830) involving the first two exons and a part of third exon of *GJB6*, and a large region of the upstream sequence. Homozygotes for this deletion and compound heterozygotes carrying del(*GJB6*-D13S1830) and a deafness causing allele variant of *GJB2* have severe to profound congenital deafness. Although, there are previous studies reporting the types of *GJB2* mutations in the Indian population (Maheswari *et al*. 2003; Ramshanker *et al*. 2003; Ramchander *et al*. 2005), no data are available on the presence of the mutation del(*GJB6*-D13S1830) from India. In the present study, we assessed the prevalence of *GJB2* and *GJB6* mutations in patients from India with nonsyndromic HI.

Materials and methods

Patients

A total of 456 cases with profound (≥90) bilateral sensorineural congenital HI gave their consent to participate in the study were evaluated for various epidemiological parameters like sex, family history, parental consanguinity, details of milestones (table 1), birth ranks of the probands (table 2), parental age at the time of conception of probands, sib mortality and audiology. Of these, 303 HI patients co-operated to give the blood samples and were tested for the presence of mutations in *GJB2* and *GJB6* genes. These cases were referred to the Government Ear, Nose and Throat (ENT) Hospital, Hyderabad, India, and schools for deaf that are in and around the district of Hyderabad, India. Hyderabad being a cosmopolitan city includes people belonging to different states who have settled here. Also, cases from surrounding districts of Hyderabad come to the city for treatment and training. Hence, the ethnicity of the cases cannot be specified. All the probands were examined by the ENT specialists and the loss of hearing was graded based on the pure-tone audiometry (PTA). Inclusion criteria for this study were deafness graded as profound sensorineural hearing loss (≥ 90) with no associated acquired etiology. Thus, patients with a history of acquired etiology, genetic syndrome, otoacoustic trauma or any neonatal disease, were excluded from the study. Also 200 healthy controls with normal hearing and without a history of deafness in their family were selected at random to compare with the data generated on the probands.

Mutation analysis

DNA from all the patients and controls was isolated from leukocytes present in 5 mL of anticoagulated whole blood using rapid non-enzymatic method (Lahiri and Nurnberger 1991). *GJB2* gene was screened for already reported common mutations (c.IVS1+1G>A (exon 1), p.W24X (c.71G>A), c.35delG, c.167delT, p.W77X (c.231G>A), c.235delC) in exons 1 and 2 using allele-specific PCR amplification (ARMS PCR) and RFLP methods, and by single-strand conformation polymorphism (SSCP) analysis for other novel mutations. Table 3 summarizes the primer sequences and techniques used for the detection of different mutations in exons 1 and 2 of *GJB2* gene. ARMS PCR amplification of genomic DNA was performed for screening of c.35delG and p.W77X mutations using primers as described by Scott *et al*. (1998) and the products were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide. To check for the presence of c.IVS1+1 G>A mutation in exon 1 of *GJB2* gene restriction enzyme digestion was performed on the PCR products amplified using primers Ex1F and Ex1R mentioned in table 3. The presence of c.IVS1+1 G>A mutation, results in loss of *Hph*1 restriction site. *Hph*1 digestion of DNA derived from mutant subjects produced a single fragment of 363 bp, normal subjects produced two fragments of 245 bp and 118 bp, and heterozygotes produced all the three fragments. Also mutations p.W24X (Ramshanker *et al*. 2003), c.167delT (Lerer *et al*. 2001) and c.235delC (Kudo *et al*. 2000) were detected by restriction digestion of PCR products using *Alu*1, *Pst*1 and *Apa*1 enzymes respectively and the products were genotyped using 8% polyacrylamide gels followed by staining with ethidium bromide.

GJB6 gene was screened for del(*GJB6*-D13S1830) using primers Cx30-1 (5′-TCAGGGATAAACCAGCGCAAT-3′) and Cx30-8 (5′-GTTGGTATTGCCTTCT GGAGAAGA-3′) to amplify the Cx30 gene and primers *GJB6*-1R (5′-TTTAGGGCATGATTGGGGTGATTT-3′) and BKR1 (5′-CACCATGCGTAGCCTTAACCATTTT-3′) to amplify the breakpoint junction of the deletion as described by del Castillo *et al*. (2002). Samples that are homozygous for Cx30 deletion would amplify with only *GJB6*-BKR1 primer pair while those heterozygous would amplify with both the primers.

SSCP analysis was performed for three overlapping regions of exon 2 of *GJB2* gene and for four overlapping regions of exon 3 of *GJB6* gene to detect point mutations if any. The regions were amplified using primers as described in table 3. The PCR products were denatured in LIS (low ionic strength buffer; 10% sucrose, 0.01% bromophenol blue and 0.01% xylene cyanol) buffer at 95°C for 10 min and electrophoresed in 12% polyacrylamide gel (37.5:1) with glycerol for ~16 h at 100 V. The gels were visualized by silver staining. All the variants were confirmed by sequencing on ABI 3100 DNA sequence analyzer.

Results and discussion

Table 1 describes the demographic features found in the patients studied. There was a higher frequency of males than females with the presence of family history, elevated consanguinity among the parents of the probands and delayed milestones (table 1). High incidence of NSHI (nonsyndromic HI) in primiparous cases (39%) was observed indicating higher risk in first born children as compared to later born children (table 2). Considering the parental age

at the time of conception of probands, it was observed that the frequency of mothers at the conception of male and female probands was maximum between the age group of 20–24 years (43.1%; 41.6%) and regarding paternal age maximum frequencies of birth of male and female probands was seen in the age group of 25–29 years (42.0% and 36.6%). Sib mortality was found to be significantly high among the sibs of male probands (19.2%) as compared to the female probands (13.2%; $\chi^2 = 7.140$; $P \leq 0.01$). All the cases had profound HI requiring decibels ≥ 90.

Table 1. Sex wise distribution of probands with NSHI based on the incidence of family history parental consanguinity and milestones.

	Males		Females		Total	
	N	%	*N*	%	*N*	%
NSHI	284	58.7	200	41.3	484	
Familial	106	59.9	71	40.1	177	36.6
Non-familial	178	58.0	129	42.0	307	63.4
Consanguineous	152	58.0	110	42.0	262	54.1
Non-consanguineous	132	59.5	90	40.5	222	45.9
Delayed milestones	94	59.5	64	40.5	158	32.6
Normal milestones	190	58.3	136	41.7	326	67.4

Table 2. Distribution of primi and other para cases with NSHI.

	Primipara		Other para		Total
	N	%	*N*	%	*N*
NSHI	189	39.0	295	61.0	484
Males	100	35.2	184	64.8	284
Females	89	44.5	111	55.5	200
Familial	68	38.4	109	61.6	177
Non-familial	121	39.4	186	60.6	307
Consanguineous	95	36.3	167	63.7	262
Non-consanguineous	94	42.3	128	57.7	222
Delayed milestones	67	42.4	91	57.6	158
Normal milestones	122	37.4	204	62.6	326

Table 3. Primer sequences and the technique used for the detection of different mutations in exons 1 and 2 of *GJB2* gene.

Mutation	Primer sequences	Technique used
c.IVS1+1 G>A	Ex1F: 5′TCC GTA ACT TTC CCA GTC TCC GAG GGA AGA G-3′ Ex1R: 5′ CCC AAG GAC GTG TGT TGG TCC AGC CCC-3′	RFLP (*Hph*1)
c.35delG	Nor: 5′-TTG GGG CAC GCT GCA GAC GAT CCT GGG GAG-3′ Mut: 5′-TTG GGG CAC GCT GCA GAC GAT CCT GGG GAT-3′ Com: 5′-GAA GTA GTG ATC GTA GCA CAC GTT CTT GCA-3′	ARMS PCR
p.W77X	Nor: 5′-TAC TTC CCC ATC TCC CAC ATC CGG CTA TTG-3′ Mut: 5′-TAC TTC CCC ATC TCC CAC ATC CGG CTA TCA-3′ Com: 5′-GAT GAC CCG GAA GAA GAT GCT GCT TGT GTA-3′	ARMS PCR
p.W24X	1F: 5′-TCT TTT CCA GAG CAA ACC GC-3′ 1R: 5′-GAC ACG AAG ATC AGC TGC AG-3′	RFLP (*Alu*1)
c.235delC	1F: 5′-TGT GTG CAT TCG TCT TTT CCA G-3′ 4R: 5′-GGT TGC CTC ATC CCT CTC AT-3′	RFLP (*Apa*1)
c.167delT	271: F 5′-GCT CAC CGT CCT CTT CAT TT-3′ 508: R 5′-CTT CTT CTC ATG TCT CGG GTA-3′	RFLP (*Pst*1)
Region 1	Cx26 1F: 5′-TCT TTT CCA GAG CA ACC GC-3′ Cx26 3R: 5′-GAC ACG AAG ATC AGC TGC AGG-3′	SSCP
Region 2	Cx26 10F: 5′-GCC GAC TTT GTC TGC AAC-3′ Cx26 15R: 5′-GAA GAT GCT GCT TGT GTA GGT-3′	SSCP
Region 3	Cx26 17F: 5′-GAA GGT GCG CAT CGA AGG CT-3′ Cx26 5R: 5′-GGG CAA TGC GTT AAA CTG GC-3′	SSCP

Results of mutation analysis are summarized in table 4. Of the 303 probands screened for Cx26, a total of 33 (10.9%) had mutations in *GJB2* gene with six different mutations (c.IVS1+1G>A, p.W24X, c.35delG, c.167delT, c.235delC and p.W77X) known to cause NSHI. The most common cause was p.W24X accounting for nearly 86.7% (table 5) of the mutated alleles. This mutation was homozygous in 24, and heterozygous in four probands with no other detected mutation in Cx26 and hence were considered as compound heterozygotes with the second allele yet to be explored. This mutation was first described in a Pakistani family (Kelsell *et al*. 1997) and later in several Asian families (Scott *et al*. 1998; Kudo *et al*. 2000; Najmabadi *et al*. 2002). This mutation was also found to be a common allele among the mutations causing autosomal recessive non-syndromic HI (ARNSHI) in previous studies from Indian population (Maheswari *et al*. 2003; Ramshanker *et al*. 2003; Ramchander *et al*. 2005), and among Slovak gypsies (Minarik *et al*. 2003) and haplotype analysis of markers flanking the *GJB2* gene suggested a possible founder effect for this mutation in Indian population (Maheswari *et al*. 2003; Ramshanker *et al*. 2003).

Table 4. Prevalence of mutations detected in probands with nonsyndromic hearing loss and in normal hearing controls.

	No. of probands	No. of controls
Cx26 mutations		
Exon 1 of Cx26:		
c.IVS1+1G>A/c.IVS1+1G>A	1	–
c.IVS1+1G>A/+	1	–
Exon 2 of Cx26:		
p.W24X/p.W24X	24	–
p.W24X/+	4	–
c.167delT/+	1	–
c.235delC/c.235delC	1	–
p.W77X/p.W77X	1	–
p.M163V/p.M163V	–	1
Cx26 polymorphisms		
p.R127H/p.R127H	4	–
p.R127H/+	77	70

Cx, connexin; +, denotes presence of a wild-type allele (no mutation detected in either Cx26 or Cx30).

Table 5. Frequency of mutated alleles in probands with nonsyndromic hearing loss.

Mutation	No. of alleles	Total (% of all mutated alleles)
c.IVS1+1G>A	3	5.0
p.W24X	52	86.7
c.167delT	1	1.7
c.235delC	2	3.3
p.W77X	2	3.3
Total	60	

In our study, 7.7% of p.W24X alleles were present as heterozygotes with no other mutation detected in Cx26 and Cx30. There are several reasons to explain this result: (i) the second mutated allele in the probands may be located in a *GJB2* region that was not analysed (promoter), (ii) the second mutated allele may be present in an entirely different gene, and (iii) the second mutated allele could be a third putative gene making the condition triallelic that has not been yet identified. None of the 200 normal hearing cases showed the presence of p.W24X mutation. The high frequency of W24X mutation in our population as compared to other populations supports the view that it is a mutation specific to Indian population.

Among the other variants identified, c.235delC reported to be a common mutation in Japanese population and p.W77X first reported in two families from Pakistan and one case from India, were found to be homozygous in one proband each (0.3%). The c.167delT mutation found to be common mutation in Ashkenazi Jewish and several other populations (Kelley *et al*. 1998; Morell *et al*. 1998; Sobe *et al*. 1999; Lerer *et al*. 2000), was detected in heterozygous state in only one proband, which is the first report from the Indian population. Regarding c.35delG mutation which is predominantly seen in Caucasians and other Mediterranean populations (Italy 88.0%, Spain 55.0%, Australia 55% and Lebanon 94.0%; Estivill *et al*. 1998; Lench *et al*. 1998), none of the NSHI and control samples showed the mutation. Absence of c.35delG and low incidence of c.167delT, c.235delC and c.231G>A in the present study might suggest a possible role of promoter methylation or other epigenetic factors modulating the expression of Cx26 in the pathogenesis of HI.

R127H was found in homozygous state in four probands (1.3%) and in heterozygous state in 77 probands (25.4%) and 70 controls. This mutation was previously described in HI patients as the sole mutation found in Cx26, affecting a residue that is not highly conserved among β-connexins (Estivill *et al*. 1998; Rabionet *et al*. 2000). The high frequency of p.R127H in cases with NSHI and controls in the present study suggests that it may be or may not be a causative mutation for hearing as supported by the earlier studies from the Indian population (Ramshanker *et al*. 2003; Ramchander *et al*. 2005).

Of the 200 controls studied for *GJB2* mutations, one was found to be heterozygous for p.M163V mutation previously reported in one French family (Marlin *et al*. 2001). The pathogenicity of this mutation is yet to be established. The mutation was detected by shift in the band by SSCP analysis and confirmed by sequencing using ABI 3100 automated DNA sequencer.

There are few reports with reference to the occurrence of mutations in the exon 1 of *GJB2* gene. c.IVS1+1G>A mutation in exon 1 of *GJB2* is a splice site mutation that was originally reported by Denoyelle *et al*. (1999) and was found to be more common in Czech population representing 4% of all pathogenic mutations in *GJB2* (Seeman and

Sakmoryova 2006). Also, a study from Netherlands showed that this mutation is the third most common in Dutch NSHL patients after the c.35delG and del(*GJB6*-D13S1830) mutations (Santos *et al*. 2005). In all the above studies, the mutation was found only in heterozygous individuals carrying one pathogenic mutation in the coding region of the *GJB2* gene. Hence it was suggested that it is not probably necessary to test for this mutation in all the patients but to screen for it in those individuals who are heterozygous for only one pathogenic mutation (Seeman and Sakmoryova 2006). In the present study, one homozygous mutant and one heterozygote were detected for c.IVS1+1G>A mutation, the frequency of which works out to be 0.3% (1 of 303 each) for both. In contrast to the earlier studies, these samples did not show any mutation in the coding region (exon 2) of *GJB2* gene. This suggests that screening for this mutation in all the probands with NSHI can be carried out instead of only in heterozygotes as recommended by Seeman and Sakmoryova (2006).

Connexin30 (*GJB6*) gene is located on chromosome 13q12, approximately 35-kb downstream of the *GJB2* gene. A 342-kb deletion (*GJB6*-D13S1830) was identified in a group of subjects with an unexplained cases of non-syndromic prelingual hearing loss (del Castillo *et al*. 2002). This deletion was found to be the second most frequent mutation causing prelingual deafness in the Spanish population (del Castillo *et al*. 2002). The deletion was also detected in *trans* in four of six hearing loss patients heterozygous for *GJB2* mutations and in homozygous state in one case of congenital profound deafness in France (Pallares-Ruiz *et al*. 2002). Digenic inheritance in those cases was supported by the findings that both genes, *GJB2* and *GJB6*, encode for gap-junction proteins and are expressed in same cells in the rat cochlea and in the cochlea of the 22-week-old human embryo (Lautermann *et al*. 1999).

Although there are studies reporting the types of *GJB2* mutations in the Indian population (Maheswari *et al*. 2003; Ramshanker *et al*. 2003; Ramchander *et al*. 2005), no data are available on the presence of del(*GJB6*-D13S1830) and other mutations in Cx30 from this population. In the present study, we assessed the prevalence of *GJB6* mutations in Indian population. None of the probands and controls screened showed mutations in Cx30 gene including the deletion mutation, which was also not detected in Chinese (Liu *et al*. 2002), Turkish (Tekin *et al*. 2003), Iranian (Riazalhosseini *et al*. 2005) and Jordanian deaf patients (Mahasneh and Al-Asseer 2005), supporting the view that the mutation is not widespread in Asia and suggesting a possible founder effect for this deletion in certain populations.

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