

## Screening of GJB6 Gene for the 342-kb Deletion in Patients from Jordan with Non Syndromic Hearing Loss

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**ABSTRACT** Hearing loss is a common congenital disorder frequently associated with mutations in the connexin 26 gene (GJB2). However, recent studies found a 342-kb deletion in another gene, connexin 30 (GJB6) that causes non-syndromic recessive hearing loss in either a homozygous monogenic inheritance of Cx30 deletion or digenic inheritance of Cx30 deletion and a Cx26 mutation. The objective of this study was to screen for the 342-kb deletion in Cx30 gene in patients with non-syndromic hearing loss from Jordan. Two different PCR conditions were used to detect the 342-kb deletion of connexin 30 gene by amplifying the deletion breakpoints using specific primers. None of the patients with non-syndromic hearing loss was found to carry deletion in connexin 30 gene indicating that the occurrence of this deletion is restricted to Spanish, Caucasians, and Ashkenazi Jews.

### INTRODUCTION

Hearing loss is the most prevalent inherited sensory disorder affecting about 1 in 1000 newborns (Cohen and Gorlin 1995). Approximately, 60% of hearing loss cases are inherited. About 80% of these inherited cases are classified as autosomal recessive nonsyndromic hearing loss (ARNSHL) (Nance 2000). More than 100 genes are estimated to be involved in hearing impairment and to date 60 loci have been mapped and 23 causative genes have been cloned (<http://www.uia.ac.be/dnalab/hhh>). Of these genes, Connexin 26 (GJB2) located on chromosome 13q12 accounts for most ARNSHL in many world populations (Kelsell et al. 1997). So far, over 90 different ARNSHL-causing mutations in connexin 26 have been reported (<http://www.cgr.es/deafness>), several of which show specific ethnic biases (Abe et al. 2000; Denoyelle 1997; Loffler 2001; Zelante et al. 1997). However, 10-42 % of the cases with GJB2 mutations have only one mutant allele (Denoyelle et al. 1997; Gabriel et al. 2001; Wu et al. 2002, 2003) indicating that there is another gene associated with the cause of the deafness. A 342-kb deletion was found in a gene known as connexin 30 (Cx30), gap junction protein beta 6 (GJB6), located approximately 35 kb downstream of GJB2 (del Castillo et al. 2002; Lerer et al. 2001; Pallares-Ruiz et al. 2002). The deletion includes the 5' region of GJB6 gene and most of its coding region and extends distally to GJB2, which remained intact.

As the deletion also comprises the microsatellite marker D13S1830, it has been termed D(GJB6-D13S1830). The locus that contains these two genes is known as DFNB1. This deletion can cause deafness in a monogenic pattern of inheritance or as a digenic pattern involving GJB2. It is unclear whether this deletion removes regulatory elements common to GJB6 and GJB2 resulting, in addition to the deletion of GJB6, in reduced expression of the wild type GJB2 gene (del Castillo et al. 2002; Lerer et al. 2001; Pallares-Ruiz et al. 2002).

Both connexin 26 and connexin 30 encode gap junction proteins. Six monomers of these proteins bind to form homo and heterohexamers called connexons (Kelley et al. 1999). Two connexons from adjacent cells then form functional channel between the cells that allow diffusion of small ions including potassium ions, as well as metabolites and secondary messenger molecules. Co-expression of connexin 26 and connexin 30 in the inner ear is thought to be a prerequisite for the maturation of the cochlea (Lautermann et al. 1998; Xia et al. 2001). Their co-expression is also detectable in human embryo cochlea after the 22<sup>nd</sup> week of the pregnancy (Lautermann et al. 1998, 1999). These gap junctions connect supporting cells of cochlear neurosensory epithelium with adjacent epithelium cells. In the absence of connexin 30 there is a deficiency of the gap junctions between cells preventing recycling of K<sup>+</sup>, leading to a decrease of potential within the cochlea, which results in hearing impairment (Teubner et al. 2003).

We have previously estimated the allele frequency for the most common cause of NSHL, the 35delG mutation in GJB2 gene, in Jordanian deaf patients and found it to be ~14% in familial cases (Mahasneh, in press). This frequency is low when compared to that of Lebanese (94%) (Mustaapha et al. 2001) or European populations (60-80%) (Gasparani et al. 2000). In addition, ~3% of familial cases were found to carry single 35delG mutation indicating that they are heterozygotes. Therefore, the present study is directed toward the newly identified 342-kb deletion mutation in GJB6 gene, the second most common mutation that causes ARNSHL. The objective of the study was to estimate the frequency of 342-kb deletion mutation in GJB6 gene in Jordanian patients with non-syndromic hearing loss.

## MATERIALS AND METHODS

**Patients:** Details describing the patients were reported elsewhere (Mahasneh 2005). Briefly, genomic DNA was extracted from blood samples obtained from 160 Jordanian patients with autosomal recessive non-syndromic hearing loss. Patients were recruited from Al-Raja School for Deaf in Zarqa and Princes Ali Club for Deaf in Irbid during the period February 2003 to March 2004. The medical history of each patient was examined to exclude the environmental and infectious factors that cause deafness.

**DNA Extraction and Multiplex PCR Analysis:** Genomic DNA from deaf individuals was extracted from whole blood using the "Wizard

Genomic DNA Purification" Kit (Promega, USA) according to the manufacturer instructions.

For each sample, polymerase chain reaction (PCR) was used to amplify DNA fragments simultaneously with each of the three sets of primers in a multiplex state. Each pair of PCR primers (10-20 pmol), Cx30d-F/Cx30d-R or GJB6-1R(F)/BKR-1(R); Cx30c-F/Cx30c-R; Actin-F / Actin- R (Table 1), was added into a 25 µl reaction mixture containing 100 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl, pH 8.8 and 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>), 200 M dNTPs and 1 U of DyNAzyme II DNA polymerase (Finnzymes, Finland). This single-tube PCR reaction was amplified in a BIO-RAD iCycler thermal cycler (Bio-Rad, USA) after an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C denaturation for 1 min, 61 °C annealing for 1 min and 72 °C extension for 2 min. The PCR products were verified by 2% agarose gel electrophoresis following staining with ethidium bromide. Gels were photographed using Gel documentation system (Bio-Rad, USA). The product sizes were determined by using DNA-*Hind* III and  $\phi$ X174 DNA-*Hae* III digest (Finnzymes, Finland) as DNA marker.

Each sample was repeated at least twice to confirm the genotype. DNA representing positive control for a heterozygous 342-kb deletion mutant was a kind gift from Dr. Delphine Feldmann, Laboratoire De Biochimie, Hôpital D'enfants, Armand-Trousseau, Paris, France.

## RESULTS

A total of 160 blood samples from Jordanian

**Table 1: Primers used in multiplex PCR reaction representing GJB6 gene**

Primer Set	Primer Sequence (5'→3')	Annealing Temp. (°C)	Expected Size (bp)
<sup>a</sup> Cx30 wild type	Cx30c-F: AGG-GAT-AAA-CCA-GCG-CAA-TG	60/62	651
	Cx30c-R: AGC-ACA-ACT-CTG-CCA-CGT-TA		
<sup>a</sup> Cx30 deletion Set 1	Cx30d-F: AGT-GCT-GGG-ATT-ACA-GGT-AC	60	405
	Cx30d-R: AGC-ACA-ACT-CTG-CCA-CGT-TA		
<sup>b</sup> Cx30 deletion Set 2	GJB6-1R: TTT-AGG-GCA-TGA-TTG-GGG-TGA-TTT	62	457
	BKR-1: CAC-CAT-GCG-TAG-CCT-TAA-CCA-TTT-T		
<sup>c</sup> Internal control	Actin-F: CGG-AAC-CGC-TCA-TTG-CC	60/62	289
	Actin-R: ACC-CAC-ACT-GTG-CCC-ATC-TA		

<sup>a</sup> sequences based on the published primers described by Wu et al. (2003)

<sup>b</sup> sequences based on published primers described by del Castillo et al. (2002)

<sup>c</sup> sequences based on the published primers described by Curran et al. (2000)

patients with non-syndromic hearing loss were tested for the presence of the newly identified 342-kb mutation in the connexin 30 gene using a multiplex PCR assay. Among the samples, there were 130 (81.3%) males and 30 (18.8 %) females in the range of 7 to 52 years (mean 27 years). The assay was based on amplifying the deletion breakpoints using specific primers as illustrated in (Fig. 1). The amplified wild type Cx30 showed a band of 651 bp, while the 342-kb deletion amplification showed a band of about 405 bp in case of the Cx30d-F/Cx30d-R primer set (Fig.2, lane hC) and a 457 bp band in case of GJB6-1R(F)/BKR-1(R) primer set (Fig.3, Lane hC). The amplified actin gene which was used as an internal control showed a band of 289 bp in all samples (Figs. 2 and 3).

Non of the tested samples showed the 405 bp or 457 bp band characteristic of the 342-kb deletion using the two different sets of primers (Fig. 2 and 3, Lanes 1 to 10) indicating the absence of this deletion in the Jordanian patients in neither homozygous nor heterozygous state. All

160 tested samples were homozygous wild type showing the characteristic 651 bp band (Fig. 2 and 3, Lanes 1 to 10 ).

**DISCUSSION**

Non-syndromic hearing loss is difficult to diagnose by molecular means owing to lack of mutational information on several genes involved (del Castillo et al. 2002). Most of the genetic analyses include routine molecular diagnosis for mutations in the GJB2 gene, since such mutations are the cause of more than 50 percent of the cases in many ethnic populations (Kelsell et al 1997). Previous studies have shown that 35delG was the most common mutation in Jordanians with NSHL (Mdelj et al. 2002; Mahasneh, 2005). The frequency of this mutation was found to be 14 % in the two studies, which is lower than that found in Lebanese population (Mustaapha et al. 2001). In addition, a substantial proportion of patients were found to carry only one mutant GJB2 allele (Mahasneh, 2005) which encouraged us to look

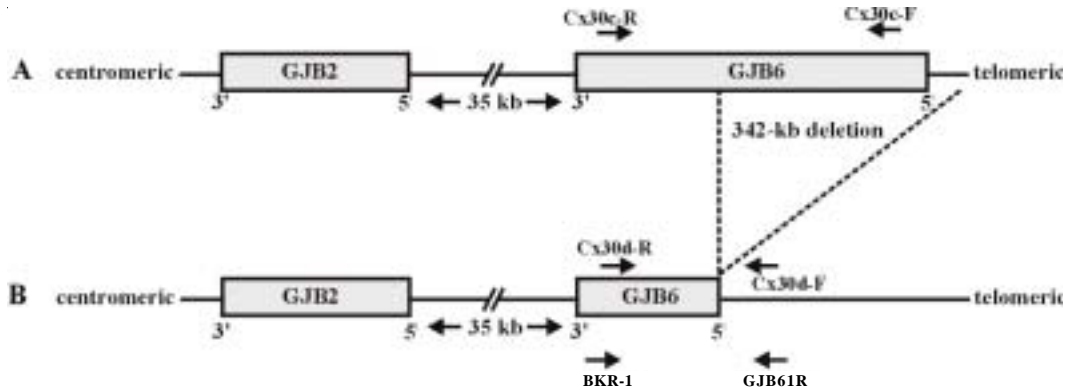


Fig. 1. Schematic diagram of DFNB1 locus on chromosome 13q showing the GJB6 deletion and the localization of primers used for the multiplex PCR (drawing not to scale). A: Part of the wild type chromosome 13 carrying GJB2 and GJB6. B: Part of chromosome 13 with the deletion in GJB6.

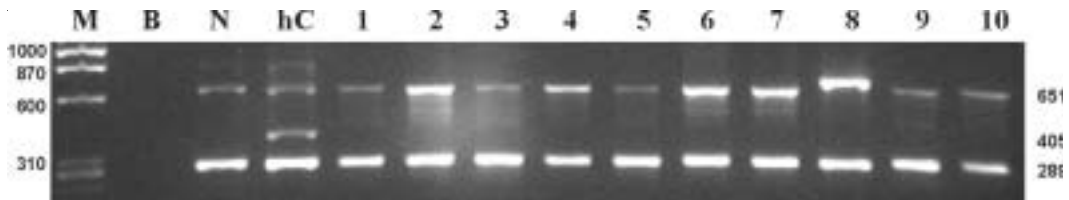
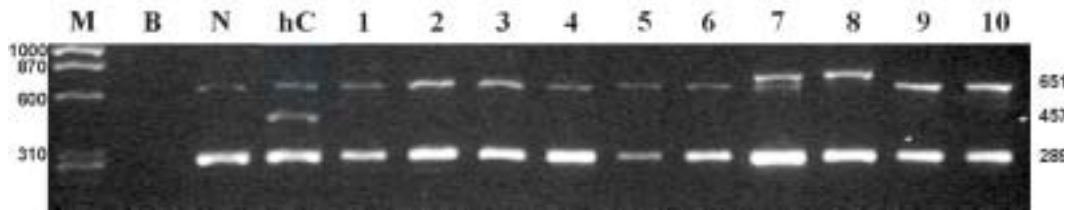


Fig. 2. 2% agarose gel of the multiplex PCR products of GJB6 gene. Lane M:  $\lambda$  DNA Hind III / $\phi$ X174-Hae III DNA marker. Lane B: PCR blank. Lane N: normal healthy individual. Lane hC: heterozygous positive control. Lanes 1-10: deaf individuals showing wild type connexin 30 but no 342- kb deletion mutation. A segment of the actin gene was amplified as an internal control which gives rise to 289 bp band.



**Fig. 3.** 2% agarose gel of the multiplex PCR products of GJB6 gene. Lane M:  $\lambda$  DNA *Hind* III / $\phi$ X174-*Hae* III DNA marker. Lane B: PCR blank. Lane N: normal individual. Lane hC: heterozygous positive control. Lanes 1-10: deaf individuals showing wild type connexin 30 but no 342-kb deletion mutation. A segment of the actin gene was used as internal control which gives rise to 289 bp band.

for other mutations in other genes. One interesting gene we decided to examine was connexin 30 (GJB6), which is located on chromosome 13q12, approximately 35-kb downstream of the GJB2 gene. A novel mutation - a 342-kb deletion that truncates the GJB6 gene but not affect the GJB2 gene- was identified in a group of subjects with unexplained cases of non-syndromic prelingual hearing loss (del Castillo et al. 2002). The 342-kb 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 4 of 6 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). Moreover, the deletion was found in seven NSHL patients being heterozygous for GJB6 mutations from four unrelated Ashkenazi Jewish families (Lerer et al. 2001). Also, it was found in 2 out of 25 patients from Germany (Bolz et al. 2004). Digenic inheritance in those cases is supported by the findings that both genes, GJB2 and GJB6, encode for gap junction proteins, connexin 26 and connexin 30, respectively, 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). Likewise, mice mutants that are homozygous for a deletion of the GJB6 coding region develop non-syndromic hearing loss (Teubner et al. 2003) representing a mouse model for this type of hearing loss.

However, in our study none of the 160 Jordanian patients tested was found to have the 342-kb deletion. Similarly, none of the 118 tested deaf Chinese patients had this mutation (Liu et al., 2002). Moreover, Günther et al. (2003) found the same results when they screened 317 Austrian, 35 Turkish, 10 Serbian and 14 Bosnian

patients. The deletion was also not found in 229 deaf patients from the Kurdish population in Iran (Mahdieh et al 2004). More recently, analysis of a large (2112) unselected newborn population group from New York State failed to detect this deletion (Fitzgerald et al. 2004). A similar situation has been observed regarding the most common cause of hearing loss, 35 del G mutation in GJB2 gene, where it was the most common mutation involved in NSHL in certain populations and was absent in other populations (Denoyelle et al. 1997; Gasparini et al. 2000; Abe et al. 2000). In conclusion, our results show the absence of the 342-kb deletion in deaf Jordanians which indicates that this deletion is restricted to certain populations, and indicating a founder effect regarding this deletion.

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