

Prevalence of Connexin 26 Mutations in Patients from Jordan with Non Syndromic Hearing Loss

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ABSTRACT Mutations in GJB2 gene are a major cause of autosomal recessive congenital hearing loss and the cause in some rare cases of the autosomal dominant form. The objectives of this study were to estimate the frequency of connexin 26 35delG and 167delT mutations in congenital deaf Jordanian population and to estimate the frequency of carriers among normal Jordanian population. PCR was used to amplify two regions of the exon 2 of connexin26 and PCR products were analyzed using *Bst* I and *Pst* I analysis followed by gel electrophoresis. Homozygous 35delG was detected in fourteen out of 114 (12.3%) of the familial group, while in the sporadic group there was one individual out of 38 (2.6%). There were five individuals with a heterozygous mutations (35delG⁺/unknown), three of them were in the familial group (2.6%) and two in the sporadic group (5.3%). Among the normal group there was one carrier sample out of 95 (1.1%). The 167delT mutation was not detected in any of the deaf individuals, while it was detected in one individual from the normal group. The allele frequency for the 35delG mutation among the familial group was 13.6 % and 5.3 % for the sporadic group. The frequency of carriers among normal individuals was 1.1%. For the 167delT mutation the allele frequency was zero for the familial and sporadic groups.

INTRODUCTION

Hearing is one of the five major senses that allow us to communicate with our surrounding environment. Hearing impairment is the most common sensory defect in human; affect one in 1000 children and whose prevalence increases with age (Rabionet et al. 2000 ; Liu et al. 2002). The understanding of genetics of hearing impairment advanced rapidly during the last decade of the 20th century, also several essential genes, involved in the hearing mechanism, were cloned and localized to human genome. The mode of inheritance (dominant or recessive) of these genes is well known nowadays.

Many of those genes can cause other complications in addition to hearing impairment and are referred to as syndromic hearing loss and account for 30% of hearing loss, whereas those causing only hearing loss are referred to as nonsyndromic hearing loss and account for 70% of hearing loss (Rachel et al. 1999). Among hereditary nonsyndromic deafness, autosomal-

recessive inheritance is predominant, accounting for about 80% of the cases (Morton 1991). Mutation in connexin26 gene account for nearly 50% of recessive nonsyndromic hearing loss (NSHL) in Caucasian populations. Up to now, more than 90 variants of connexin26 gene have been reported (Tekin et al. 2003). Connexin26 gene, coded for gap junction protein 2 (GJP2), has two exons of about 2311 bp and mapped to chromosome thirteen (13q12), the second exon contains the most coding sequence of about 226 amino acid protein (Guilford et al. 1994; Petit et al. 2001). Gap junction proteins regulate the passage of inorganic ions and small metabolites, those of less than 1000 Dalton, between adjacent cells of most animal tissues. Thus coupling cells both electrically and metabolically. This type of communication is essential for organ homeostasis during development and adult life of multicellular organisms (Bruzzone 2001; Bruzzone et al. 2003). Connexin26 gene is expressed in many tissues, but in the cochlea of the inner ear, this gene product plays an important role in the normal hearing by controlling the Potassium-recycling pathway (Kikuchi et al. 2000).

GJP2 formed by cx26 gene is used for returning potassium ions to the endolymph, which plays a key role in sensorineural hearing function (Kikuchi et al. 1995). Therefore any mutation in this gene will lead to disruption of the normal function of

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the inner ear, which leads to hearing impairment.

Many mutations have been detected in the coding region of *cx26* gene and most of them express nonfunctional gap junction proteins that cause hearing loss. World-wide 35delG mutation is the most common mutation of *cx26* gene accounting for up to 85 % of all GJP2 mutant alleles detected in Mediterranean populations (Estivill et al. 1998; Kelley et al. 1998; Lench et al. 1998; Denoyelle et al. 1997). Whereas, 167delT mutation is the second common one that cause hearing loss and was found to be the most common in Ashkenazi Jewish populations (Morell et al. 1998). Other mutations e.g. 235delC in Asian populations, R143W in Africa were also shown to cause hearing loss (Park et al. 2000).

The present study is directed toward the mutations caused along the *cx26* gene using blood samples from deaf and normal Jordanian individuals. The objectives of the study were to estimate the frequency of *cx26* 35delG and 167delT mutations in deaf Jordanian population and to estimate the frequency of carriers of the these two mutations among normal Jordanian populations.

MATERIALS AND METHODS

Collection of Samples: Genomic DNA was extracted from blood samples obtained from 152 deaf patients and 95 healthy individuals. Deaf samples were collected from Al-Raja School for Deaf in Zarqa and Prince Ali Club for Deaf in Irbid. Healthy samples were collected from students at Jordan University of Science and Technology. All samples were collected during February 2003 and March 2004. The medical history of each patient was examined to exclude the environmental and infectious factors that cause deafness. In all samples taken deafness was congenital, prelingual and range from severe to profound. Non of the subjects showed signs or other findings associated with syndromes that involve permanent hearing impairment. A questionnaire covering demographic information as well as other information related to deafness was filled for each individual.

Prior to sample collection, written consent was obtained from patients or their parents in case of a minor and normal control individuals. The internal review committee on research using human subjects cleared the project after due deliberation.

DNA Extraction and PCR Analysis:

Genomic DNA was extracted from whole blood using the "Wizard Genomic DNA Purification" Kit (Promega, USA) according to the manufacturer instructions. For 35delG, a primer set was developed with a modified reverse primer for amplification of GJB2 sequence, based on the sequence entry for GJB2 in Gene Bank (accession no. M86849), so that a new restriction site for *Bsl* I is created only when the 35delG mutation is present. *Bsl* I recognizes the sequence 5'-CCN5↓N2GG-3'. The primer set consists of a wild-type forward primer 5'-GGT GAG GTT GTG TAA GAG TTG G (sense cDNA nucleotides 48-69) and a modified reverse primer 5'-CTG GTG GAG TGT TTGTTTC C*CA C (antisense cDNA nucleotides 233-254, modified at nucleotide 236). The C at nucleotide position 236, marked with an asterisk, replaces an A in the wild-type sequence and creates a *Bsl* I restriction site when the 35delG mutation is present. The PCR conditions were as follows: An initial denaturation at 94°C for five minutes, followed by 35 cycles of 94°C for one minute, 60°C for one minute, 72°C for one minute. A final extension was carried out at 72°C for 5 minutes.

For 167delT mutation, the following primers were used to amplify a 237 bp fragment containing the mutation; Forward 5' GCT CAC CGT CCT CTT CAT TT 3' and Reverse 5' CTT CTT CTC ATG TCT CCG GTA 3'. The PCR conditions were as follows: An initial denaturation at 95°C for five minutes, followed by 32 cycles of 95°C for forty second, 65°C for thirty second, 72°C for one minute. The PCR machine used was iCycler from (BioRad, USA).

PCR reactions were performed in a 25 µl final volume containing, 25ng of genomic DNA, 10 mM Tris-HCl, pH 8.3, 1.5mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 200 µM dNTPs, 10 pmol of each primer and 0.6 U *Taq* polymerase.

Restriction Enzymes Analysis: For detection of 35 delG mutation, PCR products were digested with *Bsl* I (New England Biolabs, USA) in a 20 µl total reaction volume containing: 4.5 µl of the PCR product, 2 µl of 10X NB buffer, 0.5 µl of the *Bsl* I enzyme (10U/µl) and 13 µl of nuclease free water. The reaction mixture was incubated in at 55°C for 16 hours.

For detection of 167delT mutation, PCR precuts were digested with *Pst* I (Promega, USA) in a 20 µl volume containing: 4 µl of PCR product, 2 µl of (10X) buffer H, 0.2 µl acetylated bovine

serum albumin, 1 μ l of Pst I (10U/ μ l) and 12.8 μ l of nuclease free water. The mixture was then incubated at 37°C for 4 hours. Fifteen μ l of the digested PCR products were added to 3 μ l (6X) loading dye and loaded on 2% agarose gel and run at 80V for three hours. The product sizes were determined by using 50 bp ladder marker (Promega, USA).

For the 35delG mutation, the primer pair generated a PCR product of 207 bp. When the 35delG mutation is present (homozygous mutant), the *Bst* I digested PCR product will give two fragments (181 bp and 26 bp). In the absence of the 35delG mutation (homozygous normal), the PCR product is not digested (one band at 207 bp). Heterozygous 35delG carriers will give three bands (207 bp, 181 bp and 26 bp). For the 167delT mutation, the primer pair used generated a PCR product of 237 bp. Upon digestion with *Pst* I, homozygous normal will give three fragments (97 bp, 72 bp, 68 bp), homozygous mutant will give two fragments (165 bp and 72 bp) and heterozygous carriers will give four fragments (165 bp, 97 bp, 72 bp and 68 bp). Each sample was repeated at least twice to confirm the genotype. DNA representing positive controls for homozygous and heterozygous 35delG and 167delT mutants was a kind gift from Dr. Ssa Francesca Gualandi, University of Ferrara, Italy.

RESULTS

A total of 152 blood samples from deaf individuals and 95 blood samples from healthy individuals were tested for the presence of mutations in the connexin26 gene using PCR-restriction enzyme analysis. Among deaf samples, there was 123 (80.9%) males and 29 (19.1%) females, the age was ranged from 7 to 52 years (mean 27 years). Whereas among healthy samples

there was 67 (70.5 %) males and 28 (29.5 %) females, and the age ranged from 18-35 (mean 26 years). Based on medical history and pedigrees constructed based on the information given in the questionnaire, deaf individuals were divided into two groups; familial group, defined as individuals who have at least one deaf person in his family whether brother, sister, or parent. A second group called sporadic is defined as deaf with no affected family members. Normal group, defined as healthy individuals with no deaf individuals in his/her family or relatives. In the familial group, there were 114 deaf individuals (75%) and in the sporadic group, there were 38 (25%) deaf individuals.

The two mutations (35delG and 167delT) in exon two were detected using restriction enzyme analysis of the PCR products of the amplified sequences as shown in (Fig. 1 and Fig. 2), respectively. Homozygous 35delG was detected in fourteen out of 114 (12.3%) of the familial group, while in the sporadic group there was one individual out of 38 (2.6%). There were five individuals with a heterozygous compound mutations (35delG⁺/unknown), three of them were in the familial group (2.6%) and two in the sporadic group (5.3%). Among the normal group there was one carrier sample out of 95 (1.1%), (Table 1).

The 167delT mutation was not detected in any of the deaf individuals, while it was detected

Table 1: Frequency of 35delG mutation among the studied Jordanian groups.

Parameter/ Group	Homozygous 35delG	Compound mutations (35delG ⁺ /35delG ⁻)	Frequency (%)
Normal	0/95	1/95	(1.1)
Familial	14/114	3/114	(13.6)
Sporadic	1/38	2/38	(5.3)

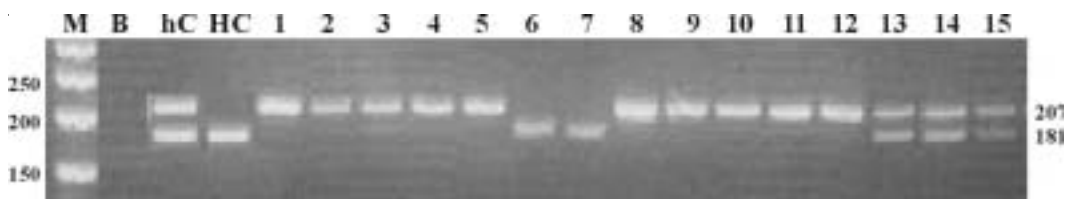


Fig. 1. 2% agarose gel of exon 2 (35delG mutation) where PCR products were digested with *Bst* I restriction endonuclease. Lane M: 50 bp DNA ladder, B: PCR Blank, Lane hC: heterozygous positive control, Lane HC: Homozygous positive control, Lanes (1-5): normal individuals, Lanes (6-7): deaf individuals with homozygous 35delG, Lanes (8-12) deaf individuals with no 35delG mutation, Lanes (13-14): deaf individuals with heterozygous compound 35delG. Lane 15: normal individual carrier for 35delG.

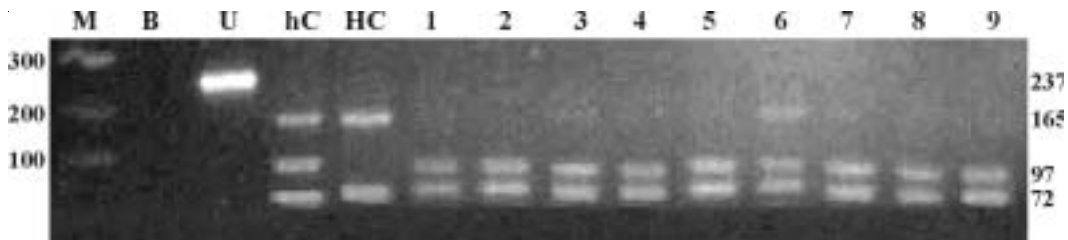


Fig. 2. 2% agarose gel of exon 2 (167delT mutation) where PCR products were digested with *Pst* I restriction endonuclease. Lane M: 100 bp DNA ladder, B: PCR Blank, U: uncut PCR product, Lane hC: heterozygous positive control, Lane HC: Homozygous positive control, Lanes (1-5): normal individuals, Lane 6: heterozygous normal carrier individual Lane (7-9): deaf individuals with no 167delT.

in one individual from the normal group as shown in (Fig.2, lane 6). By using Hardy Weinberg equation, the allele frequency of the 35delG mutation was calculated and found to be 13.6 % for the familial group, and 5.3 % for the sporadic group. The frequency of heterozygous carriers among normal individuals was 1.1%. For the 167delT mutation the allele frequency was zero for the familial and sporadic groups, but the frequency of heterozygous carriers among the normal group was 1.1% (Table 2).

Table 2: Frequency of 167delT mutation among the studied Jordanian groups.

Parameter/ Group	Homozygous 35delT	Compound mutations (35delT ⁺ /35delT ⁻)	Frequency (%)
Normal	0/95	1/95	(1.1)
Familial	0/114	0/114	-
Sporadic	0/38	0/38	-

DISCUSSION

Until recently deafness was thought to be a highly complex problem due to extreme genetic heterogeneity. This complexity has now been greatly reduced since the description of a very common mutation in the most common form of genetic hearing loss. To date, four connexin genes have been demonstrated to underlie different forms of non-syndromic deafness. Mutations in Connexin 26 (*GJB2*) have been detected in many ethnic populations being tested in the world and are the most common cause of deafness in many populations (Kelsell et al. 1997). Connexin 31 (*GJB3*) is also expressed in the cochlea and is the cause of deafness in several Chinese pedigrees (Xia et al. 1998; Liu et al. 2000). Likewise, mutations in *GJB6* (connexin 30) are the apparent common cause of deafness in Southern European populations (Grifa et al. 1999;

del Castillo et al. 2000). Recently, *GJA1* (connexin 43) has been shown to cause non-syndromic recessive deafness and appears to be a common form of deafness in African Americans (Liu et al. 2001). It seems to be clear that specific mutations in a connexin gene or certain connexin genes play a predominant role in different ethnic populations.

In this study we investigated the association between two connexin 26 mutations and non-syndromic recessive hearing loss among Jordanian population. Our results showed that the frequency of the 35delG mutation (13.6%) among deaf Jordanians was similar to that for Palestinian population (14%) and lower than that for Lebanese population (94%) and to European Populations (60-80%) (Shahin et al. 2002; Mustapha et al. 2001; Wilcox et al. 2000). However, this mutation has not been detected in Omani, Japanese and Korean populations (Simsek et al. 2001; Abe et al. 2000; Park et al. 2000).

As the frequency of the 35delG mutation was small, testing 190 control chromosomes was necessary to assess the frequency of this mutation in the normal Jordanian population. Only 2 of these chromosomes (1.1%) has the 35delG mutation, an indication of its relatively low frequency in this population, which is comparable to its prevalence in other populations such as Palestinians, Lebanese, Omani and Egyptian populations (Shahin et al. 2002; Mustapha et al. 2001; Park et al. 2000; Van Laer et al. 2001). Therefore, its rare allelic frequency in normal Jordanian population and the subsequently rare 35delG involvement in Jordanian non-syndromic recessive deafness (13.6%) are not in favour of a common founder for the 35delG mutation. Indeed the absence of a common founder for this mutation could be

expected since Jordan was always been a cross-road in the region and was occupied successively by various populations throughout history (Medlej-Hashim et al. 2002). However, high carrier frequencies of the GJB2 35delG mutation have been reported in many countries, with the highest carrier frequency so far being found in Greeks (3.5%) and Italians (3.2%) (Antoniadi et al. 1999; Gasparani et al. 2000).

It has been shown that 10–42% of patients with connexin 26 mutations have only one mutant allele (Liu et al. 2000). In our study, 2.6% of familial cases and 5.3% of the sporadic cases were found to carry single 35delG mutation (i.e., they are heterozygotes). Our genotyping results showed the absence of 167delT mutation in both familial and sporadic groups. This is in agreement with what has been known about this mutation, mainly observed in Ashkenazi Jews (Morell et al. 1998). However, the carrier frequency for 167delT mutation among normal Jordanian population was 1.1%.

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