Identification of Novel Mutations in GJB6 Gene in Deaf Individuals of District Mansehra Pakistan

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Abstract- Deafness is one of the common sensory impairments in humans, affecting approximately 1 to 3 individuals in every 1000 live human births. Genetic mutations in the gap junction proteins, Cx26 and Cx30 are the major contributors to sensorineural, non-syndromic and prelingual hearing loss in humans. The GJB6 gene encoding connexin (Cx30) protein is expressed in mesenchymal and epithelial structures of the inner ears. Mutation in GJB6 gene results in loss of function mutation that causes hearing loss in humans. The current study was thus designed to explore genetic mutations in GJB6 gene in deaf individuals from District Mansehra, Pakistan. The DNA of non-syndromic deaf-individuals and normal controls were isolated from their saliva. The whole coding area of GJB6 gene was amplified by using PCR specific primers and subsequently sequenced by Sanger method. After sequence data analysis we identified 3 mutations in the coding area of GJB6 gene. It was observed that at position 242, Guanine (G) is substituted by Thymine (T), at position 338 Thymine (T) is substituted by Cytosine (C), and at position 381 Adenine (A) is substituted by Guanine (G). The identification of these novel mutations in coding region of GJB6 gene has enabled us to establish the genetic bases for hearing loss in deaf individuals. The occurrence of higher frequency of genetic mutations in GJB6 gene in some populations suggests the use of molecular markers as tools for diagnosis, carrier detection, and prenatal diagnosis of deafness.

***Index Terms*-** Deafness, Mutations, GJB6 gene, Pakistan

I. INTRODUCTION

Deafness is a common congenital disease in the US which affect 1 to 3 per 1000 infants at birth while 1 to 6 have milder impairments (Wu *et al*., 2022). Hearing impairment is defined as, "the inability of a person to perceive sound either partially or completely" (Varshney, 2016). Hearing loss is the third chronic disorder world's wide. About 2.5 billon people around the globe are expected to have some level of hearing impairments by 2050 and 700 million will need hearing rehabilitation while 1 billion teenagers are at risk of permanent deafness due to dangerous listening practices (WHO, 2022). About 250 million peoples suffer from deafness worldwide. More than 4000 new-born children conceived loss of hearing every year and most of them are genetic (Porter *et al*., 2009). According to the Centre for disease control (CDC), the most common types of deafness are conductive, sensorineural, and central and mixed that affect about 3% of school age children. Causes of hearing loss are both acquired and congenital (Shave et al., 2022). The acquired hearing loss mostly occur due to exposure to pollution, formation of fluid behind the eardrums, ear infections, and head injuries (Chang *et al*., 2020). The congenital hearing loss occur due to family history of deafness, problems during pregnancy such as (rubella, Rh factors or maternal diabetes) (WHO, 2006). Like other inherited disorders, deafness too has high rate among consanguineous population (Martines *et al*., 2012). Inherited hearing impairments occurs due to

single or multiple mutations throughout the genetic material which express at birth or later in life. Approximately 50 % reasons of hearing impairments are genetic. It is estimated that approximately 1% of total human genes (around 300) are responsible for hearing process in humans (Jacoszek *et al*., 2017), and the same genes are responsible for hearing loss when mutated. The inherited hearing loss is further divided into syndromic and non-syndromic types. Virtually, about 130 different loci has still been identified which are responsible for syndromic and/or non-syndromic hearing impairments (Friedman *et al*., 2007). It is believed that mutation in Gjb2 gene has the major contribution in autosomal recessive hearing loss which account for about 50% and the remaining are due to mutations in other genes (Tin *et al*., 2017). In 1994, the first locus for autosomal recessive hearing loss, DFNB1, was identified located on chromosome 13q12 (Li *et al*., 2005). DFNB1 locus contains two connexin genes, GJB2 (or connexin 26) and GJB6 (or connexin 30). Both of these genes are lengthy with two exon, of which only one contain coding region (Buonfiglio et al., 2020). Connexins are a family of genes that code for gap junction protein. Gjb6 is also known as connexin 30. Connexin 30 (CX30) is a protein in human being that is encoded by the GJB6 gene (Huang *et al*., 2023). Other names of this gene are DFNA3, gap junction protein or beta-6, which is 30kDa in size (Smith & Ranum, 2016). The cytogenetic location of this gene is 13q12.11, which means the gene Gjb6 is the located on long (q arm) of chromosome # 13 at position 12.11 (Figure 1).

The human Gjb6 gene encoding connexin CX30 demonstrated that it differs from other connexin-encoding genes. The Gjb6 gene has six exons, some of which can be alternatively spliced. One of the non - coding exons of GJB6, which has been described in brain CX30 CDNA, was not found in CDNA obtained from human keratinocytes suggesting tissue-specific splicing (Essenfelder *et al.*, 2005). This gene has four trans-membrane domains connected by two extracellular loops and a cytoplasmic loop, along with cytoplasmic amino -terminus and cytoplasmic carboxyl-terminus tails. Mutation in CX30 protein have been identified in autosomal dominant (DFNA3) and autosomal recessive (DFNB) non-syndromic sensorineural hearing impairments, and in at least one case of the keratitis-Ichthyosis deadness (KID) Syndrome (Baris *et al*., 2008). The GJB6 gene (AJ005585) encodes a 261-amino acid long protein. The human CX30 protein shares 93% homology with mouse Cx30 and 76% identity with human CX26 (Wang *et al.*, 2011). A 342-kb deletion in GJB6 genes was directly correlated to recessive non syndromic hearing loss in humans (Lerer *et al*., 2001). The large deletion in GJB6 gene could affect an undiscovered upstream regulatory element of GJB2 (Bykhovskaya *et al*., 2001). This deletion, named del(GJB6-D13S1830), was found in affected subjects either in homozygosity or in double heterozygosity with a GJB2 mutation (Pallares-Ruiz et al., 2002). Genetic mutations in the gap junction proteins, Cx26 and Cx30 are the major contributors to sensorineural, non-syndromic and prelingual hearing loss in humans. The GJB6 gene encoding connexin (Cx30) protein is expressed in mesenchymal and epithelial structures of the inner ears. Mutation in GJB6 gene results in loss of function mutation that causes hearing loss in humans. The current study was thus designed to explore genetic mutations in GJB6 gene in deaf individuals from District Mansehra, Pakistan.

II. MATERIAL AND METHODS

Ethical Approval and Sample Collections

This research was permitted by the Institutional Bioethical Committee, Hazara University Mansehra Pakistan. Samples were collected randomly from deaf individuals of district Mansehra through buccal swabs. All the participants were briefed about the study and informed consent forms were signed from them. Swabs were used to get epithelial cells from cheeks inside mouth. Samples were kept in fridge before the extraction of DNA.

DNA Extraction from Buccal Swabs

The swab head was cut with sterile blade into Eppendorf tube, about 600 microliters of CTAB or SEB buffers were added to the sample or as much required to dip it completely then 20 microliter of proteinase K was added into the same sample and vortexes (Ghatak *et al*., 2013). Keep it in water bath for 1 to 3 hours at 65°C for incubation and vortex after every 30 minutes. After incubation the samples were centrifuged for 3 to 4 minutes at 14000rpm. The swabs were Basketed and centrifuged again for 5 minutes at 14000rpm.

For basketing tips of the Eppendorf tubes were cut with sterilized blades and the swabs were transferred to cut tubes. Because of the centrifugation all the solution from the tubes containing swabs would go down to the intact tubes and the cut tubes having swabs were discarded. 600 µl phenol chloroform Isoamyl alcohol (PCI) having ratio of (25:24:1) was added and vortexed till milky colors appeared. The samples were again centrifuged at 14000rmp for 8 minutes. After centrifugation the supernatant was transferred to a new tube and properly labelled. Then, 800 µl of chilled absolute ethanol (100%) was added for precipitation and then kept in freezer for 20 minutes. Tubes were centrifuged for 5 minutes at 14000rpm. Supernatant was removed carefully and 1000 µl of chilled 70 % ethanol was added to the pellet and centrifuged again for 3-4 minutes. Then, the supernatant was discarded and the pellet was kept for drying. Then 40 µl of nuclease free water was added to each sample. For checking the quality and quantity of purified DNA, the DNA samples were checked on agarose gel electrophoresis. 1% agarose gel was prepared by using 50X TAE (stock solution). About 2ml of TAE was taken and dissolved in 98ml of distilled water. Then, 1g powder of agarose was blended in 100ml of 1X TAE and the solution was heated in oven for one minute. After cooling the solution 20µl of Ethidium Bromide was added. The gel was then poured carefully into the comb fitted gel caster and was left at room temperature until the gel solidified, combs were removed. Running buffer was made by taking 5ml of 50X TAE which was mixed with 245 ml distilled water. The gel was put in a gel tray and poured running buffer in such a way that gel is fully covered. Bromophenol (2µl) was mixed with 5µl sample and loaded into well. At 80V electrophoresis was carried out for 20 minutes. When the gel moved 2-3 cm away from the initial point, remove it carefully. Under UV light the gel was visualized and picture was taken.

PCR amplification

For amplification of target gene, the primers were designed with Forward sequence: '5- ATGAAGCTTA TGGATTGGGGACG CTG-3' and Reverse: '5-ATGCTCGAGGCGCTTGGGAAACCTGTGATTG-3'. The extracted genomic DNA was used as a template for PCR amplification. The 25ul of PCR master mix was made by combining reagents including 2µl of forward and reverse primers ,2µl dNTPs, 2.5ul Mgcl2, 2.5ul of 10X Taq Buffer, 0.5ul Taq DNA polymerase (Thermo Scientific), 1.5ul of DNA template and 12ul ddH2O). PCR amplification was done at 95 °C for 5 min (initial denaturation), then, 35 cycles at 95 °C for 30 seconds (Denaturation), 52 °C for 40 sec (annealing), 72 °C for 40 sec (elongation), and a single step at 72 °C for 5 min (final extension), then hold at 4 °C. To analyze the desired amplified product of PCR, again gel electrophoresis was performed for PCR product. The PCR products were purified from agarose gel, by cutting the portion of gel having DNA bands and put in Eppendorf tubes. In each tube 300 µl of GB solution was added. The tubes were then kept in the water bath at 50 to 60ºC until the gel melted. The gel dissolved in GB solution was moved to spin columns and centrifuged for 1 and half minutes at 12,000 rpm. From the spin columns the materials were discarded and 700 µl washing buffer was added in column. After this the columns were again centrifuged for 30 seconds at 12,000 rpm. Material from the columns were discarded and again centrifuged at 12,000 rpm for one minute. Lower tubes were discarded, and columns were placed in newly labeled Eppendorf tubes. Then, 40 µl

S/No	Nucleotide position	Normal Nucleotide	Nucleotide Substituted
1	242	**G**	**T**
2	338	**T**	**C**
3	381	**A**	**G**

Table 1. Representation of mutation through Clustal W analysis

of illusion buffer (EB) was added and left for 1 minute. The tubes were centrifuged at 12,000 rpm for 1 minute and discard spin columns. The tubes were packaged properly to be sent for commercial sequencing. For sequencing the Gene clean product was sent to MACROGEN South Korea. For identifying and visualizing the target mutation the sequences were further analyzed. Clustal W and Bio Edit software were used for multiple sequence alignment. For alignment the human reference genome (hg19) from NCBI was used as reference sequence.

III. Result and Discussion

The current study was designed to explore genetic mutations in GJB6 gene in deaf individuals from District Mansehra, Pakistan. The DNA of non-syndromic deaf-individuals and normal controls were isolated from their saliva. Good quality DNA was isolated (Figure 2). For amplification of target gene, the primers were designed having Forward sequence: '5-ATGAAGCTTATGGATTGGGGGACGCTG-3' and Reverse: '5-ATGCTCGAGGCGCTTGGGAAACCT GTGATTG-3'. The extracted genomic DNA was used as a template for PCR amplification (Figure 3). The whole coding area of GJB6 gene was amplified by using PCR specific primers and subsequently sequenced by Sanger method. After sequence data analysis we identified 3 mutations in the coding area of GJB6 gene. It was observed that at position 242, Guanine (G) is substituted by Thymine (T), at position 338 Thymine (T) is substituted by Cytosine (C), and at position 381 Adenine (A) is substituted by Guanine (G) (Figure 4 and Table 1). The identification of these novel mutations in coding region of GJB6 gene has enabled us to establish the genetic bases for hearing loss in deaf individuals.

The purpose of the current study was to find mutations connected to deaf individuals. Retrospectives studies indicates that major deletion occur in GJB6 gene like del(GJB6-D13S1830) which coexist with Gjb2 35delG and constitute about 1% and is the second most frequent after 35delG mutation in GJB2 (Rodriguez-Paris et al., 2011). Similarly other deletion that occur in GJB6 is 232 kb deletion (GJB6-D13S1854) which is less common than 342 kb deletion (Castillo *et al*., 2005). Similarly other deletion that occur in GJB6 is 232 kb deletion (GJB6-D13S1854) which is less common than 342 kb deletion (Castillo *et al*., 2005). Our study indicates that we have found point mutation at three different locations which may lead to non-syndromic hearing loss. According to the importance of GJB6 gene for normal hearing, it is recommend that the homozygous absence of this gene is responsible for severe deafness and therefore DFNB1, as DNFA3, should also include the GJB6 gene. Additionally, this deletion appear in Trans of a GJB2 deficient allele (35delG or E47X) also leads to profound hearing loss. We also need to identify the effect of point mutation upon GJB2.

IV. Conclusion and Recommendations

It is concluded that mutations in GJB6 gene has strong association with deafness in Pakistani individuals. The mutations identified in this study can be further used for developing personalized medicine for the above population. It is also recommended that there is need of a cohort base study of all the populations living in Pakistan to screen all variants linked with hearing loss. Whole exome or whole genome study would also give us clear picture of the genetic information of the deafness.

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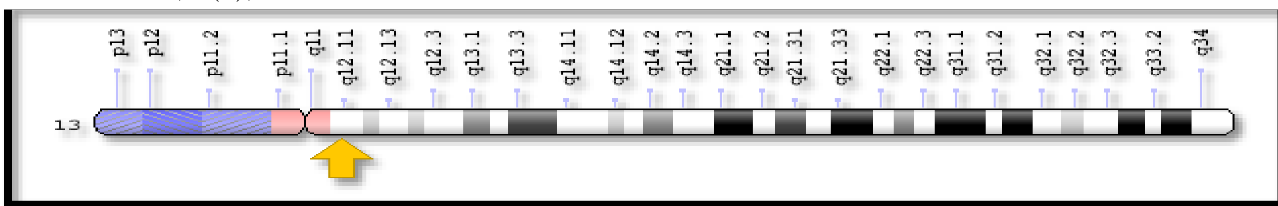

Figure 1. Location of Gjb2 and Gjb6 genes on human chromosome 13 (Ambarkova, 2017).

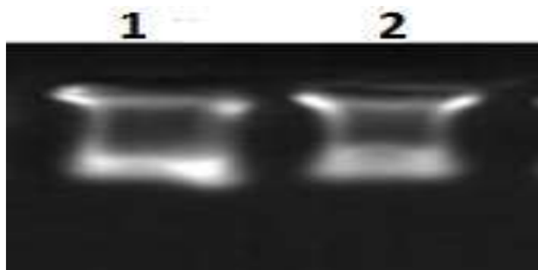

Figure 2. Genomic DNA isolated from deaf individuals.

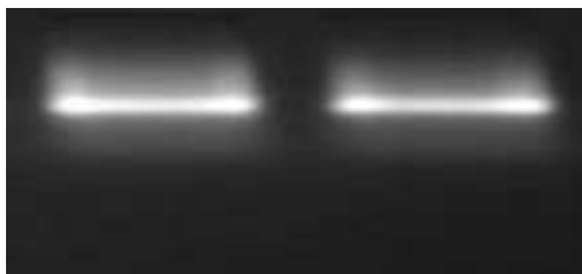

Figure 3. Bands of targeted gene after PCR amplification

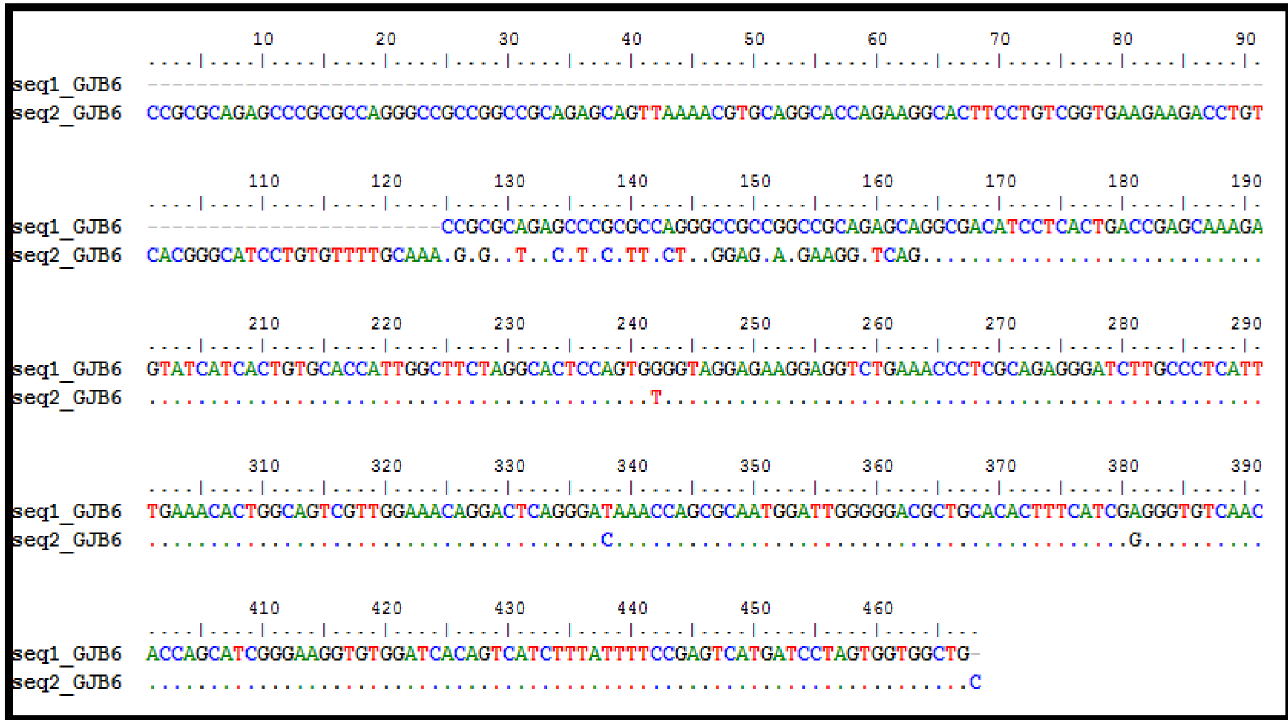

Figure 4. Graphical view of Clustal W Result (in graph the sequence 1 is control. In sequence 2 there are three mutations at positions 242,338, and 381).