

# The Association Between GJB2 Mutation and GJB6 Gene in Non Syndromic Hearing Loss School Children

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## SUMMARY

Recently, molecular testing for GJB2 mutations has become the standard of care for the diagnosis of patients with non syndromic hearing impairment of unknown cause. The aims of this study are to determine the association between GJB2 mutation and GJB6 and to report the variation of mutations in deaf students who have heterozygous GJB2. This retrospective study was conducted at Universiti Kebangsaan Malaysia Medical Center (UKMMC). Data was collected from previous files and records from Tissue Engineering and Human Genetic Research Group Laboratory. Approval from Ethical Committee was obtained prior to the study. A total of 138 students have been screened in previous studies in UKMMC for the presence of GJB2 mutations as a cause for hearing loss. Thirty four of the 138 subjects have GJB2 mutations; 2 showed homozygous mutations whereas another 32 were heterozygous for GJB2 gene mutation. Only 31 DNA samples of students presented with sensorineural hearing loss with heterozygous mutation in GJB2 gene were included in this study. The sequencing results obtained were analyzed. The degree of hearing loss of those students with association GJB2 mutation and GJB6 mutation will be discussed. Five out of 31 subjects (16.2%) have mutations in their GJB6 gene, suggesting a digenic inheritance of GJB2/GJB6 mutation. In total, four novel mutations were identified; E137D (n=1), R32Q (n=1), E101K (n=1) and Y156H (n=1) and one mutation deletion; 366delT (n=1). All students with association GJB2 mutation and GJB6 showed severe to profound hearing loss in both ears. Interestingly this study not detected the large deletion of 342 kb in GJB6 gene suggesting that the mutation is very rare in this region compared to certain parts of the world.

## KEY WORDS:

*Mutation, Homozygous, Gap junction, Deletion, Gene*

## INTRODUCTION

Genetic deafness is generally divided into two categories; syndromic and non syndromic hearing loss. Some forms of syndromic hearing impairment are autosomal recessive; such as Usher syndrome, Pendred syndrome, branchio-oto-renal

syndrome, Jervell syndrome and Lange-Nielsen syndrome. For autosomal dominant-based syndromes, the few examples are Alport syndrome, Treacher-Collins syndrome and Stickler syndrome. The underlying cause of all these syndromes is mutation in a single gene. However, the gene may be different from one patient to another (e.g. at least ten different genes cause Usher's syndrome) and the phenotypic expression of defects in the same gene may be variable among affected individuals (e.g. Waardenburg's syndrome).

More than 400 clinical syndromes are recognized to have hearing loss as a component, and hearing loss in a child may be the first symptom of a more serious clinical disorders. However, approximately 70% of hereditary hearing impairment is non syndromic, where abnormal auditory function is the only obvious clinical outcome resulting from gene mutation<sup>1</sup>. Genetic hearing impairment also can be classified according to the mode of inheritance; autosomal recessive inheritance in approximately 75% of patients, autosomal dominant inheritance in approximately 20% of patients and X-linked inheritance in the remaining 5%<sup>2</sup>.

In 1994, Guilford *et al.* mapped the first single locus for non syndromic autosomal recessive, prelingual hearing impairment is (DFNB1) on chromosome 13q11. This was discovered from a study conducted on Tunisian families. Three years later, the deafness-causing gene at this locus was identified as gap junction beta 2 (GJB2)<sup>3</sup>. Gap junctions (GJs), composed of proteins from the connexin family, are the only channels that directly connect the cytoplasm of adjacent cells to allow for the intercellular transfer of small hydrophilic molecules. Several different connexins have been shown to participate in these gap junction systems. Expression of at least four different connexins has been reported in the inner ear; Cx26 (GJB2), Cx30 (GJB6), Cx31 (GJB3) and Cx43 (GJA1). These Cx26 and Cx30 appear to have more widespread and similar patterns of distribution in the inner ear and are present together in regions where neither of the other two connexins Cx31 and Cx43 is identified. Thus, these other connexins may not be able to compensate for defects in either Cx26 or Cx30.

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A GJB2 mutation is one of the causes of non-syndromic hearing loss. However, the finding of a large number of affected subjects with only one GJB2 mutant allele complicates the molecular diagnosis of DFNB1 deafness. It was hypothesized that there could be other mutation residing in the DFNB1 locus which is in close proximity with GJB2 gene. This hypothesis gained support by the reported findings of 342kb deletion in the DFNB1 locus truncating the GJB6 gene which encodes connexin30 protein. Another hypothesis was that physiopathology of the hearing impairment due to the association between GJB2 mutation and GJB6 deletion is not a digenism, but a position effect with deletions of a regulatory element of GJB2 located closed to GJB6. The mutations of GJB6 are responsible only for dominant form of isolated hearing loss and several deletions of the GJB6 regions have been described associated with GJB2 mutations.

### MATERIALS AND METHODS

A retrospective study was conducted at Universiti Kebangsaan Malaysia Medical Center (UKMMC). All data were collected from previous files and records from Tissue Engineering and Human Genetic Research Group Laboratory. Approval from Ethical Committee was obtained prior to the study (Code Number: FF-099). A total of 138 students have been screened in previous studies in UKMMC for the presence of GJB2 mutations as a cause for hearing loss<sup>4</sup>. DNA fragments containing the entire coding region were amplified using polymerase chain reaction (PCR) technique from genomic DNA samples using two sets of primers; the first set is forward 5'-CAC TTT CCC AAG GCC TCT TCC AC-3' and reverse 5'-GTA CGT CCA CCA CAG CGA CC-3' and the second primer set is as follows; forward 5'-TGG TGG CCA TGC ATG TGG CCT AC-3' and reverse 5'-CAG AAG TCT CCT TAT GAC GCA GC-3'. The same primers were used as a template for sequencing.

The step for optimum amplification conditions were as follows; pre-denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s and elongation at 72°C for 20 s. After the final elongation at 72°C for 10 min, the PCR products were electrophoresed in a 1.5% agarose gel. DNA bands of 478 bp for the first primer set and 561 bp for the second primer set were excised and purified using QIA quick Gel Extraction Kit, (Qiagen, USA). For the sequencing, approximately 400 ng PCR products was used as template with DYEnamic (TM) ET Terminator Cycle Sequencing Kit (Amersham Pharmacia, USA). The products were run on an ABI377 DNA sequencer (Applied Biosystem, USA).

Thirty four of the 138 subjects have GJB2 mutations; 2 showed homozygous mutations whereas another 32 were heterozygous for GJB2 gene mutation. Only 31 DNA samples of students presented with sensorineural hearing loss with heterozygous mutation in GJB2 gene were included in this study. PCR products were sequenced from both ends by the dideoxy chain termination method by using DYEnamic (TM) ET Terminator Cycle Sequencing Kit. Fluorescence colour used to detect DNA chain. The reactions were analyzed on ABI377 DNA sequence machine (Applied Biosystem, USA).

The sequencing results were analyzed using descriptive analysis. The chronological order of mutation detection is shown in the (Figure 1).

### RESULTS

There were 31 students involved in this study. Out of these students, 18 students were females and 13 males. For the race distribution; 22 were Malays, 6 Chinese and 3 Indian (Table I). Seven subjects had family history of deafness. Non syndromic features were found in all these thirty one students. All students had bilateral sensorineural hearing loss. In this study, 5 out of 31 subjects (16.2%) have GJB6 gene mutation in digenic mode of inheritance. Two of the students who have digenic GJB2 and GJB6 mutation have family history of deafness. The rest of the students have no other family members with deafness.

#### *Mutation and Race*

The GJB6 mutation in Malay children was 22.7% (5/22) and Indian 33.3% (1/3). No mutation was found among Chinese students. There was no significant difference in the number of mutation in relation to race (p value =0.642) (Figure 2).

#### *Types of Mutation*

There were four types of mutation detected; E137D, R32Q, E101K and Y156H and one deletion mutation; 366delT. All of these mutations detected in the GJB6 were heterozygous which indicates for the presence of this second mutation in combination with heterozygous GJB2 mutation (in digenic mode of inheritance). In R32Q mutation, an R to Q transition at residue 94 was involved. This mutation which converted Arginine to Glutamine at codon 32 was found in one subject (Figure 3). Missense mutation, Y145H, was due to a transversion of Y to H at nucleotide 463, converts Tyrosine to Histidine at position 145 (Figure 4). This mutation was found in one subject. In E148D mutation, E to D transversion was involved at residue 444. This missense mutation converts Glutamic acid to Aspartate at codon 148 (Figure 5). In E101K mutation, an E to K transition at residue 301 was involved. This mutation converts amino acid Glutamine to Lysine at position 101 (Figure 6). This mutation was found in one subject. 366delT mutation had a deletion of base (T) at nucleotide 366 (Figure 7), inducing a frame shift mutation, causing all of the codons occurring after the deletion to be read incorrectly during translation producing a severely altered and potentially non functional protein. This deletion was found in one subject.

#### *Mutation and Degree of Hearing Loss*

The audiological data collected on the students were categorized according to their severity; mild hearing loss (less than 40dB), moderate (40 to 69dB), severe (70 to 90dB) and profound (more than 90dB). The degree of hearing impairment was determined by averaging the air-conducted pure tone thresholds over 500–4000 Hz in the better ear. The degree of hearing loss in different type of mutations is shown in Figure 8. Three of the students have bilateral profound hearing loss. Two students have bilateral with severe to profound hearing loss (Table II).

Table I: Distribution of students according to gender and race

Race	Male	Female	Total
Malay	8	14	22
Chinese	3	3	6
Indian	2	1	3
Total	13	18	31

Table II: The type of mutations and degree of hearing loss in the GJB6 and GJB2 genes detected in the 5 students

Genotype		Level of hearing loss		Age	Subject
Allele (GJB2)	Allele1 (GJB6)	Left	Right		
V37I	R32Q	Profound	Profound	12	1
V37I	E101K	Profound	Profound	12	2
V95L	366delT	Profound	Profound	17	3
V37I	E148D	Severe to Profound	Severe to Profound	11	4
W24X	Y145H	Severe	Severe	17	5

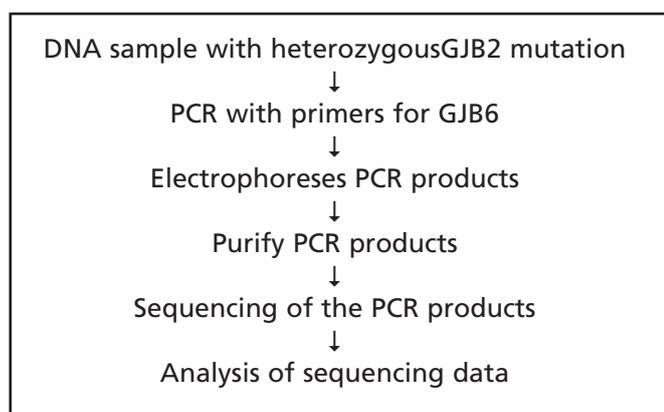


Fig. 1: The chronological order of mutational analysis.

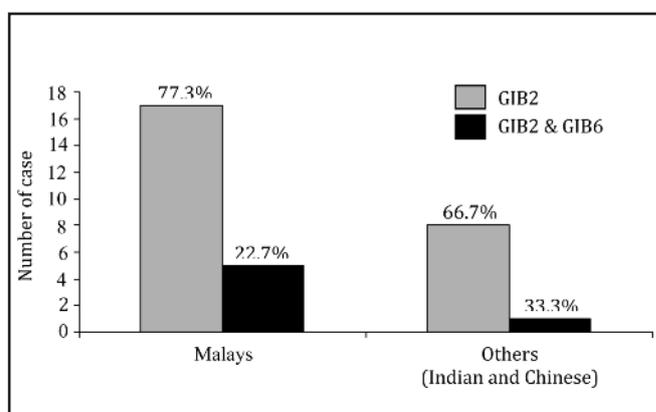


Fig. 2: The number of cases of individuals for the affected genes according to race. The percentage of association between GJB2 and GJB6 mutation according to race is also highlighted.

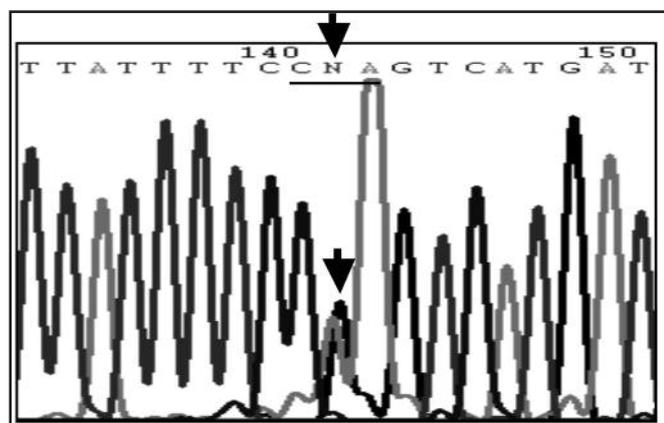


Fig. 3: R32Q mutation. G is substituted by A (CGA→CNA) resulting in change of amino acid from arginine to glutamine at codon 32.

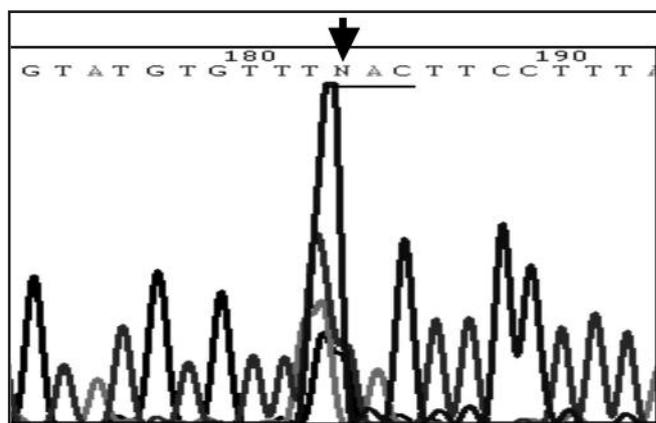


Fig. 4: Y145H mutation. T is substituted by C (TAC→NAC) resulting in change of amino acid from tyrosine to histidine at codon 145.

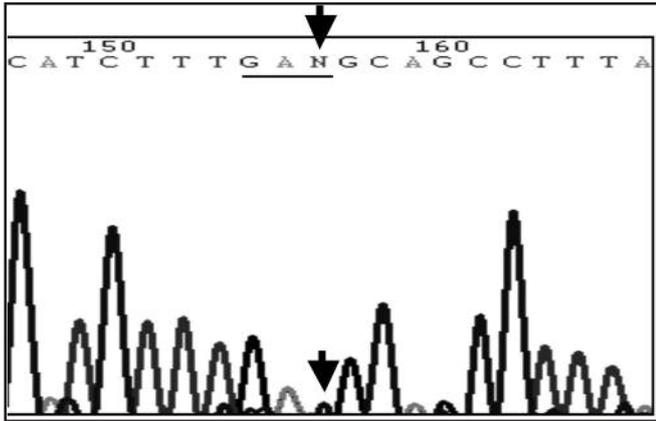


Fig. 5: E148D mutation, A is substituted by C (GAA→ GAN) resulting in change of amino acid from glutamic acid to aspartate at codon 148.

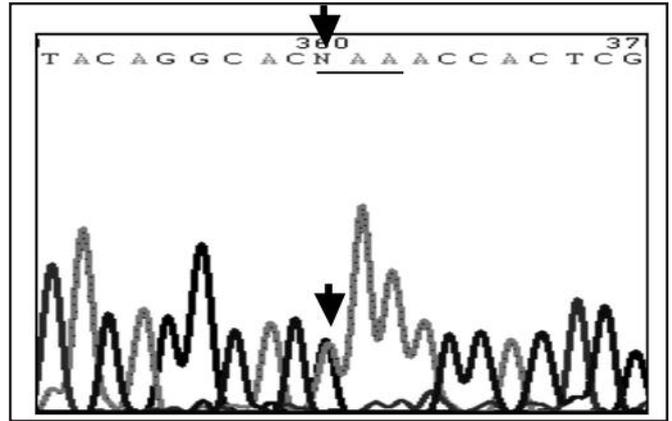


Fig. 6: E101K mutation, G is substituted by A (GAA→NAA) resulting in change of amino acid from glutamic acid to lysine at codon 101.

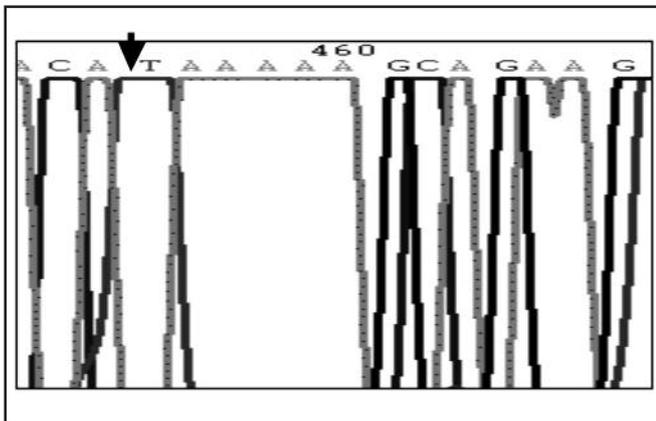


Fig. 7: 366delT mutation, deletion of a single base (T) at nucleotide 366 inducing a frameshift mutation in amino acid coding.

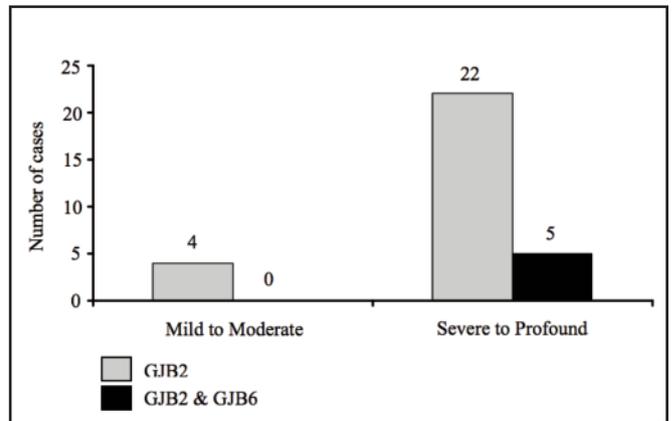


Fig. 8: The degree of hearing loss in different type of mutations.

**DISCUSSION**

The prevalence of association between GJB2 mutation and GJB6 was 5/31 (16.2%). Association between these GJB2 and GJB6 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<sup>5</sup>. Although mutations in GJB2 have been shown to be the major cause of autosomal recessive non syndromic sensorineural hearing loss (ARNSHL) in many populations, other connexin gene also have been demonstrated to cause recessive non-syndromic deafness: a large deletion involving GJB6 called the 342-kb is a common cause of deafness in Southern European populations<sup>6</sup>.

Other study has shown that the 342-kb has been identified as the second most frequent mutation causing prelingual deafness in the Spanish population<sup>6</sup>. 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<sup>7</sup>. The deletion was also found in 2 out of 25 patients from German<sup>8</sup>. It also was found in seven NSHL patients being heterozygous for GJB6

mutations from four unrelated Ashkenazi Jewish families<sup>9</sup>.

Interestingly, 342kb has not been detected in the Chinese deaf population. The deletion was also not found in 229 deaf patients from the Kurdish population in Iran<sup>10</sup>. Moreover, Günther *et al.* (2003) found the same results when they screened 317Austrian, 35 Turkish, 10 Serbian and 14 Bosnian patients<sup>11</sup>. Our study showed that 342kb mutation was not found in our population suggesting that the mutation is very rare in this region compared to certain parts of the world.

Similarly, none of the 118 tested deaf Chinese patients had this mutation<sup>12</sup>. In this study, no mutation was found among Chinese students. These finding may due to the small number of Chinese students involved in this study 5/31. Günther *et al.* (2003) reported that del(GJB6-D13S1830) deletion also appears to have an ethnic specific origin as it is absent from the Siberian, Chinese, Austrian and Italian populations<sup>11</sup>.

However interestingly in this study, a novel of five different GJB6 mutations; E137D, R32Q, E101K, Y156H and 366delT were identified. All of these students were heterozygous for

both GJB2 and GJB6 mutation. With regards to the hearing level, Ruszymah *et al.* (2005) found that heterozygous V371 mutation in GJB2 causing a severe to profound sensorineural hearing loss<sup>4</sup>. Variable degree of hearing loss was also found in GJB2 mutation which ranges from moderate to profound hearing loss. Biallelic GJB2 mutations and combined GJB2/GJB6 anomalies were more frequent in profoundly deaf children<sup>13</sup>. Christel *et al.* (2004) reported that there was no significant difference in hearing impairment between the patients with the homozygous 35delG mutation in GJB2 and those who are heterozygous for both the 35delG mutation and the deletion encompassing part of GJB614.

Marlin *et al.* (2005) observed that there was no difference in deafness severity with GJB6 deletion or homozygous GJB2 mutation<sup>15</sup>. Similarly, in our study, two of the student had severe to profound hearing loss and three had profound hearing loss. Two of these students with profound hearing loss, found to have deletion in GJB6 which is reported as a severe type of mutation. Hearing loss in patients with biallelic (i.e. deletions, nonsense and splice site) mutations was significantly worse than in heterozygotes. The presence of at least one missense mutation in compound heterozygotes tends to lead to better hearing thresholds compared to biallelic mutations and digenic GJB6 and GJB2 mutation<sup>16</sup>. Recent study reported that 35delG as the most frequent mutation causing in Italian populations with prevalence 68.6% of detected Cx26 mutated alleles. They detected two novel mutations: V1561 and the C>A change at nucleotide 684 in the in 3'UTR (untranslated region) of the gene<sup>17</sup>.

On the basis of these findings, genetic testing for GJB2/GJB6 mutations in hearing loss is recommended as it could be a diagnosis tool for future intervention therapy and genetic counseling especially in cases of inherited hearing loss. In this study we found 2 cases with association between GJB2 mutations and GJB6 have family history of deafness. We proposed that in the future, all patients with non syndromic sensorineural hearing loss should undergo PCR-based genetic test to screen for the presence of GJB2 mutations. Those patients with heterozygous GJB2 mutation should be encouraged to undergo further genetic testing as to confirm the basis of the hearing loss. However it again depends on the availability of the test as it is not a routine screening test available in the hospital but rather, based on the request from the clinicians.

Further local study with larger sample size need to be conducted to establish data on GJB2/GJB6 specific mutation as the mutation might be regional and also ethnic specific.

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