

Molecular characterization of autosomal recessive non syndromic hearing loss in selected families from District Mardan, Pakistan

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Abstract: Deafness is the most common sensory disorder, which affects 1/1000 neonates globally. Genetic factors are major contributors for hearing impairment. This study was conducted to explore the linkage of DFNB4 loci and their mutations with NSHL in selected Pakistani families. We included 10 families with history of deafness from district Mardan, Pakistan. Blood sample (5ml) along with personal and clinical information was collected from the available family members including both diseased and un-affected individuals. Genomic DNA was amplified using loci specific STR markers to investigate the linkage of DFNB4 loci. Family found linked with DFNB4 locus was screened for SLC26A4 mutations. One out of the ten explored families was found linked with DFNB4 locus which was further investigated for SLC26A4 gene mutation through direct DNA sequencing. Two novel mutations were observed in the studied family, one at splice donor site (164+2T>G) and the other at position 164+5C>G only in the affected members of the linked family. DFNB4 locus was found linked in the present study which harbors SLC26A4 gene. The novel mutation of SLC26A4 gene at the splice donor site results in skipping of the first coding exon and thus can lead to loss of expression of SLC26A4 product in the inner ear.

Keywords: Genetic hearing loss, linkage analysis, DFNB4, SLC26A4, Mutation.

INTRODUCTION

Deafness is the most common sensory disorder affecting 1/1000 neonates worldwide (Lombardi, Garofoli *et al.*, 2010). It is a complex condition which occurs either due to environmental or genetic factors (Collin, Kalay *et al.*, 2007). Autosomal recessive non-syndromic hearing loss is one the most common congenital disorder, affecting 1/500 neonates (Morton and Nance, 2006). Like other genetic disorders, hearing impairment too has high incidence among consanguineous populations (Martines, Salvago *et al.*, 2012).

The Pakistani population are found to be the richest source of genetically inherited disease due to high incidence of consanguineous marriages (Riaz and Iqbal, 2012). Consanguineous marriage is customary in many societies, but leads to an increased birth prevalence of infants with severe recessive disorders (Modell and Darr, 2002), central to the kinship system and are used to strengthen ties within the kin group (Agha, 2016). Consanguineous marriages promote the replication of harmful recessive alleles in the next generation and thus increase the risk of a disease (Weatherall, 2009).

Genetic exploitation of hearing loss was started in an autosomal dominant form in a multi generation effected

family from Cost Rica (Leon *et al.*, 1992), while non-syndromic type was studied and mapped in Tunisian family (Guilford *et al.*, 1994). Several studies have been conducted on deafness in Pakistani population and almost 35 autosomal recessive non syndromic deafness loci have been identified by using Pakistani genetic resources. Large cohort consanguineous families with hereditary deafness showed that DFNB4 locus is the predominant cause of autosomal recessive non-syndromic hearing loss in Pakistani population (Riaz and Iqbal, 2012).

SLC26A4 gene located at 7q22-q31 (DFNB4 locus), is the second leading cause of deafness. It encodes Pendrin protein which is an iodide/chloride transporter and is expressed mainly in kidney, cochlea and thyroid (Mahdieh, Rabbani *et al.*, 2010). Approximately, more than 200 mutations have been identified in SLC26A4 gene, scattered in the whole gene and are mostly missense in nature (Reiisi, Sanati *et al.*, 2014). This study was aimed investigate the possible linkage of DFNB4 locus with autosomal recessive non-syndromic deafness and SLC26A4 gene mutations in Pakistani families from district Mardan.

MATERIALS AND METHODS

Ethics

The study was conducted at the Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan and

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was approved by the Ethics and scientific Research Committee of IBGE.

Enrollment of families

Families with three or more individuals having congenital deafness were included in the study from different areas of district Mardan. A total of ten families were enrolled in the study. Institutional IDs were allotted to each family as, 1DFN, 2DFN, 3DFN, 4DFN, 5DFN, 6DFN, 7DFN, 8DFN, 9DFN and 10DFN respectively. All families were clinically confirmed to be with non-syndromic hearing loss. The studied families collectively included more than 115 individuals. They consist of more than 25 second generation individuals, 38 third generation individuals and 52 fourth generation individuals. Sixty one individuals at total have been affected with the disease, majority of them were from the fourth generation.

Sample collection

All patient and normal samples with clinical information was obtained with informed signed consent. Samples were collected from the radial vein by using disposable Syringe and were transferred to ACD (acid citrate dextrose) vacutainer tubes. Pedigrees were drawn by using Cyrillic software.

Genotyping

DNA extraction from blood samples of the participating individuals was carried out using the organic method (Maniatis *et al.*, 1982). UV Spectrophotometer (U-3210, Hitachi, Japan) was used to determine DNA concentration and purity. Each sample was amplified with the following PCR conditions, 2 μ l MgCl₂ (4mM), 1.5 μ l Taq PCR buffer (10X), 1 μ l of each of the primers (10PM), 0.3 μ l Taq DNA polymerase (5U/ μ l), 1 μ l dNTP mix (10mM) and 1 μ l DNA (about 100 ng). The reaction was adjusted to the volume of 10 μ l by adding 2.2 μ l ddH₂O. Standard cycling conditions was performed in a thermo cycler as follows, 95°C for 5 min; 35 cycles; 95°C for 1 min', 57°C for 2min, 72°C for 45 seconds and finally 72°C for 7 minutes by using five STR markers (D7S2420, D7S425, D7S2456, D7S2459, D7S496) on chromosome 7q31 to detect linkage with DFNB4.

PCR products were run on Polyacrylamide Gel Electrophoresis (PAGE) at 100W and were visualized by using ethidium bromide stain with UV light. The family in which linkage was observed was screened for mutation through sequencing analysis. Details of DFNB4 locus were explored through the Hereditary Hearing loss Homepage, which aims to give an up-to-date overview of the genetics of hereditary hearing impairment for researchers and clinicians. It lists links and data for all known gene loci related to hearing loss. It was confirmed that there were multiple genes at the DFNB4 locus, but only SLC26A4 was reported linked with congenital non-syndromic hearing impairment (Van Camp and Smith,

2002). The SLC26A4 gene encodes an anion transporter known as pendrin, located on Chromosome 7q31: 107,660,635-107,717,809 forward strand having a total of 21 exons (coding exons=20), Transcript length of 4,930 bps and codes for 780 residues polypeptide. Exon specific primers (Wu, Lu *et al.*, 2009)(Table 1) were used for the amplification of all the 21 exons of SLC26A4 (Ensembl id: ENST00000265715) along with intron-exon boundaries. Subsequently, DNA sequencing of the PCR-amplified product from the linked family deaf and normal members was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems) using the same primers with the ABI BigDye Terminator v3.1 cycle sequencing kit.

RESULTS

Peripheral blood samples were obtained from ten unrelated families with multiple deaf members. The families were identified from District Mardan, Khyber Pakhtunkhwa (KPK), Pakistan. The pedigree of the largest family with reference to affected individuals (01-DFN) is shown (fig. 1a). All families were from different villages in District Mardan. Only one family (02-DFN) was linked to the deafness locus DFNB4 (Fig. 1b), out of the ten families studied. This family was collected from College Road, Main Mardan city, Tehsil Mardan, district Mardan, KPK, Pakistan. The family belongs to Yousafzai tribe. Blood samples of eight individuals (6 males and 2 females) were collected containing 3 affected and five normal members. Age of the affected individuals ranges from 5-40 years.

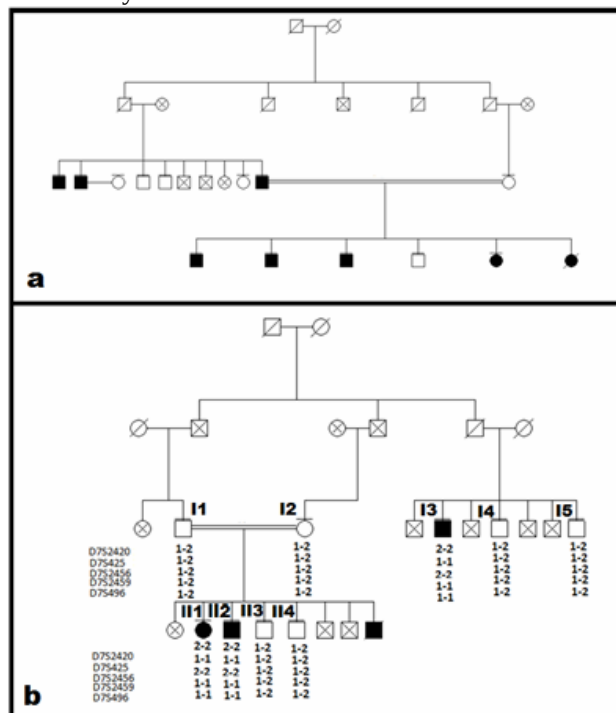


Fig. 1: Selected pedigrees of Pakistani families. Patients are shown in black.

Table 1: Sequences of the primers used for the amplification of SLC26A4 exons

S.NO	Exons	Sequences 5-3 (Forward/Reverse)	Product size bp
1	Exon 1	F-CCCTTCGACCAAGGTGTCTGT R-ACCCTTCCGCTGCCTTTATAG	80
2	Exon2	F-TCTTCCCCTCCGATCGTCCT R-CCTCCCCAAGGCGTGGAC	292
3	Exon3	F-AAGAGAGCCTTTGGTGTGCTAAA R-TACTAAGCAGCCATTCCTTGA	85
4	Exon4	F-CATATGCCCTACTAGCTGCAGTTC R-GATGTTCCAAAGATAAAGTATGTCAGGAT	87
5	Exon5	F-GGTGAGTTTAATGGTGGGATCTG R-GCTGCTGGATACGAGAAAGTGT	69
6	Exon6	F-GTGGCTTGACAGATTGGATTTCAT R-TGGAAGGCAGCAGCTGTTG	78
7	Exon7	F-GCGTGTAGCAGCAGGAAGTA R-CCTTGTTTGTCACCAAATAATG	250
8	Exon8	F-GAAAGTTCAGCATTATTTGGTTG R-GGAGTATCAGTGAAATGAAGCTTG	257
9	Exon9	F-TCACTAGGTTTTGCCTCCTGAA R-CAGCGATGGAATAATGATGCA	77
10	Exon10	F-CAGTCTCTTCCTTAGGAATTCATTGC R-GGCCGTGCGGGAAAG	98
11	Exon11	F-GACACAAGGGAGAAGGACGA R-AGGGAATGGTTTTCCATGTG	236
12	Exon12	F-AACAATCATCACATGGAAAACC R-TTATTTTCAGATAAATATAGGC	193
13	Exon13	F-TTTTCCCTAGGTTATCTGGGTGTTT R-AACTCTCAGGACCACAGTCAACAG	111
14	Exon14	F-TCCAAAATACGGCTGTTC R-ATGGAGCTGCTGAAACTTCAGG	187
15	Exon15	F-CCTTGCTAAGTAGCCAGAAATG R-TTGGACCCAGTAAATACTTGT	254
16	Exon16	F-CCTTTGAGAAATAGCCTTTCCAG R-GCTCTCATCAGGGAAAGGAA	241
17	Exon17	F-AAATAATGCTTTTGAGCCTGATGA R-CCAATCCACTTGAATCTCTATTTCCT	87
18	Exon18	F-TCCTGAGCAAGTAACTGAATGC R-GAAAGGGCTTACGGGAAAGT	190
19	Exon19	F-ACGACAACATTAGAAAGGACACATTC R-ACCTTGACCCTCTTGAGATTTCAC	91
20	Exon20	F-CAGTGGAGCATCAGGTGGG R-GTTCCCTGACAGTTCTTAATCAG	246
21	Exon21	F-ACAGGCTATGCGTACACTTGCA R-CATTGAGGAAGTTTGTCTTGTATTCC	83

a. Pedigrees of non-linked 01-DFN.

b. Pedigree and haplotypes of 02-DFN family.

Samples were amplified through five STR markers spanning in the region of DFNB4 locus. Polyacrylamide gel electrophoresis (PAGE) was run for about 2-3 hours at 150 volts and was visualized for the allele's homozygosity and heterozygosity. The alleles were then read manually to determine the pattern of inheritance. The STR marker D7S2456 showed heterozygosity in both parents and normal children while homozygosity in their entire affected children. The affected individuals (I3, II1 and II2) were homozygous while normal four (I1, I2, I4, I5, II3

and II4) were heterozygous (fig. 2). LOD score was calculated and was found 2.154.

The homozygous individuals for D7S2456 were sequence analyzed and for SLC26A4 mutations. Two novel transversions were observed in intron 2, one at splice donor site (164+2T>G) and the other at position 164+5C>G shown in fig. 3. In both cases pyrimidine was substituted into purine. The T>G mutation at 164+2 position altered the splice donor site in intron-2 of wild-type sequence which leads to the skipping of first coding exon (ex-2) of SLC26A4. The starter codon ATG is

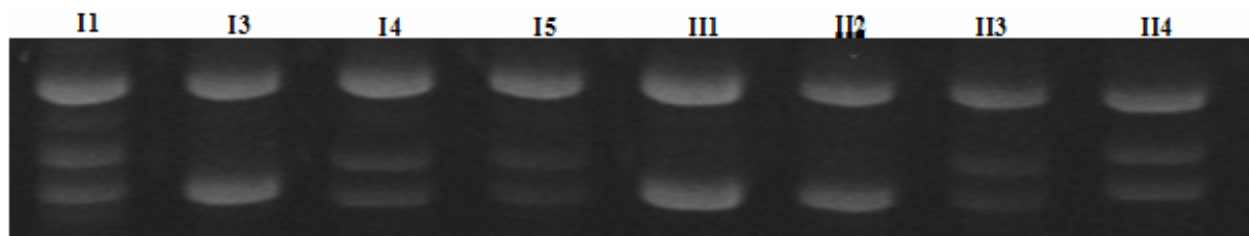


Fig. 2: Poly Acrylamide Gel Electrophoresis of PCR products of all with STR marker D7S2456 with the linked family 02-DFN. The name of the targeted amplicon is given above each panel.

positioned in exon-2 and thus its skipping will lead to loss of its product expression.

All affected patients of the linked family (2DFN) carried the observed novel mutations in contrast to their unaffected relatives. It was clear from the results that the mutations only co-segregated with the disease phenotype and affect the expression of the SLC26A4 gene. We therefore concluded that, the mutations are disease-specific and are not the polymorphic variant of the gene.

DISCUSSION

Linkage analysis was the major tool for genetic mapping of complex and Mendelian traits for many years. It was succeeded by the genome wide association studies but with the introduction of whole genome sequencing, it is again emerging as an important method for the identification of genes involved in disease etiology (Ott, Wang *et al.*, 2015). Almost 35 autosomal recessive non syndromic deafness loci or genes have been identified by using Pakistani genetic resources. Large cohort consanguineous families with hereditary deafness show DFNB4 locus as the predominant cause in Pakistani population (Anwar, Riazuddin *et al.*, 2009, Riaz and Iqbal, 2012). DFNB4 was first described in a deaf Israeli Druze family with pre-lingual, severe deafness linked to a 5-cM region on human chromosome seven (Petersen and Willems, 2006). In the present study all of these loci were investigated in ten families from District Mardan, of Khyber Pakhtunkhwa, Pakistan. Only one family (02-DFN) out of the ten found to be linked with DFNB4 locus (SLC26A4 gene), while rests of the families were not linked with any marker used. Several other investigations have also been carried out to analyze linkage analysis of DFNB loci and have showed linkage to the DFNB4 locus (Jamal, Anwar *et al.*, 2012, Noori-Daloi, 2010, Reisi, Sanati *et al.*, 2014, Yazdanpanahi, Tabatabaiefar *et al.*, 2013).

SLC26A4 gene is located on DFNB4 locus and is causative agent for hearing loss. From various reports of East-Asia DFNB4 is involved in 5% cases of prelingual deafness similarly in 5% at South Asia with recessive deafness (Park, Shaikat *et al.*, 2003). It provides blueprint for the translation of pendrin protein, transporting anions

including chloride, iodide and bicarbonates across the cell membrane (Scott, Wang *et al.*, 1999). Though, the exact function of the protein is not fully understood, but it is known as an important protein for the normal functioning of the inner ear, kidney and thyroid gland.

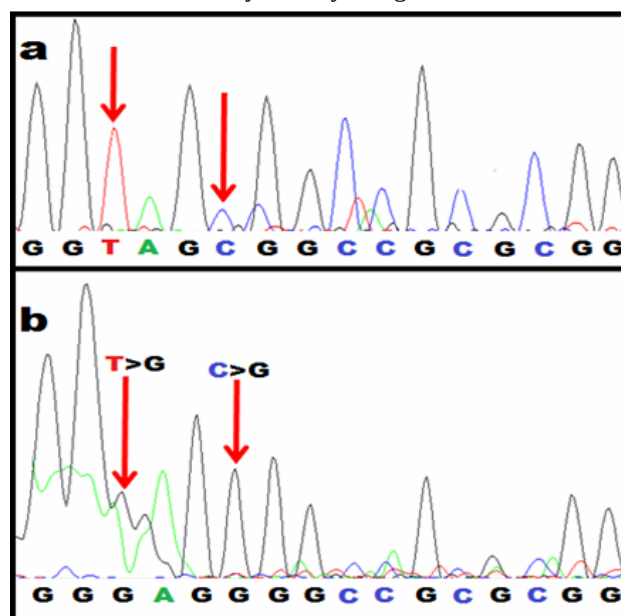


Fig. 3: Sequence chromatogram of SLC26A4

- Wild sequence from normal un-affected member of DFNB2 family
- Mutant sequence from affected member of DFNB2 family

Mutations in SLC26A4 gene alters the structure or function or both of the pendrin and thus disturbs the transport of anions (Abbasi, Ramadan *et al.*, 2007). SLC26A4 is commonly expressed in a variety of epithelial cells in the vestibular labyrinth, cochlea and the endolymphatic sac, enclosing the endolymph, which is the luminal fluid of the inner ear. It also get expressed in the outer sulcus, spiral prominence and transitional cells in the utricle, ampullae, saccule and mitochondria-rich cells of the endolymphatic sac (Kim and Wangemann, 2010). Slc26a4D/D mice are lacking pendrin and found to develop excessive enlargement of the membranous labyrinth, results in failure to acquire hearing and balance (Li, Sanneman *et al.*, 2013). Enlargement of the vestibular aqueduct (EVA: OMIM#600791) is a deformity of the temporal bone, commonly observed in youngsters with

sensorineural hearing impairment (Griffith and Wangemann, 2011, Ito, Choi *et al.*, 2011).

We hypothesized that our reported novel mutations especially the one at the splice donor site results in loss of pendrin expression and thus will lead to the enlargement of endolymph volume or swelling of the inner ear during embryonic development causing failure of the cochlea and the vestibular organs, resulting in deafness and loss of balance. Our results are also supported by the previous studies in a mouse model (Kim and Wangemann, 2011).

CONCLUSION

Despite of the extensive studies on DFN4 locus and SLC26A4 gene, its mutations and frequency is poorly been explored in Pakistani population. Our investigation demonstrates that, out of the 10 studied families only one family was linked with DFN4 locus and having two novel mutations in the affected members of the family. One of the two mutations is at the splice donor site results in skipping of the first coding exon which leads to the loss of starting codon and thus lack of SLC26A4 expression. It is therefore recommended to further characterize the said mutation to validate our results. We are hopeful that, this study will help to further our understanding about SLC26A4 gene for counseling and clinical decision making of the affected families.

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