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Low prevalence of Connexin 26 (*GJB2*) variants in Pakistani families with autosomal recessive non-syndromic hearing impairment

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Abstract

The Pakistani population has become an important resource for research on autosomal recessive non-syndromic hearing impairment (ARNSHI) due to the availability of large extended and highly consanguineous pedigrees. Here is presented the first report on the prevalence of gap junction beta-2 (*GJB2*) variants in Pakistan. One hundred and ninety-six unrelated Pakistani families with ARNSHI were recruited for a study on the genetics of NSHI. DNA sequencing of the *GJB2* coding region was done on two affected individuals per family. Evolutionary conservation and predicted effect on the protein product were studied in order to hypothesize whether or not a variant was potentially deleterious. Homozygous putatively functional *GJB2* variants were identified in 6.1% of families. None of the putatively functional *GJB2* variants were observed in the compound heterozygous state. The six putatively causative variants noted were 231G > A(W77X), 71G > A(W24X), 167delT, 95G > A(R32H), 358–360delGAG(delE120), and 269T > C(L90P), with 231G > A(W77X) and 71G > A(W24X) being the most common. In addition, five benign polymorphisms, 380G > A(R127H), 457G > A(V153I), 493C > T(R165W), 79G > A(V27I), and 341A > G(E114G), were identified within this population. In a few individuals, benign polymorphisms were observed to occur on the same haplotype, namely [457G > A(V153I); 493C > T(R165W)] and [79G > A(V27I); 341A > G(E114G)]. The spectrum of *GJB2* sequence variants in Pakistan may reflect shared origins of hearing impairment alleles within the Indian

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Database information

On *A R* genes and loci: Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. URL: <http://dnalab-www.uia.ac.be/dnalab/hhh/> Accessed 31 August 2004.

On *GJB2* mutations and polymorphisms: Calvo J, Rabionet R, Gasparini P, Estivill X. The Connexin-deafness Homepage. URL: <http://www.crg.es/deafness> Accessed 31 August 2004.

On *GJB2* sequence: URL: <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=42558282> Nucleotide NM_004004.

For homology search: URL: <http://usexpasy.org/tools/blast/> Accessed 10 June 2004.

For multiple sequence alignment: URL: <http://www.ebi.ac.uk/clustalw/> Accessed 10 June 2004.

For prediction of effect of non-synonymous SNPs on protein: URL: <http://www.bork.embl-heidelberg.de/PolyPhen/> Accessed 20 May 2004.

For prevalence rates of consanguinity: Bittles A. URL: <http://www.consang.net/> Accessed 21 May 2004.

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subcontinent. The high degree of consanguinity within Pakistan may have maintained the *GJB2* prevalence at a much lower rate than within India and other populations.

Keywords

autosomal recessive non-syndromic hearing impairment; Connexin 26; consanguinity; *GJB2*; Pakistan

In 1997 Kelsell et al. (1) identified the first non-syndromic hearing impairment (*NSHI*) gene, the gap junction beta-2 gene (*GJB2*), which encodes for Connexin 26 (*Cx26*). Since then, 21 genes have been identified and 37 loci mapped for autosomal recessive (AR) NSHI (2). At present there are >100 known sequence variants for *GJB2*, of which 56 are associated with ARNSHI (3). Some *GJB2* mutations have high prevalence rates in specific populations, namely: 35delG among people of European descent, 167delT in the Ashkenazim, 235delC among East Asians, and 427C > T(R143W) in the Ghanaian population (4–7).

The elevated frequency of *GJB2* alleles in large populations has been attributed to relaxed selection and assortative mating in the past two centuries, i.e. a rapid increase in survival and reproductive fitness and higher rates of intermarriage among the deaf (8). Alternatively, being a carrier might confer a selective advantage for better survival, such as thicker epidermis and greater salt concentration in sweat (9). On the other hand, consanguinity was associated with a decreased risk of hearing impairment (HI) due to *GJB2* (10).

The Pakistani population has become an important resource for research on ARNSHI due to the availability of large extended pedigrees. In addition, about 60% of marriages are consanguineous, of which 80% are first-cousin unions (11). Here, we describe the allele frequency and spectrum of *GJB2* sequence variants in Pakistan using data on families with ARNSHI.

Materials and methods

The study was approved by the Quaid-I-Azam University Institutional Review Board and by the Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals. Informed consent was obtained from all family members who participated in the study.

One hundred and ninety-six unrelated Pakistani families with at least two ARNSHI individuals were ascertained from various regions of Pakistan. Medical and family history and information on pedigree structure were obtained from multiple family members. Pure-tone audiometry at 2500–8000 Hz was performed for selected subjects. All hearing-impaired (HI) family members underwent physical examination. No clinical features, including mental retardation, that would indicate that the HI was part of a syndrome, were observed. In addition, no gross vestibular involvement was noted. The HI phenotype was prelingual, severe to profound, and was not known to be caused by inflammatory middle ear disease or specific environmental factors.

DNA was isolated from venous blood samples following a standard protocol (12) and stored at –20 °C. Genomic DNA was diluted to 5ng/μl and optimized under standard polymerase chain reaction (PCR) conditions with primers 5'-TGTGCATTCGTCTTTTCCAG-3' and 5'-GGGAAATGCTAGCGACTGAG-3'. The PCR products were then separated by electrophoresis to check proper amplification with genomic DNA. When the desired *GJB2* coding region was amplified, DNA from at least two affected individuals from each family were diluted to 5ng/μl, amplified, and purified with ExoSAP-IT® (USB Corp., Cleveland,

OH, 1999–2004). The second exon of *GJB2* was sequenced using either the forward or the reverse primer and the Big-Dye[®] Terminator v3.1 Cycle Sequencing Kit together with an Applied Biosystems 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, 2004). Single nucleotide polymorphisms (SNPs) were identified via SEQUENCHER[™] Version 4.1.4 software (Gene Codes Corp., Ann Arbor, MI, USA, 1991–2002).

To determine the evolutionary conservation of identified substitutions, the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics was used to look for homologs of the *Cx26* protein. ExPASy uses the NCBI BLASTP 1.5.4-PARACEL program (13) to search the ExPASy/UniProt database. To perform the BLASTP search, the default settings were used, except that the threshold for expected random matches (E) was set to a more conservative value of 10^{-4} in order to minimize false-positive results. Of the 256 query matches (score range 50–470), one match per species was chosen for further alignment. The match that was selected for each of 36 non-human species was least similar to human *Cx26* protein sequence in order to decrease the bias for mammalian *Cx26* protein sequences. All 37 proteins, including the human *Cx26* sequence, were then submitted for multiple sequence alignment via ClustalW (14) at the European Bioinformatics Institute using default settings.

The web server PolyPhen from the European Molecular Biology Laboratory was used to assess the functionality of identified substitutions. It annotates non-synonymous SNPs through the identification of sequence changes in comparison with the HGVS v.12 database (15). It uses both mathematical algorithms and an empirically derived set of prediction rules to predict the phenotypic effect of the substitution.

For all point estimates 95% confidence intervals (CI) were calculated using the binomial distribution. To determine the effect of ethno-linguistic origin on the distribution of *GJB2* alleles, chi-square tests were applied.

Results

Of 196 families studied, 94% presented with consanguineous matings, with an average of three consanguineous matings per pedigree. In total, 430 affected subjects were screened for *GJB2* variations. Forty-eight of the families had 11 different variants within the *GJB2* gene. Six of the identified variants are putatively functional variants while five other identified polymorphisms are presumably benign (Table 1). No 35delG, 235delC, or 427C > T(R143W) alleles were detected. One pedigree presented with *HI* individuals who were homozygous for 167delT. The allele frequency in this study population for both putatively functional and benign *GJB2* variants is 16.6% (95% CI: 13.0, 20.6) while the allele frequency for functional variants alone is 6.6% (95% CI: 4.4, 9.6). There were two families for which only heterozygous individuals were identified with putatively causative variants 71G > A(W24X) and 269T > C(L90P). It was considered that these families did not have *HI* due to *GJB2*. Therefore, only 6.1% (95% CI: 3.2, 10.4) of the families had *GJB2*-related *HI*. In two of these families, one affected family member was homozygous for a functional *GJB2* variant while another *HI* family member was homozygous wild type. This suggests that for these families there is either intrafamilial heterogeneity and/or phenocopies present (Table 1).

Table 2 enumerates the families who had an affected individual who was heterozygous for two different putatively benign variants. For these individuals the DNA from their normalhearing parents was sequenced for *GJB2* in order to determine the haplotype for the *HI* individual. It was found that the individuals who had polymorphisms 380G > A(R127H) and 457G > A(V153I) were compound heterozygous while the [79G > A(V27I); 341 A >

G(E114G)] and [457G > A(V153I); 493C > T(R165W)] polymorphisms occurred on the same haplotype. The [79G > A(V27I); 341 A > G(E114G)] haplotype was previously reported in the Japanese population (20) while the [457G > A(V153I); 493C > T(R165W)] haplotype was observed in a large Sri Lankan pedigree (21) (Table 2).

When multiple sequence alignment was done using non-Cx26 proteins from 36 species, residues for 95G > A(R32H) and 231G > A(W77X) were shown to be highly conserved. Residues for 71G > A(W24X) and 79G > A(V27I) were also conserved. The 269T > C(L90P) variant was at a semiconserved residue, while the other variants, 41A > G(E114G), 380G > A(R127H), 457G > A(V153I) and 493C > T(R165W) were at residues that were not conserved.

The results of the homology search were mostly supported by the functional annotation from the PolyPhen web server, wherein the *GJB2* variants 71G > A(W24X), 95G > A(R32H), 231G > A(W77X), and 269T > C(L90P) that occurred at conserved residues were deemed possibly damaging, while those at non-conserved residues, variants 341A > G(E114G), 380G > A(R127H), 457G > A(V153I), and 493C > T(R165W) were considered benign. The only exception was 79G > A(V27I), which occurred at a conserved residue based on the homology search but was predicted to be functionally benign. This can be explained by its location at a transmembrane region which, for hydrophobic residues, is variable in conservation according to a predicted hydrophobic and transmembrane matrix (PHAT) (22). In contrast 231G > A(W77X), 95G > A(R32H), and 71G > A(W24X), which also occur at the transmembrane region, were considered conserved and damaging due to polarity of residues. The 269T > C(L90P) substitution at the transmembrane region, though with a hydrophobic residue, results in a negative PHAT matrix score and was thus considered possibly damaging.

Of the families that were studied, 70% come from the Punjab province, the largest province in Pakistan. The native languages of these families are Punjabi and Sairiki. The remaining 30% of the families in the study were equally representative of the other provinces and their corresponding language groups. No significant association was found between the presence of a particular *GJB2* variant and the region of origin or linguistic background. Interestingly, four of five 71G > A(W24X) families spoke Sairiki.

Discussion

Allele frequency and spectrum of *GJB2* variants

The prevalence of putatively functional *GJB2* variants is in general 3–4 times higher in Europeans and Middle Easterners than in Pakistanis (Table 3). There was one exception in the Middle East, Oman, where the prevalence of HI-associated *GJB2* variants was reported to be 0.0 (95% CI: 0, 0.04). This observation could be due to the mixed population and that only 35delG and 167delT were screened (28). The prevalence rates of functional *GJB2* variants within Africa varied greatly depending on country, with Kenya and Sudan having lower prevalence rates than Pakistan, while in Tunisia and Ghana the prevalence rates were twice higher than those observed in Pakistan (7,29,30). When 95% CI were estimated for the prevalence rates of the putatively functional variants, there was overlap of the 95% CI for the Pakistani prevalence rate and the CIs of the prevalence rate for several Mid-east, African, and East Asian Countries and Brazil (18,19,26,27,29–34). It should be noted that most of the studies from these countries had small sample sizes (Table 3).

More importantly, however, the spectra of sequence variations in these studies were essentially different from those observed in Pakistan (Table 3). The commonly described mutations 35delG, 427C > T(R143W), and 235delC were not found in the Pakistani sample.

Conversely, the 231G > A(W77X) variant has only been reported in people originating from the Indian subcontinent (17,21,35). The 71G > A(W24X) variant has been observed in Europeans, but its prevalence is about three times higher in Pakistanis and at least 20 times higher in Indians (16,17,23,35). In addition, 380G > A(R127H) and 457G > A(V153I) had much higher frequencies among Indians and Pakistanis than in Europeans. The 493C > T(R165W) polymorphism has only been observed in families from the Indian subcontinent (17,21).

Thus, the spectrum of *GJB2* variants in Pakistan may reflect shared origins of HI alleles within the Indian subcontinent. For example, the Slovak Romanies who migrated from India demonstrated a similar pattern of *GJB2* allele distribution, with the 71G > A(W24X) and 380G > A(R127H) variants as most common (36). Although there was similarity in the *GJB2* variants that were observed in Pakistanis and Indians, absolute frequencies were not similar. The allele frequency of *GJB2* sequence variants is two times higher in India than in Pakistan (16). For putatively functional variants, the prevalence rate in India is three times higher than in Pakistan. Notably the 71G > A(W24X) variant is seven times more common in India than in Pakistan whereas 231G > A(W77X) comprised a bigger proportion of *GJB2* alleles among Pakistanis (16,35). It could be hypothesized that the elevated consanguinity rates in Pakistan vs the rest of the subcontinent have depressed the *GJB2* prevalence in this country.

Identified sequence variants

Based on the criteria of evolutionary conservation, predicted effect of amino acid substitutions on protein, and published frequencies among controls in different populations, there is strong evidence that 71G > A(W24X), 231G > A(W77X), 95G > A(R32H), and 358–360delGAG(delE120) are damaging mutations, whereas 380G > A(R127H), 457G > A(V153I), 493C > T(R165W), and [79G > A(V27I); 341A > G(E114G)] are benign polymorphisms (Table 1). This was corroborated by articles on profound hearing loss among 71G > A(W24X) and 231G > A(W77X) homozygotes, and in a patient with the [95G > A(R32H)+35delG] genotype (1,16,37). For 167delT homozygotes, HI is usually prelingual, bilateral, and non-progressive, but severity may range from mild to profound (5,24). The mutations 358–360delGAG(delE120) and 269T > C(L90P) failed to form functional gap junction channels in cellular studies (38–40) though the phenotype was less severe than in 35delG homozygotes (41,42).

On the other hand, 380G > A(R127H) and 341A > G(E114G) were not different from wild type in functional studies on transfected HeLa cells (38,39,43). Furthermore, 380G > A(R127H) and 457G > A(V153I) were mostly observed in the heterozygous state among the hearing-impaired, and in addition occurred with a relatively high frequency in the hearing control population (16,17). Polymorphisms 79G > A(V27I) and 341A > G(E114G) have been observed independently and as a haplotype. These variants have high frequencies among East Asians (19,20). Also, the 79G > A(V27I) polymorphism was observed in a hearing individual in the homozygous state (44). The 493C > T(R165W) variant has not been noted among hearing controls (17), nevertheless its predicted effect on the protein product point to its benignity.

All putatively functional *GJB2* variants occurred on the wild-type haplotype but for putatively benign variants there were a few cases in which individuals were observed to have two *GJB2* polymorphisms. Two individuals were identified to be compound heterozygous for the polymorphisms [380G > A(R127H)]+[457G > – > A(V153I)]. Higher allele frequencies for these SNPs among individuals from the Indian subcontinent as compared to other populations tend to support the existence of a founder effect (17,35). However, mutational hotspots cannot be ruled out. Similarly, the observation in the

Pakistani sample of haplotypes [457G > A(V153I); 493C > T(R165W)] and [79G > A(V27I); 341A > G(E114G)] which were reported in populations both within and outside the Indian subcontinent may suggest inheritance from common ancestors (20,21).

Effect of consanguinity on allele frequency

It is hypothesized that the increased *GJB2* frequency in some countries is due to assortative mating among the deaf (8). Only five of our families (2.6%) had matings between *HI* subjects, and within four of these families the couples were related. These unions may not have occurred due to the couples' hearing-impaired status but because of their kinship. The lower rate of *HI* due to *GJB2* variants in this sample of families with ARNSHI may be due to the increased occurrence of other recessive deafness loci in multiple inbred lineages that have been maintained for generations. Currently 14 loci and 9 genes for ARNSHI have been reported in Pakistani pedigrees (2). Notably, consanguinity is about twice as frequent in Pakistan than in India, which lends further support to the idea that the lower *GJB2* prevalence in Pakistan compared to India may be due to a higher consanguinity rate. Additionally, random genetic drift may explain the difference in *GJB2* prevalence between India and Pakistan. Ascertainment of more *HI* families may allow deeper scrutiny of the ethno-linguistic background, pedigree structures, and genetic constitution of the Pakistani population and further elucidation of the relationship between inbreeding and genetic *HI*.

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Table 1Gap junction beta-2 (*GJB2*) sequence variations identified in Pakistani families

Sequence variation	Number of families with homozygous/heterozygous genotype*	Evolutionary conservation [†]	Functional effect [‡]	Allele frequencies among controls in other populations [§]
71G > A(W24X)	4 homozygous 1 heterozygous	Conserved	Possibly damaging	0.0007 [French (16)], 0.01 [Indian (17)]
231G > A(W77X)	5 homozygous	Highly conserved	Possibly damaging	Not available
95G > A(R32H)	1 homozygous	Highly conserved	Possibly damaging	0.0001 [French (16)]
167delT	1 homozygous	–	Frameshift→truncated	0.001 [Palestinian (18)] 0.04 [Ashkenazim (5)]
358–360delGAG(delE120) Frameshift→truncated	1 homozygous 0.0009 [French (16)]			
269T > C(L90P)	1 heterozygous	Semi-conserved	Possibly damaging	Not available
380G > A(R127H)	1 homozygous, 18 heterozygous	Not conserved	Benign	0.007 [French (16)], 0.18 [Indian (17)]
457G > A(V153I)	1 homozygous 12 heterozygous	Not conserved	Benign	0.004 [French (16)], 0.06 [Indian (17)]
457G > A(V153I)	1 homozygous	Not conserved	Benign	R165W = 0 [Indian (17)]
493C > T(R165W)	2 heterozygous			
79G > A(V27I)	1 heterozygous	V27I: Conserved	Benign	V27I=0 [Indian (17)], 0.8 [Korean (19)]; E114G = 0
341A > G(E114G)		E114G: Not conserved		[Indian (17)] 0.4 [Korean (19)]; Haplotype = 0.05 [Japanese (20)]

* Only benign polymorphisms 380G > A(R127H) and 457G > A(V153I) were found in the compound heterozygous state.

[†] Human *GJB2* sequence was compared with various connexin and connexin-like protein sequences of 36 species via the NCBI BLASTP (13) program and the ExPASy/UniProt databases. Species include four primates, 14 other mammals, one avian, two reptiles, three amphibians, nine bony fishes, one cartilaginous fish and two urochordates.

[‡] Possible effect of amino acid substitutions on the protein structure and function were predicted via the web server PolyPhen (20).

[§] References cited in brackets.

Table 2

Pakistani families with compound heterozygosity (CH) or haplotypes (Hap) of gap junction beta-2 (*GJB2*) polymorphisms

Family ID	Genotype of HI individual	CH or Hap	Genotype of parents with normal hearing	Genotype of other HI relatives
4055	(R127H) het + (V153I) het	CH	Mother: (R127H) het + (V153)-Wt Father: NA	Half-sib by father: Wt
4063	(R127H) het + (V153I) het	CH	Mother: (R127)-Wt + (V153I) het Father: (R127H) het + (V153)-Wt	Sib: Wt
4078	(V153I; R165W) hom	Hap	Not tested	A Sib and a 3rd-cousin: (V153I) hom + (R165W) het
4126	(V153I; R165W) het	Hap	Mother: (V153I; R165W) het Father: Wt	Sib: Wt
4193	(V27I; E114G) het	Hap	Mother: Wt Father: (V27I; E114G) het	Sib: Wt
4218	(V153I; R165W) het	Hap	Mother: (R127H) + (V153; R165)-Wt Father: NA	1st-cousin once removed: Wt

HI, hearing-impaired; het, heterozygous; hom, homozygous; Wt, wild-type; NA, DNA not available.

Nucleotide changes: 79G > A = V27I; 341A > G = E114G; 380G > A = R127H; 457G > A = V153I; 493C > T = R165W.

Table 3

Overview of gap junction beta-2 (*GJB2*) variants and prevalence among the hearing-impaired in different populations

Place of origin	Probands (n)	Frequencies of homozygous and compound heterozygous probands with putatively functional <i>GJB2</i> variants (95% CI)*	Most common putatively functional <i>GJB2</i> Variant(s)	References
EUROPE ^{†a}	2146	0.28 (0.26, 0.30)	35delG	23
MIDDLE EAST				
Israel	64	0.39 (0.27, 0.52)	35delG, 167delT	24
Palestine [‡]	48	0.23 (0.12, 0.37)	35delG, 167delT	18
Lebanon ^{†b}	48	0.33 (0.20, 0.48)	35delG	25
Jordan	68	0.16 (0.08, 0.27)	35delG	26
Iran ^{†c, ‡}	83	0.11 (0.05, 0.20)	35delG	27
Oman ^{†d}	95	0 (0, 0.04)	–	28
AFRICA				
Ghana [‡]	365	0.16 (0.12, 0.20)	427C > T(R143W)	7
Tunisia ^{†e}	70	0.17 (0.09, 0.28)	35delG	29
Sudan [‡]	183	0.03 (0.009, 0.06)	35delG	30
Kenya [‡]	406	0 (0, 0.009)	–	30
SOUTH AMERICA				
Brazil [‡]	62	0.11 (0.05, 0.22)	35delG	31
EAST ASIA				
Japan	35 + 39	0.18 (0.10, 0.28)	235delC	20, 32
China [‡]	118	0.16 (0.10, 0.24)	235delC	33
Korea	147	0.05 (0.02, 0.10)	235delC	19
Thailand	12	0.17 (0.02, 0.48)	235delC	34
INDIAN SUBCONTINENT				
British Asian ^{†f}	13	0.23 (0.05, 0.54)	231G > A(W77X)	21
India ^{†g, ‡}	45 + 215	0.17 (0.13, 0.22)	71G > A(W24X)	17, 35
Pakistan	196	0.06 (0.03, 0.10)	231G > A(W77X), 71G > A(W24X)	This article

* Frequencies are based on number of probands and sporadic HI individuals tested. If subjects with prelingual and postlingual HI were specified, only prelingually HI individuals were included.

[†] Mutation detection strategies that were not by direct sequencing of the second exon of *GJB2* as follows:

^a Greece – initial screening for 35delG by allele-specific polymerase chain reaction (ASPCR) then sequencing for 35del heterozygotes.

^b Initial screening via polymerase chain reaction (PCR)-mediated site-directed mutagenesis and BsiYI digestion then sequencing for non-35delG subjects.

^c Initial screening for 35delG by ASPCR then single strand conformational polymorphism (SSCP) for 35delG heterozygotes.

^d Screening only for 35delG and 167delT by restriction fragment length polymorphism (RFLP) method.

^eInitial denaturing gradient gel electrophoresis (DGGE) screening then sequencing for samples with shifts.

^fdenaturing high performance liquid chromatography (DHPLC) screening then sequencing if with altered elution peaks.

^gInitial ASPCR analysis then confirmation by sequencing (35).

[‡]Only these samples [including (16)] from India) were tested for exon 1 of *GJB2*.