

Haplotype Analysis for DFNB4/PDS Locus in Hearing Impaired Families of Punjab (Pakistan)

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Abstract: Deafness is one of the most common genetic disorders affecting 1 in 1000 newborns worldwide, while in Pakistan, its prevalence is 1.6/1000. The present study was conducted to map reported autosomal recessive deafness locus DFNB4/PDS in highly consanguineous families in Punjab. For this purpose families with deafness were identified. Blood samples of these families were studied for linkage analysis of common reported deafness locus DFNB4/PDS. Genomic DNA was isolated from the blood samples of these families. Linkage analysis was then performed by amplifying microsatellite markers through PCR. Genotyping was done by ABI PRISM® 3730 Genetic Analyzer. The Deaf Family was found linked to microsatellite markers of DFNB4/PDS. Linkage analysis showed that all three affected were homozygous for three STR markers for DFNB4/PDS while individuals V: 3 was heterozygous i.e., is carrier for DFNB4. Therefore individuals V: 3 were phenotypically normal but genotypically he is carrier. As the ages of that affected individuals' ranges from three years to seven years they might develop goiter at a later age. Therefore at present this family is linked to an overlapping nonsyndromic/ syndromic locus DFNB4/PDS.

Keywords: Consanguineous, genotypically, microsatellite, phenotypically

INTRODUCTION

Deafness may be complete or partial hearing loss and has dramatic effects on language acquisition and educational progress of infant. Deafness is one of the most common hereditary disorders in humans and mutations in many genes lead to its development (Tamhankar and Solomon, 2004). The incidence rate is 1 in 1000 newborns, with severe to profound hearing loss (Fettiplace and Hackeny, 2006). Another 1/1000 becomes deaf before their post lingual in a progressive and less severe manner. In developed countries, hearing loss affects 6-8% of the population when all causes are combined and is the most common birth defect (Petit *et al.*, 2001a). The prevalence of profound bilateral hearing loss in Pakistan is 1.6 per 1000 associated due unique socio-cultural practices and consanguineous marriages (Hussain and Bittles, 1998; Elahi *et al.*, 1998).

Hearing loss is caused by a variety of factors, including genetic, noise trauma, age-related and drug induced damage resulting in a wide variations and severity (Marazita *et al.*, 1993; Atar and Avraham, 2005; Petit *et al.*, 2001b). Environmental factors include bacterial and viral infections, acoustic trauma and ototoxic drugs etc., (Robson, 2006). Genetic deafness can

be classified as syndromic or non-syndromic. In syndromic deafness other clinical manifestations occur along with hearing loss e.g., Wardenburg's, Usher's, Pendred's, Alport's syndrome etc., (Gorlin *et al.*, 1995) while in non syndromic deafness no other clinical manifestation occurs. Non-syndromic deafness includes autosomal recessive (80%), autosomal dominant (20%), X-linked (1%), mitochondrial (<1%) (Morton, 1991) and Y-linked deafness has also been reported (Wang *et al.*, 1998)

Sensorineural deafness represents extreme genetic heterogeneity and 154 non-syndromic deafness loci have been mapped on different chromosomes, 59 of these loci are inherited in an autosomal dominant pattern (DFNA) and 91 loci are inherited in autosomal recessive mode (DFNB) (Van Camp and Smith (2000) www.uia.ac.be/dnalab/hhh). A total of 300 genes are expected to involve in hearing that accounts for 1% of human coding genes (Friedman and Griffith, 2003). Mutations in different genes cause the same clinical phenotypes in deaf individuals, even within the same family. On the other hand, different phenotypes exist for mutations in the same gene (Masmoudi *et al.*, 2000).

The DFNB4 critical interval at 7q31 encodes a transmembrane protein known as *pendrin*, which

functions as an anion (chloride/iodide) transporter. It contains 780 amino acids codified by gene *SLC26A4* (21 exons) and mutations in this gene are responsible both for *Pendred syndrome* (7q21-34) and *DFNB4* (gene *SLC26A4-7q31*). The *PDS/Pds* gene is expressed in the inner ear, thyroid, kidney and placenta (Bidart *et al.*, 2000; Adato *et al.*, 2000).

The present study was initiated to know the genetic basis of hearing impaired families in Punjabi population. For that purpose, families segregating pre-lingual profound deafness were identified. DNA was isolated from these families segregating autosomal recessive deafness. Linkage analysis of these families was done for common autosomal recessive deafness locus *DFNB4/PDS* by using at least three microsatellite markers. The Deaf Family was found linked to *DFNB4/PDS*.

In Pakistan, marriages within families are quite common and using linkage analysis as a tool for carrier screening and genetic counseling will help to reduce the incidence of inherited deafness in the population. This work of genetics can provide empowering knowledge to member of this family to make informed decision for future marriages to avoid the birth of affected individuals.

MATERIALS AND METHODS

Identification and enrollment of families: First of all, the schools for special children were visited. Informed consent was obtained from participant in study. Detailed history was taken from each family to minimize the presence of other abnormalities and environmental causes for deafness. Families were questioned about skin and hair pigmentation, problems relating to balance, vision, night blindness, thyroid, kidneys, heart and infectious diseases like meningitis, antibiotic usage, injury and thyroid. Pure tone audiometry with air conduction with 250, 500, 1000, 2000, 4000, 8000 Hz, respectively was performed with Siemens, SD-25 or Beltone 112 audiometer. Pedigrees of the enrolled families were drawn using Cyrillic[®] program (Cyrillic for Windows 3.1) and Macromedia[®] FreeHand[®] software.

Blood samples were collected from the identified families. DNA were extracted using Helsinki protocol.

Agarose gel electrophoresis estimation with a known standard DNA dilution: This method uses the UV-induced fluorescence of ethidium bromide dye intercalated into the nucleic acid. The amount of

fluorescence is proportional to the amount of nucleic acid present. Fluorescence from the test DNA and from a known amount of a DNA standard was compared visually. It also allowed the assessment of the integrity of the nucleic acid.

PCR amplification of microsatellites: For haplotype analysis, microsatellite markers were used. For PCR amplification of microsatellites, 5 mL reaction volume was used. For linkage analysis of *DFNB4/PDS*, nine fluorescently labeled primers (with forward primers labeled with one of the three fluorescent dyes, FAM, NED, or VIC) were used. In the same way, five and three microsatellite markers, fluorescently labeled with one of the three dyes (FAM, NED, or VIC) were used for the linkage analysis of other deafness loci. PCR reactions were performed with 50 ng of template DNA in 5 mL reaction mixture containing different volumes of forward and reverse primer, 0.5 mL of 10X PCR buffer (100 mM Tris-Cl, pH 8.4, 500 mM KCl, 25 mM MgCl₂ and 1% Triton), 0.5 mL of 1.25 mM dNTPs, 0.25 uL of spermine (0.5 M) as PCR enhancer and 0.5 unit of Taq DNA polymerase. The samples were amplified using Multiplex (54 and 52°C) PCR programs as shown in (Table 1). Gene Amp PCR system 9700 & 2700 (Perkin Elmer) were used for PCR. All the markers for linkage analysis were dinucleotide repeats and were chosen from the Marshfield Comprehensive Human Genetic Maps (<http://www.marshmed.org/genetics/>) for chromosome 11. The primer sequences for amplification of each marker are listed in the genome database (<http://gdbwww.gdb.org>).

Genotyping:

Principle of automated fluorescent genotyping: PCR amplicons labeled with four different fluorophores were combined and subjected for automated genotyping in ABI PRISM[®] 3100 Genetic Analyzer. Sample sheets in the Genescan are made to identify the lane number and contents of each sample like file name, sample name, dye and internal size standard. When the fluorescent labeled DNA fragments electrophoreses through the polymer, they are separated according to their size and at the lower portion of the gel they pass through a region where a laser beam continuously scans across the gel. The laser excites the fluorescent dyes attached to the fragments and they emit light at a specific wavelength for each dye. A spectrograph collects and separates the lights according to wavelength, thus all four types of fluorescent emissions can be detected with one pass of the laser. With the help

Table 1: STRs markers used for linkage analysis of *DFNB4/PDS* locus

Locus	Markers	cMdistance	DYE	PCRprogram	PCRconditions	Productsize (bp)
PKNB4/PDS	D7S2420	119.81	FAM	Multiplex 54	0.2 mL Primers, 2.5 mM MgCl ₂ , Spermine	272-292
	D7S2459	119.81	FAM	Multiplex 52	0.2 mL Primers, 2.5 mM MgCl ₂ , Spermine	140-152
	D7S2456	120.6	NED	Multiplex 54	0.2 mL Primers, 2.5 mM MgCl ₂ , Spermine	238

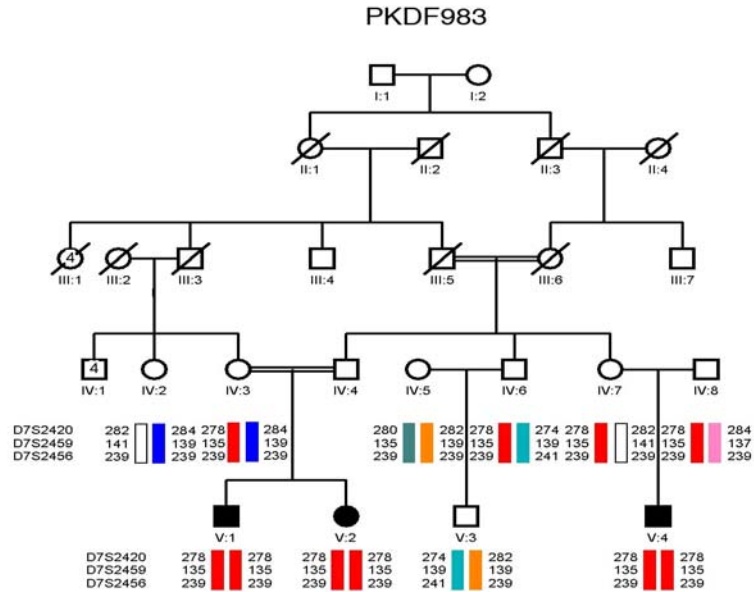


Fig. 1: Pedigree drawing of deaf family showing linkage to DFN4/PDS. Filled boxes shows affected individual. Circles are for females and square for males

of data collection software light intensities are stored as electrical signals (Lee *et al.*, 1997). Automated allele assignment was performed using the Genescan analysis and Genotyper 3.7 NT software (Applied Biosystem). The Genescan analysis software uses the automated fluorescent detection capability of the ABI genetic analysis instrument to size and quantitate DNA fragments and displays the result of the experiment as a reconstructed gel image, electropherogram or tabular data or a combination of electropherogram and corresponding tabular data. The Genotyper import Genescan sample files and converts data from Genescan files into user application results. Genotypic results include dye color, sample fragments with identifying labels, quantitative data, sample information and comments. The Genotyper software also screens out peaks resulting from PCR related artifacts fragments detected during electrophoresis. The Genotyper results are transferred to data spread sheets for haplotype and statistical analyses.

Haplotype analysis: A haplotype representing an individual's chromosomal segment is the set of genotyped alleles arranged according to the cM distance along a chromosome. Alleles were arranged in ways that confirm the inheritance pattern of segregating disease. If three polymorphic (fully informative) markers located in the linkage interval of a DFN4 locus did not show homozygosity among the affected of a family, the locus was considered unlinked. Linkage to a particular locus was confirmed when homozygous data of affected members correlates with the disease pattern in the family tree.

Lod score calculations: EASYLINKAGE and other related software were downloaded from the ftp sites at the Rockefeller University (<http://www.rockefeller.edu>) and Rice University (<http://www.softlib.cs.rice.edu>). Two-point LOD scores (Ott *et al.*, 1999) were calculated (Terwillger and Ott, 1994) for deafness linked markers. Deafness was assumed to be inherited in an autosomal recessive manner with complete penetrance. Recombination frequencies were assumed to be equal in both males and females. Genetic distances were based on Marshfield human genetic map.

All the study was carried out from March 2011 to September 2011 at Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad.

RESULTS

All affected members of the family had pre-lingual, severe to profound sensorineural deafness. Physical and clinical evaluation was performed to rule out any extra-auditory phenotype. Detailed medical history was taken to exclude environmental causes. The family Deaf Family showed linkage with DFN4/ PDS. This family was enrolled from district Haroon Abad Punjab having three affected individuals in two loops. This family belongs to the Arian caste of Punjabi ethnic group. Detailed pedigree was drawn after interviewing multiple members of the family (Fig. 1). At the time of enrolment three deaf and seven normal individuals were enrolled. Written informed consents were obtained from all participants.

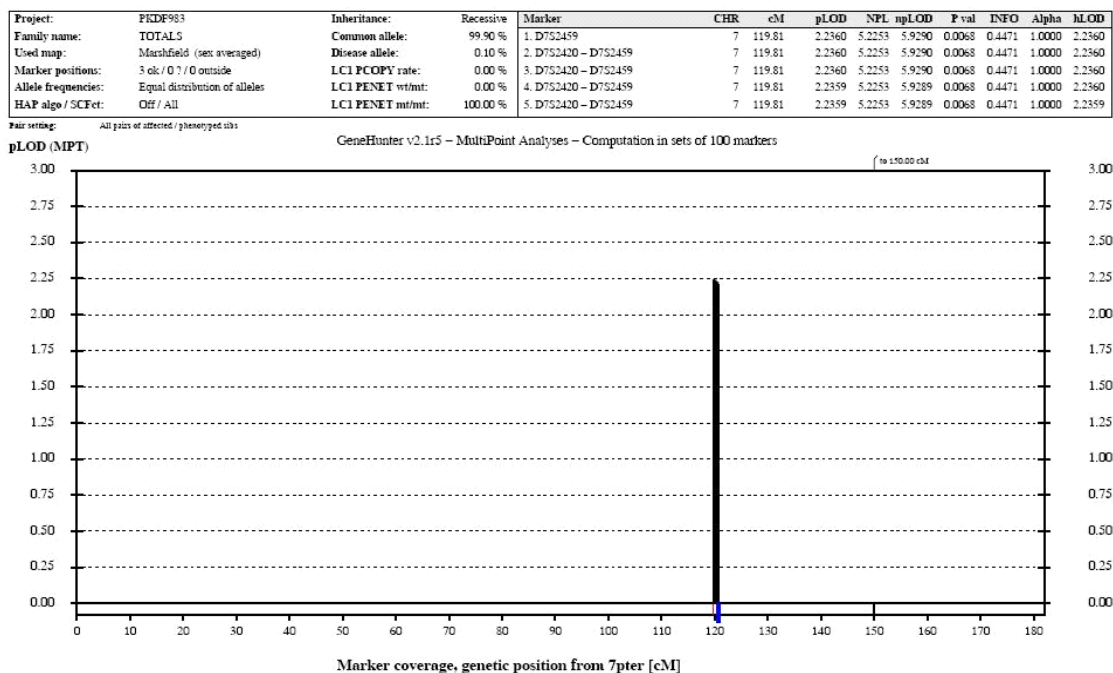


Fig. 2: Multipoint LOD score of deaf family

Medical profile and linkage analyses: A detailed medical history of each of the enrolled family member was taken with special attention to disease, medical treatment or environmental condition that might have caused hearing impairment. Tandem gait test was performed to rule out balance dysfunction in affected individuals. Affected individuals of pedigree (Fig. 1) were between age years to seven years and have no problem of night vision loss, goiter, heart and diabetes etc at the time of enrollment.

During exclusion analysis this pedigree shows linkage to the markers of DFNB4/PDS. To define the boundaries of critical region at 7q31, a set of three STRs markers, D7S2420 (119.81cM), D7S2456 (120.6cM) and D7S2459 (119.81cM) were typed in the family (Table 1). All affected individuals were homozygous for the markers, D7S2420 (119.81cM), D7S2456 (120.6cM) and D7S2459 (119.81cM) (Fig. 3), Maximum multipoint lod score of 2.236 obtained for the marker D7S2459 which suggests this family is linked to DFNB4/PDS (Fig. 2).

DISCUSSION

Hearing disorders are highly heterogeneous and it is anticipated that a large number of genes (approximately 1% of total human coding genes) are responsible for controlling the anatomic and physiologic function of the ear (Friedman and Griffith, 2003). Phenotypically, deafness is classified as syndromic and non-syndromic.

Non-syndromic forms of deafness transmitted as a recessive trait are the most common cause of hereditary hearing loss and often exhibit the most severe hearing phenotype (Cohen *et al.*, 1995).

Recessive deafness is more prevalent in endogenous and isolated populations (Friedman and Griffith, 2003) and Pakistan represents a true treasure for molecular dissection of hearing disorder because 60% marriages are consanguineous and out of those approximately 80% are between first cousins (Hussain and Bittles, 1998). To date, more than 91 recessive loci have been mapped/ reserved and out of these, 28 were mapped first time in Pakistani deaf population (hearing loss homepage). These findings highlight the genetic heterogeneity of Pakistani population.

The present study was carried out to map known autosomal recessive locus DFNB4/PDS in inbred Punjabi families of Pakistan and for this purpose, eight Punjabi families from different areas of Punjab were ascertained and included in the linkage analysis. During screening, one family (Deaf Family) showed linkage to DFNB4/PDS.

DFNB4/PDS is second most common locus in Pakistani population. 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 (Baldwin *et al.*, 1995). A large consanguineous family from India with congenital, profound, non-syndromic deafness showed linkage to the same region (DFNB4) (Li

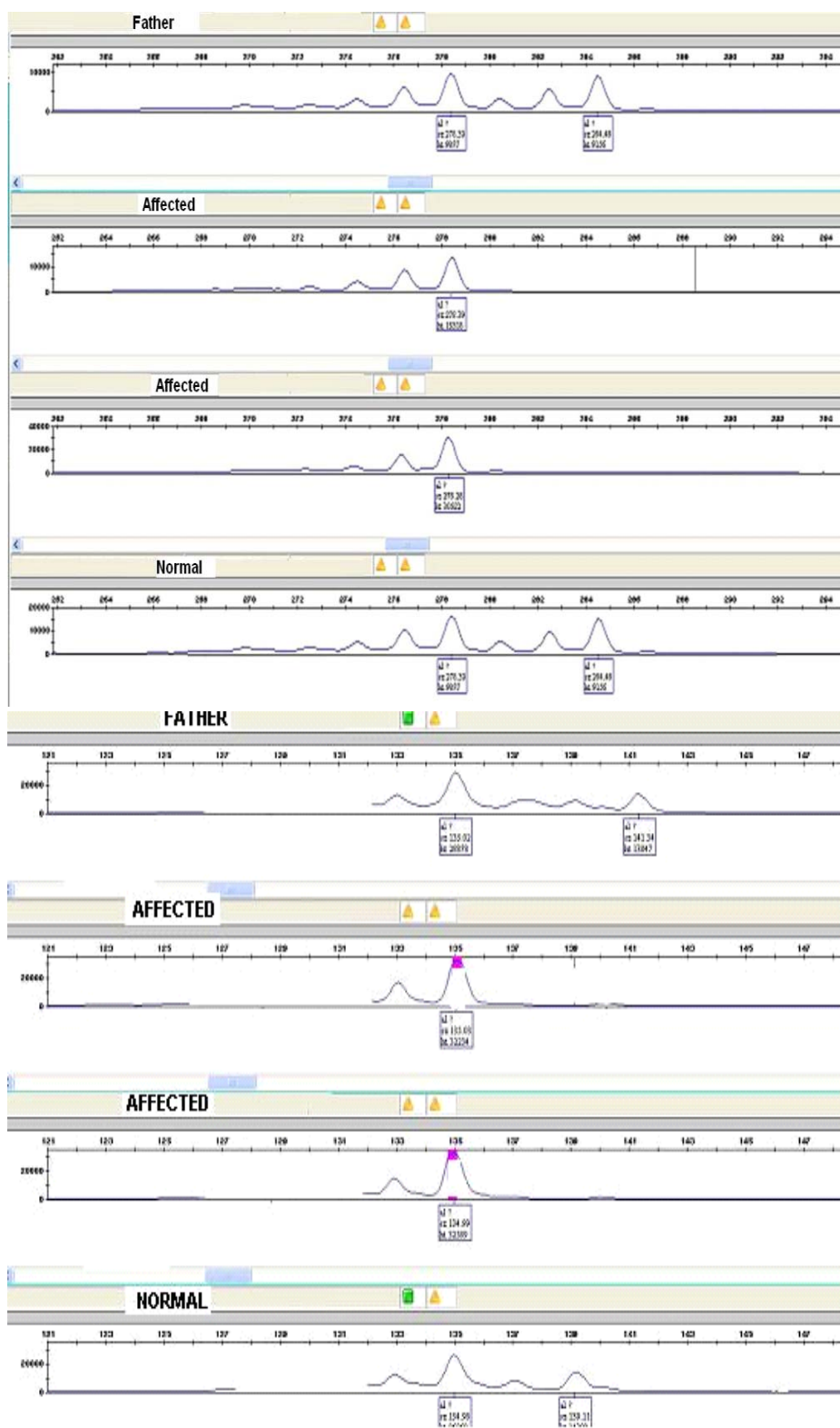


Fig. 3: Chromatograms showing the homozygous alleles for affected and heterozygous alleles for normal individuals. A) D7S2420
B) D7S2456

et al., 1998; Everett *et al.*, 1997). Mutant alleles of SLC26A4 are responsible for non-syndromic, DFNB4 (Li *et al.*, 1998), Pendred syndrome (Everett *et al.*, 1997) as well as Enlarged Vestibular Aqueduct (EVA) syndrome (Usami *et al.*, 1999). SLC26A4 mutations account for approximately 10% of hereditary deafness in diverse populations that include eastern and southern Asians (Park *et al.*, 2003).

One consanguineous family, Deaf Family, with profound hearing loss was found linked with DFNB4/PDS with multi-point LOD score of 2.236 for the marker D7S2459 (Fig. 2). Although affected individual did not show any sign of goiter at the time enrollment, but keeping in view the age of affected individual, they may show the sign of goiter at older age. Therefore at present this family is linked to an overlapping locus DFNB4/PDS.

The benefit of this study is to help in reducing the incidence of deafness in the affected families by providing knowledge and awareness through carrier screening of the families with multiple affected individuals to identify the persons at a high risk. Marriages within the family are quite common in Pakistan, therefore identification of carriers, increasing awareness about the possible effects of consanguineous marriages, offering the genetic counseling and prenatal diagnosis to the families can help to reduce the incidence of hereditary deafness in our population. For obtaining this objective, the need is to further characterize the deafness at molecular level and identify the particular loci contributing to hearing loss in the concerned country and this study being a small part of that investigation. At present, the linkage of this deaf family with locus DFNB4/PDS shows that DFNB4/PDS is one of the most common loci that occur in Punjabi families more than families from other part of Pakistan.

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