

Mutations of the Connexin 26 gene in families with non-syndromic hearing loss

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Abstract. Autosomal recessive non-syndromic hearing impairment (ARNSHI) is caused by mutations in the gap junction gene GJB2 (Connexin 26; Cx26) in numerous human populations. The aim of this study was to determine the frequency of six GJB2 mutations in 50 Syrian families with congenital deafness and in 180 controls. PCR-RFLP was used to detect the 35delG, 167delT, M34T, W24X, W77R and E47X mutations, and direct sequencing was performed for the 35delG mutation. The data revealed a high prevalence of the 35delG mutation among deaf families. Homozygous 35delG was detected in fifteen of the Syrian families (30%). A compound heterozygous genotype was observed in two families: one with the 35delG/167delT mutation (2%) and one with the 35delG/M34T mutation (2%). Nine families were heterozygous with no second identified mutation in Cx26: four with 35delG+/unknown (8%), four with 167delT/unknown (8%) and one with M34T/unknown (2%). The W24X, W77R and E47X mutations were not detected in any of the study subjects. Three individuals with the heterozygous 35delG genotype (1.66%) and five with the heterozygous 167delT genotype (2.77%) were detected among the controls. No other mutations were found among the controls. These results have important implications for the diagnosis and counseling of families with Cx26 deafness.

Introduction

Hearing impairment is the most common sensory defect in humans and affects one in 1,000 children, with its prevalence increasing with age (1). More than 60% of cases of hearing impairment are due to genetic causes (2). Over 130 loci for non-syndromic deafness have been identified in humans (3,4), and it is estimated that more than 100 genes are involved in

hearing loss (5). Despite this heterogeneity, mutations in the GJB2 (Connexin 26; Cx26) gene are responsible for approximately 60% of prelingual non-syndromic recessive hearing loss, with a carrier frequency ranging between 2 and 5% depending on the ethnic group (9).

DFNB1 (MIM 220290) was the first locus implicated in autosomal recessive deafness and prelingual hearing impairment, and was mapped to the 13q12 region (6). This locus additionally contains the genes GJB2 (MIM 121011) and GJB6 (MIM 604418), which encode the gap-junction proteins connexin 26 and connexin 30, respectively. Both genes are expressed in the cochlea in the inner ear (7), and are thought to be important for the passage of small metabolites, ion trafficking, homeostasis and the maintenance of endocochlear potential by providing a cell-cell pathway for the entry of potassium to the stria vascularis and back to the endolymph (8).

The GJB2 gene belongs to a family of transmembrane proteins with approximately twenty members in humans, and has a simple genomic structure comprising two exons. More than 90 different mutations of the GJB2 gene have been reported (10). Many are 'private' mutations observed in only one or a few pedigrees. However, commonly occurring alleles have been identified in several populations, including the 35delG allele, which is found in Caucasian (11), Mediterranean (12) and, less frequently, Egyptian (13), Turkish (14) and Jordanian (15) populations. Other frequently occurring mutations include 235delC among the Japanese and Koreans (16), 167delT among Ashkenazi Jews (17), R143W in an African village (18) and W24X in Indian populations (19).

The aim of this study was to determine the prevalence of six GJB2 gene mutations in Syrian patients with recessive non-syndromic congenital hearing impairment. To our knowledge, this is the first report on recessive autosomal deafness at a molecular level in Syrian families.

Materials and methods

Subjects and selection criteria. Fifty families were enrolled in this study. The patients were students attending the Educational Institute for the Hearing Impaired in Damascus. The age of the patients ranged from 2 to 18 years. Parents had normal hearing, with one or more affected children in each family. Written informed consent was obtained from all participants or from the parents of patients younger than 18 years.

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Table I. Parameters for PCR-RFLP used to detect point mutations.

Mutation	Primers sequences (5'-3')	Amplified fragment size (bp)	Restriction enzyme	Allele size (bp)		
				Normal	Homozygous	Heterozygous
35delG	F: GGTGAGGTTGTGTAA GAGTTGG R: CTGGTGGAGTGT <u>TTGTTCCAC</u>	207	BseLI	207	181+26	207+181+26
167delT	F: GCTCACCGTCTCTTCATTT R: CTTCTTCTCATGTCTCCGGTA	237	PstI	97+72+68	165+72	165+97+72+68
W24X	F: GAGGTATAATTGACAGATGAA R: CAAACCGCCCAGAGTAGAAG	114	XbaI	114	88+26	114+88+26
M34T	F: CCTTTGCAGCCACAACGAT ^a R: CAAACCGCCCAGAGTAGAAG	144	BclI	121+23	14ww4	144+121+23
E47X	F: GCAAAGGAGGTGTGGGGAGAC ^a R: GGATGTGGGAGATGGGGAAGTA	106	BfaI	106	85+21	106+85+21
W77R	F: CCATCTCCCACATCCGGCTC ^a R: GCCTTCGATGCGGACCTTCT	182	MspI	100+82	182	182+100+82

Underlined nucleotides correspond to cDNA nucleotide positions 233-254, with a mutation at nucleotide position 236. ^aBases modified to be compatible with the selected enzyme.

A detailed medical history and pedigree structure were obtained through personal interviews with the affected individuals or their unaffected relatives. The medical history included obstetric and perinatal data (whether the mother experienced toxoplasmosis, diabetes or other infections, or was exposed to medication, drugs or any vaccinations during pregnancy) and information on area of origin, consanguinity, onset, course and duration of hearing loss, symmetry of the hearing impairment, history of chronic diseases such as middle ear infections, medical treatment, noise damage, trauma, meningitis, ototoxic agents, head trauma, fever and ear operations.

Careful clinical examination was conducted to exclude patients with syndromic deafness and other associated anomalies. Only patients with a bilateral hearing disorder and congenital onset were included in the study.

Pure-tone audiometry and speech audiometry were performed to determine the degree of hearing in both ears. Auditory brain stem response was evaluated to confirm the hearing threshold, and otoacoustic emission was used to exclude patients with central auditory system injury.

Genetic analysis.

DNA extraction. Anticoagulated venous blood (5 ml) was obtained from the patients and controls. DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Purified DNA was run on a 0.8% agarose gel. The quality and quantity of the DNA was determined spectrophotometrically.

Polymerase chain reaction. Primer sets easily differentiated by restriction enzyme and direct sequencing were designed to amplify the fragment containing the mutation (Table I) (20). Polymerase chain reaction (PCR) was performed in a final

volume of 25 μ l containing 30 ng of genomic DNA, 12.5 μ l AmpliTaq Gold PCR Master Mix (Applied Biosystems, USA), 1 μ l (10 pmol) of each primer and 9.5 μ l of distilled water. Amplification was carried out in a thermocycler (TC-512; Techne, UK). The amplification procedure for the 35delG, 167delT, M34T, E47X and W77R mutations was: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. For the W24X mutation, the same amplification protocol was followed at one exception: the annealing temperature was 57°C for 1 min. The amplification products were loaded on a 2.5% agarose gel with 1X TAE running buffer and visualized using ethidium bromide fluorescence under ultraviolet light. The band size was determined using a 100-bp DNA ladder (Fermentas, Lithuania).

Restriction fragment length polymorphism technique. Mutations were detected using PCR-restriction fragment length polymorphism (RFLP) (21). PCR products were digested using the restriction enzymes (Fermentas, Lithuania) listed in Table I. The total reaction volume contained 15 μ l of the PCR product, 2 μ l of 10X NB buffer, 1 μ l of a specific enzyme (10 U/ μ l) and 2 μ l of nuclease-free water. The reaction mixture was incubated according to each restriction enzyme. Digested PCR products (15 μ l) were added to 3 μ l 6X loading dye and loaded on a 5% 3:1 agarose gel (Eurobio, France) and run at 100 V for 2 h. The product size was determined using a 25-bp DNA ladder (Pan-Biotech, Germany).

Sequencing. Direct DNA sequencing for the 35delG mutation was carried out in both directions using forward and reverse primers (Table I) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The cycle-sequencing reaction was performed in a 10- μ l volume containing 1 μ l of the terminator ready reaction, 5 pmol of

Table II. Frequency of mutations in exon 2 of Connexin 26 in families with non-syndromic hearing loss.

Mutation	Genotype	Frequency	
		Families (n=50) no. (%)	Controls (n=180) no. (%)
35delG	35delG/35delG	15 (30)	-
	35delG/167delT	1 (2)	-
	35delG/unknown	4 (8)	3 (1.66)
	35delG/M34T	1 (2)	-
167delT	167delT/unknown	4 (8)	5 (2.77)
M34T	M34T/unknown	1 (2)	-
W24X	-	-	-
E47X	-	-	-
W77R	-	-	-

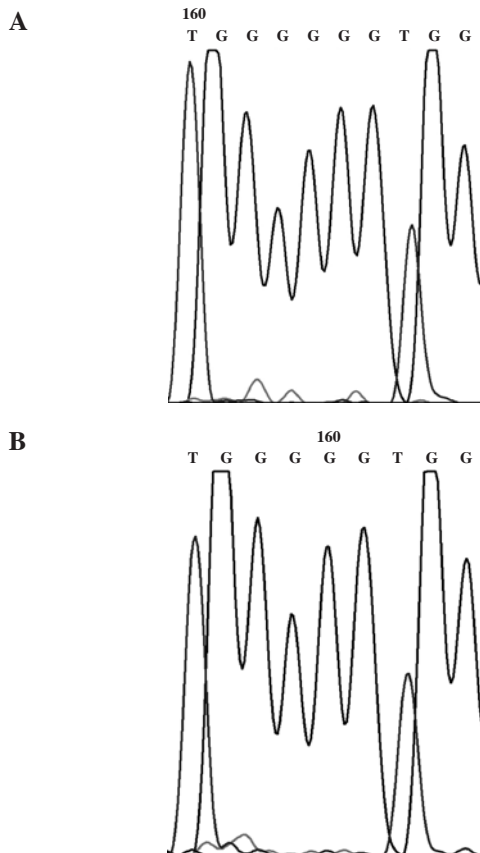


Figure 1: DNA sequencing of the GJB2 coding exon for the 35delG mutation. (A) Wild type DNA; (B) mutant DNA.

either the forward or reverse primer and 10 ng of purified PCR product (ExoSAP-IT kit; Amersham BioSciences, Piscataway, NJ). The thermal cycle protocol was 95°C for 4 min followed by 30 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min (ABI GeneAmp PCR System 9700, Applied Biosystems). Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) were used for the effective and reliable removal of

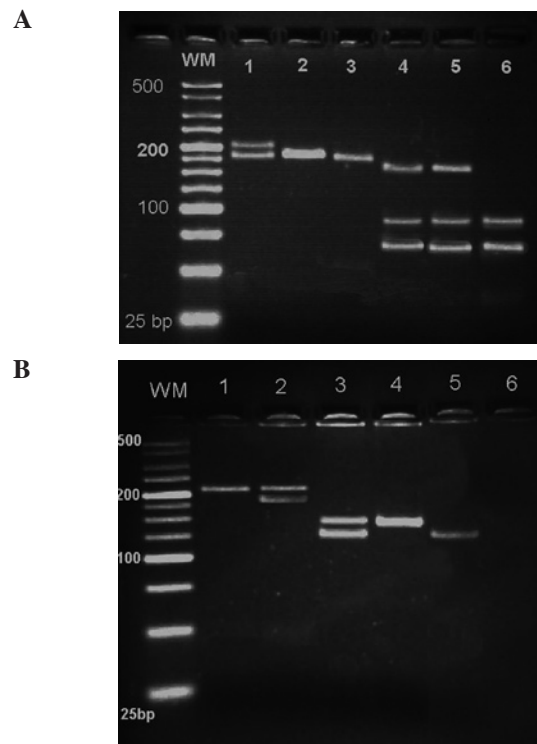


Figure 2: PCR-RFLP for mutations in exon 2 of Cx26. PCR products were digested in the restriction enzymes (A) BseI and PstI for the 35delG and 167delT mutations, respectively, or (B) BseI and BclI for the 35delG and M34T mutations, respectively. (A) Lane WM, 25-bp DNA ladder; lanes 1-4, deaf patient with compound heterozygous 35delG/167delT; lanes 2-3, positive control and deaf patient with homozygous 35delG; lane 5: positive control with heterozygous 167delT; lane 6, normal control for 167delT. (B) Lane WM, 25-bp DNA ladder; lane 1, normal control with 35delG; lanes 2-3, deaf patient with compound heterozygous 35delG/M34T; lane 4, PCR product for M34T mutation; lane 5, normal control for M34T; lane 6, PCR blank.

excess dye terminators (DyeEx2.0, Qiagen) from completed DNA sequencing reactions. Data were compared and aligned with different sequences using the NCBI BLAST Assembled Genomes tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results

This study investigated gene mutations associated with prelingual non-syndromic hearing loss in 50 Syrian families and 180 controls. Among the deaf patients, 38 were male and 52 female. All had profound hearing loss. Positive parental consanguinity was present in 50% of the studied families. Screening for six mutations in Cx26 was performed in two steps. First, the 35delG and 167delT mutations were screened in the deaf patients using restriction enzyme analysis of the PCR products. Second, when no 35delG and 167delT mutations were detected in deaf patients nor the heterozygous form of these two mutations, screening was conducted for the W24X, M34T, E47X and W77R mutations. Data for the patients and controls are presented in Table II. 35delG was the most common mutant allele in Cx26 in the Syrian families. Homozygous 35delG was detected in fifteen families (30%), as confirmed by direct sequencing (Fig. 1). A compound heterozygous genotype was observed in two families: one with the 35delG/167delT mutation (2%) (Fig. 2A) and one with

the 35delG/M34T mutation (2%) (Fig. 2B). Nine families were heterozygous with no second identified mutation in Cx26: four with 35delG+/unknown (8%), four with 167delT/unknown (8%) and one with M34T/unknown (2%). The W24X, W77R and E47X mutations were not detected in any of the study subjects. Three individuals with the heterozygous 35delG genotype (1.66%) and five with the heterozygous 167delT genotype (2.77%) were detected among the controls. No other mutations were found among the controls.

Discussion

GJB2, the gene encoding the gap-junction protein connexin 26, has been revealed to underlie distinct forms of hearing impairment, in particular autosomal recessive non-syndromic hearing impairment (ARNSHI). Mutations in GJB2 are the most common cause of moderate-to-profound congenital inherited hearing impairment in numerous populations (22). Among the genotypic variants identified was 35delG, which causes a frameshift mutation generating a premature stop codon and is reported to be more prevalent among Caucasians (11,23). In populations of European origin, the 35delG mutation accounts for up to 80% of GJB2 allelic mutations, and is most frequent in southern European countries around the Mediterranean (24-25). In this study, we investigated the association between six Cx26 mutations and non-syndromic recessive hearing loss in Syrian families. The basis of the selection of these mutations was dependant on their reported presence in the Middle East and neighboring countries (12,14,28).

Our results revealed the frequency of the 35delG mutation among deaf patients. 35delG was homozygous in 15/50 families (30%). This result was higher than that of previous studies in Jordanian (13.6%) (26), Palestinian (14%) (27), Egyptian (16%) (13) and Iranian (5.66%) (28) populations, and lower than that in Lebanese (94%) (29) and European (60-80%) (30) populations. The high level of consanguinity in many Syrian families (50%) explained the high frequency of 35delG homozygosity (30%). The carrier frequency of the 35delG mutation in the controls was 1.66%. This frequency was higher than that in the Jordanian population (1.1%) but lower than that in carriers reported in many other countries. The highest carrier frequency of 35delG found to date was in Italians (3.2%) (9).

The second most frequently occurring variant found in this study after 35delG was the 167delT mutation, which has also been found to be a cause of ARNSHI (31). This mutation was predominantly identified in Ashkenazi Jews and was attributed to a founder effect based on haplotype analysis (31). Our genotyping results revealed the presence of the compound heterozygous form of 167delT and 35delG (35delG/167delT) in one family (2%) and a 167delT/unknown mutation in four families (8%). The carrier rate of this mutation in a control group of Ashkenazi Jews was 4.03% (31). In our study, the carrier frequency was 2.77% among the Syrian controls.

The M34T variant was first described as an autosomal dominant mutation (32). Other reports have defined the M34T allele as an autosomal recessive mutation in the presence of other GJB2 mutations or homozygosity (33,34). If M34T is indeed a polymorphism, patients with the 35delG/M34T genotype are carriers of only one GJB2 mutation (35delG)

and their hearing impairment should be caused by different unidentified mutations in GJB2 or other genes. M34T is reported to have a high frequency in the general Caucasian population, comparable to that of 35delG (35). In the present study, only one family (2%) had a 35delG/M34T genotype, and another had a M34T/unknown genotype.

W24X is another commonly found allele, and has been associated with ARNSHI in the north and south of India (36,37). The incorporation of a stop codon in this mutation at codon 24 of Cx26 causes the formation of a protein that is just 1/10 the length of the wild-type protein (32). This mutation was not found in our study, indicating a relatively low frequency of this mutant allele in Syria.

The W77R missense allele encodes a GJB2 polypeptide with residual gap-junction assembly and intercellular coupling activity similar to that of M34T (38). W77R was initially detected in a large pedigree co-segregating recessive deafness DFNB1 with the 35delG and W77R alleles of GJB2 (39). Affected individuals were homozygous for 35delG and W77R, and compound heterozygous for W77R/35delG, indicating that W77R is indeed a mutant allele of GJB2 (40). The W77R and E47X mutations were reported by Moreno *et al* at a frequency of 3.7% and 5.1% in Spanish and Cuban patients, respectively (41). In the present study, the W77R and E47X mutations were not detected in any of the deaf families, suggesting the absence or low incidence of this mutation in Syria.

The results of the present study revealed the need to focus on the screening of newborns for various mutations in Cx26 for early intervention and language development, taking advantage of residual hearing. Screening for the 35delG mutation among others found in this study may be used to identify carriers among the general population, and thus aid in the counseling of families with Cx26 deafness.

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