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Analysis of the *GJB2* Gene and its Mutated Protein in Non-Syndromic Hearing Loss Patients of Gilgit-Baltistan

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ABSTRACT

Pakistani population is unique for the study of recessive genetic diseases due to a higher rate of consanguinity and Hearing Loss (HL) is one of them that affects more than 466 million people worldwide; will increase to 900 million by 2050. *GJB2* mutation is one of the main causes of hearing loss in different populations, including Pakistan, that encodes a gap junction protein involved in the homeostasis of the inner ear by recycling potassium ion. This research aimed to find out mutations in the *GJB2* gene and its protein structure. Both control and patient samples were collected from Gilgit-Baltistan for DNA isolation and PCR was done by using a specific primer while sequencing was done by Sanger sequencing. Mutations were detected by Mutation Surveyor and BLAST. Protein structures of both control and mutated samples were constructed by PHYRE2 and visualized by the software PyMOL. The detected mutations were 380G>A (R127H), 457G>A (V153I), 36T>C (G12G), 496C>T (L166L), 650 delA and 79G>A (V27I). This study revealed that the prevalence of *GJB2* mutations in hearing-impaired patients of Gilgit-Baltistan is 11.66 %. The mutation rate is higher as compared to other populations of Pakistan. This study can help deaf patients via gene therapy and also through drug designing.

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Gap junction protein, *GJB2* mutations, Hearing loss, Sensory disorder

INTRODUCTION

Hearing loss (HL) or hearing impairment is the loss of ability to hear normal sounds (Pavithra *et al*., 2017). According to the World Health Organization (WHO, 2018), 5 % of the world population are suffering from hearing loss worldwide, and it is estimated that by 2050 it will increase to 900 million. It is one of the most common disorders that affect 1in 500-1000 newborns (Naz *et al*., 2017; Arai *et al*., 2017). By the age of 5 years, the prevalence increases up to 2.7 per 1000 and 3.5 per 1000 in the adult (Morton and Nance, 2006). HL is caused by environmental (high-intensity sounds, viral infections, and drug usage), immunological and genetic factors, which significantly contribute to hearing loss. Almost 50 % of HL is caused by genetic and immunological factors (Wang *et al*., 2017).

Inheritance of HL can be recessive, dominant, X-linked, and mitochondrial (Wang *et al*., 2017). HL due to genetic factors is classified into two categories, which are syndromic hearing loss (SHL), constituted of 20-30%, and non-syndromic hearing loss (NSHL) constitutes of 70-80% (Subaşıoğlu *et al*., 2017). The NSHL may be an autosomal recessive non-syndromic hearing loss (ARNSHL) or autosomal dominant hearing loss (ADNSHL) (Atik *et al*., 2015). The gene locus for NSHL is known as DFN (DeaFNess). The loci for a gene, which is inherited in an autosomal dominant pattern, is referred to as DFNA, in an autosomal recessive pattern as DFNB, and gene inherit in X-linked pattern as DFN (Ali, 2010). It is estimated that more than 200 genes are responsible for hearing; loss; however, only 90 genes have been identified (Singh *et al*., 2017). One of the main genetic causes is mutations in *the GJB2* gene, which encodes a gap junction protein; involved in the homeostasis of the inner ear through the recycling of potassium ions (Koohiyan *et al*., 2018).

The *GJB2* gene is present at DFNB1 locus on chromosome 13q12.11 and structurally contains two exons, coding (exon 2) and non-coding exon (exon 1) (Kashef *et al*., 2015). *GJB2* gene is the most common cause of NSHL, and almost 300 different variants have been reported. The most common hereditary type of hearing impairment is ARNSHL and more than 700 different causative mutations have been reported in over 80 loci known as DFNB. DFNB1 locus contains the most common ARNSHL gene (*GJB2*) and more than 50% ARNSHL is linked to the *GJB2* mutation. More than 100 *GJB2* mutations have been

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reported, responsible for ARNSHL (Naseri *et al*., 2018).

The main aim of this study was to find out the mutations in the coding region of the *GJB2* gene in a hearing loss population of Gilgit-Baltistan, Pakistan. Other populations of Pakistan have been screened for *GJB2* gene mutations, but the region of Gilgit-Baltistan was not studied yet. This study will significantly add some knowledge to the scientific literature. In future, the present study can help deaf patients via gene therapy and through drug designing.

MATERIALS AND METHODS

This project was designed to find out the association of the *GJB2* gene with hearing loss in the population of Gilgit-Baltistan. Blood samples were collected using sterilized syringes and vacutainer (EDTA Vials). DNA was isolated by a method of Sambrook and Russell (2001). PCR was done with forward and reverse primers to amplify the coding sequence of the *GJB2* gene. The PCR condition consisted of initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, annealing at 60 for 1 min, initial extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. This PCR was set for 30 cycles. 1.3 % agarose gel electrophoresis was done to check the desired DNA band after PCR. PCR products were mostly cleaned with ethanol precipitation. All PCR products were purified with 76 % ethanol. Sequencing of the PCR product was done after purification by Sanger sequencing. Genetic Analyzer 3730. The sequencing results were analyzed by using Chromas version 2.6.4. Low-quality sequences on both sides were trimmed. All nucleotides were also checked manually to ensure any wrong base call. The similarity of the sequence was checked by the NCBI nucleotide blast. The mutations were noted by a software Mutation Surveyor and codons and amino acid changes were studied by software PyMOL. The Institutional Review Board of School of Biological Science, University of the Punjab, Lahore, Pakistan, approved this study and the Ethical Committee issued an approval letter.

RESULTS

A total of 67 HL patients and 67 controls were enrolled. The total number of male HL patients in the study was 42 (62.66 %), while female HL patients were 25 (37.31 %). The mean age of male HL patients was 43.28±11.64 and female HL patients were 39.12 ± 12.41. The patients above the age of 65 years were excluded from the study. There were 14 patients in 1st age group (10-30 years), 39 patients in 2nd age group (31-50 years) and 14 patients in 3rd age group (51-65). Thus most of the patients lie within the age group of 13 to 50 years. Cousin marriages among hearing loss patients were only 14 (20.89%), while 53 (79.10%) patients had no cousin marriages.

The onset of hearing loss varies among patients. Only 22 patients suffered from HL after birth at different ages, while 45 patients were deaf by birth. The patients with a family history of hearing loss were 18 (26.86%), while patients with no family history of hearing loss were 49 (73.13%). Only 3 patients had moderate HL 15 patients had severe HL and 59 patients had a profound HL. All samples were screened for the mutation to check out the association of the *GJB2* gene with HL in the population of Gilgit-Baltistan, Pakistan. It is already known that the mutation in the *GJB2* gene contributes around 6 % of severe to profound HL in the population of Pakistan. Sixty-seven random HL patients were enrolled from different villages of district Skardu and Ghanche of Gilgit-Baltistan. Sequence analysis of the coding exon of the *GJB2* gene revealed that 10 out of 67 patients carried only 06 different mutations in the *GJB2* gene.

Mutation 380G>A (R127H)

The mutation 380G>A (R127H) was found in 4 samples (Accession # MH234062, MH234063, MH234115). Sequencing of coding exon revealed a heterozygous mutation 380G>A (R127H) in the affected individuals. This mutation converts arginine residue into histidine at codon 127 (Fig. 1).

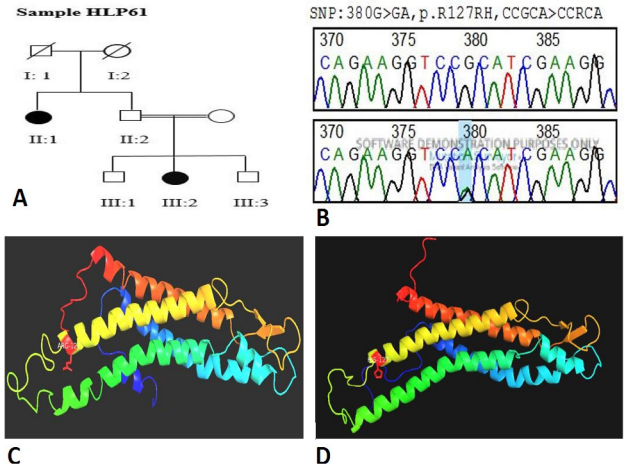
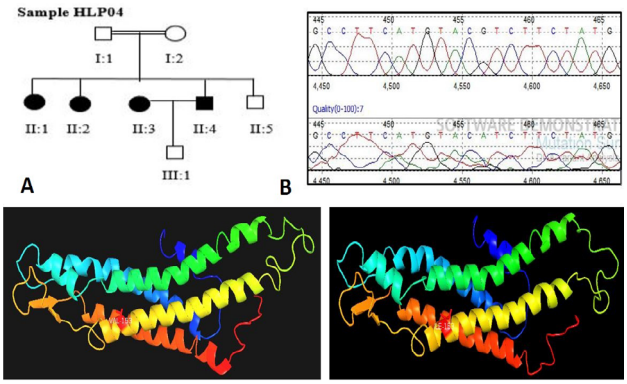


Fig. 1. A, Pedigree of sample HLP61; B, chromatogram showing mutation 380G>A; C, Protein structure of the control sample; D, Protein structure of the mutated sample.

Mutation 457G>A (V153I)

The sequencing of the coding sequence revealed a heterozygous mutation 457G>A (V153I) (Accession # MH234065). Valine residue at codon 153 was converted

to isoleucine due to this mutation (Fig. 2).

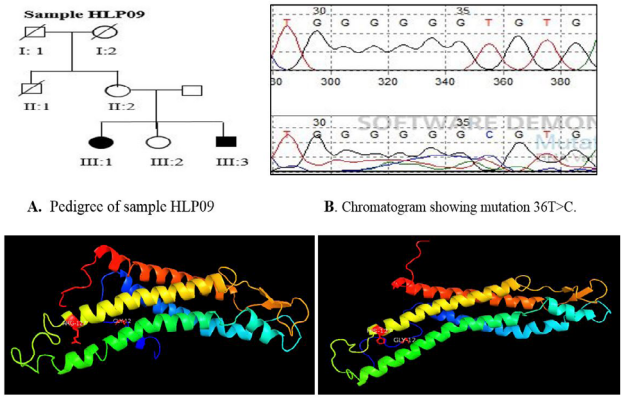


C. Protein structure of the control sample. D. Protein structure of the mutated sample.

Fig. 2. A, Pedigree of sample HLP04; B, chromatogram showing mutation 457G>A; C, Protein structure of the control sample; D, Protein structure of the mutated sample.

Mutation 36T>C (G>12G), 380 G>A (R127H)

The screening of coding exon revealed two mutations in the affected individual (III:3). There was a mutation 36T>C (G12G) (Fig. 3) and 380G>A (R127H) (Accession # MH234070, MH234077, MH234080). Due to the nucleotide change, 36C>T, there was no amino acid change at codon 12 but at codon 127 the arginine residue was converted to histidine because of the nucleotide change at position 380 (Fig. 3).



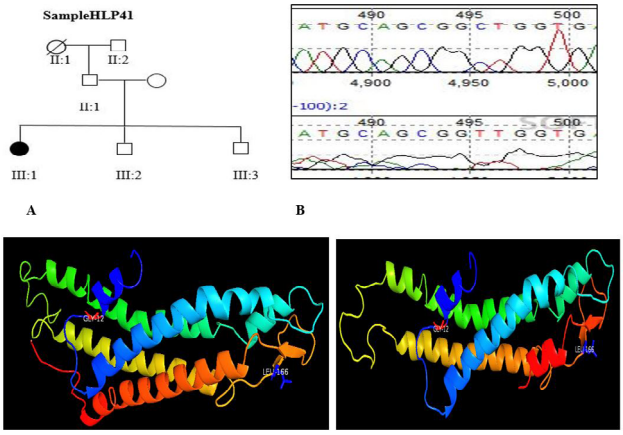
A. Pedigree of sample HLP09 B. Chromatogram showing mutation 36T>C.

C. Protein structure of the control sample. D. Protein structure of the mutated sample.

Fig. 3. A, Pedigree of sample HLP09; b. chromatogram showing mutation 36T>C; C, Protein structure of the control sample; D, Protein structure of the mutated sample.

Mutation 496 C>T (L166L), 36C>T (12G12)

The screening of coding exon revealed two mutations in the affected individual. At position 36 the cytosine was replaced by thymine (Fig. 4) and at position 496, cytosine was also replaced by thymine (Accession # MH234097). There was no effect of both mutations on the amino acid change. The glycine residue at codon 12 and leucine residue at codon 166 remained same (Fig. 4).



C. Protein structure of the control sample. D. Protein structure of the mutated sample.

Fig. 4. A, Pedigree of sample HLP41; B, chromatogram showing mutation 36T>C; C, Protein structure of the control sample; D, Protein structure of the mutated sample.

Mutation 79 G>A (V27I)

The screening of coding exon of the *GJB2* gene revealed a mutation 79G>A (Accession # MH234113) which converted Valine residue into Isoleucine at codon 27 (Fig. 5).

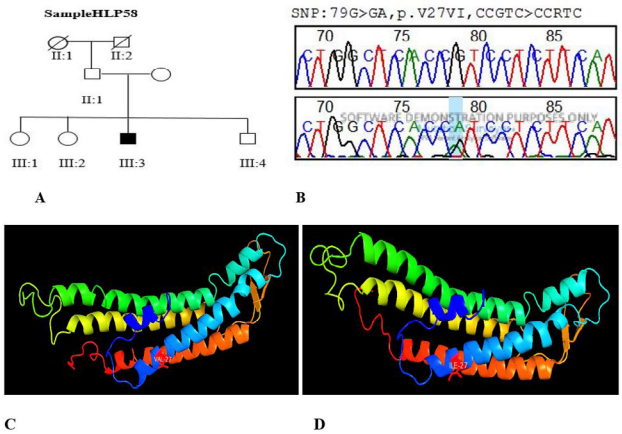


Fig. 5. A, Pedigree of sample HLP58; B, chromatogram showing mutation 79G>A; C, Protein structure of the control sample; D, Protein structure of the mutated sample.

Mutation 650 del A, 380 G>A

The sequencing result of coding exon revealed

two mutations in the affected individual (Accession #MH234105). There was a deletion of Adenine base at position 650 and substitution of Adenine in place of Guanine at position 380. Due to mutation 380 G>A, arginine residue was replaced by histidine. The deletion of 650 delA caused a change in protein structure. Many amino acid residues were converted at the end of the chain (Fig. 6).

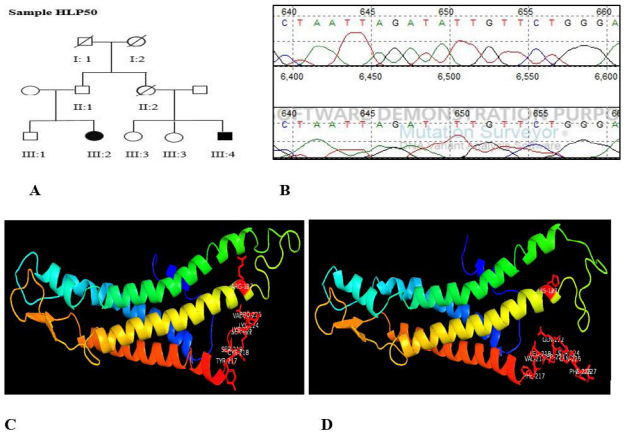


Fig. 6. A, Pedigree of sample HLP50; B, chromatogram showing mutation 650 del A, C, Protein structure of the control sample; D, Protein structure of the mutated sample.

DISCUSSION

This is a systematic genetic study of moderate to a severe HL in a population of Gilgit-Baltistan, Pakistan. HL is one of the most common sensory disorder that affects 1-3 newborn per 1000 population (Bakhchane *et al*., 2016) and 1 newborn per 500 populations (Sloan-Heggen *et al*., 2016). *GJB2* gene mutation is one of the common causes of NSHL in different ethnic groups (Mishra *et al*., 2018). In Pakistani population, the prevalence of *GJB2* mutations is 6.1% (Santos *et al*., 2005; Wang *et al*., 2017), 4 % and 53.33 % (Bukhari *et al*., 2013; Mahdieh *et al*., 2016) and (Fang *et al*., 2015). Different populations of Pakistan were screened for *GJB2* mutations, but this is the first study of the HL in Gilgit-Baltistan population of Pakistan. Tibet of China is the neighbor of Gilgit-Baltistan and the people of Baltistan region are descendent of the Tibetan race. The rate of *GJB2* mutations in Tibet was reported as 11.43% (Duan *et al*., 2015). This study revealed that the prevalence of *GJB2* mutations in hearing-impaired patients of Gilgit-Baltistan is 11.66 %. The mutation rate is higher as compared to other populations of Pakistan.

More than 100 mutations in *GJB2* have been identified for HL. However, the prevalence is different in different populations. This study revealed seven mutations which were 380G>A (R147H), 36T>C (G12G), 496C>T (L166L), 650 delA, 79G>A (V27I) and 457G>A (V153I). Three mutations are already reported in Pakistani population and other four mutations are the novel mutation in this population. 380G>A (R147H) was also reported by Santos *et al*. (2005) and Shaikh *et al*. (2017). Similarly, 79G>A (V27I) is reported by Santos *et al*. (2005) and 457G>A (V153I) was reported by Santos *et al*. (2005) and Anjum *et al*. (2014).

The most common mutation in Pakistani populations is 231G>A (W77X) and 71 G>A (W24X) (Salman *et al.,* 2015). Unlike Salman *et al*. (2015) this study did not show such mutations which are common in other populations of Pakistan. In this study, the most common type of mutation was 380G>A (R147H) which was 8.33% (5/60) and 36 T>C (G12G) which was 6.66% (4/60) while the frequencies of other mutations were low. The rate of *GJB2* mutations in Pakistani population is different in different areas, but this study showed a high rate (11.66%) as compared to 6.1% and 6-7 % by Santos *et al*. (2005) and Salman *et al*. (2015), respectively. The causes may be the non-familial study and other environmental factors. The familial study by Shafique *et al*. (2014) showed a higher rate (53.33%). Other immuno-genetic factors may also cause high rate of mutation in this population. Cousin marriages are common in Gilgit-Baltistan population, so the genome must be unique, however, in deaf population cousin marriages were only 21%. The deaf people are usually married to a deaf partner so the chances of mutation had increased. The hearing loss may be induced by noise, but the region of Gilgit-Baltistan is noise free area and very close to nature. The socioeconomic status of the deaf population is poor in Gilgit-Baltistan and the literacy rate of the deaf population is almost nil.

The most common mutation responsible for hearing loss in different populations worldwide is 35delG. This study did not show any such mutation; however, this mutation was reported in neighboring countries like Iran and India (Mahdieh *et al*., 2016). Mutations in nucleotide sequence change amino acids also. The protein structure may be changed due to a mutation in DNA sequences. The findings of this study showed seven different types of mutation. 380G>A (R127H) was a heterozygous mutation because both peaks of G and A were presented in the chromatogram. Amino acid residue Arginine was replaced by Histidine but the total protein structure was not clearly changed only the area of mutation was changed. The mutation 36T>C (G12G) was also a heterozygous mutation because both peaks of T and C were present in the chromatogram but the amino acid Glycine at codon 12 was not replaced by another amino acid. The mutation 457G>A (V153I) was reported in a family having a

consanguineous marriage. The nucleotide change resulted in an amino acid change at codon 153. Valine residue was replaced by Isoleucine. The amino acid sequence was complete in the mutated sample and the protein structure at the mutated area slightly changed. The mutation 496 C>T (L166L) was also reported in a sample having no cousin marriages in the family. The nucleotide change did not affect the amino acid. The nucleotide C was replaced by T but the amino acid residue Leucine remained the same at codon. However, the structure of the protein changed. The mutation 650 del A changed the sequence of amino acids and add an extra residue at the end of the protein. The protein structure was changed due to this mutation. The 79G>A (V27I) was a heterozygous mutation. The nucleotide change resulted in an amino acid change, but the protein structure did not change completely.

Statement of conflict of interest

The authors have declared no conflict of interest.

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