

### Linkage Analysis For Dfnb23 / Usher1f Locus

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**Abstract:** Consanguineous families with multiple affected individuals of different casts were studied for the molecular basis of hereditary hearing loss in Pakistani population. Among the screened families, for linkage to recessive deafness locus DFNB23/USH1F, three families (PKDF338, PKDF608 and PKDF627) were found to linked to DFNB23/USH1F locus. PKDF338 was enrolled from Sialkot while PKDF627 and PKDF608 were enrolled from Balochistan. In families PKDF338 and PKDF627 deafness was profound without any history of vestibular dysfunction or retinitis pigmentosa. The ages of the affected individuals were from 5 to 17 years. Therefore these two families were linked to DFNB23 which is non syndromic locus. Family PKDF608 had the history of vestibular dysfunction .The ages of the affected individuals ranges from 12 to 30 years. Fundoscopic evaluation was not there but due to the presence of balance problem this family will be categorized as syndromic and therefore linked to USH1F. It is suggested that further genetic analysis should be done by sequencing the PCDH15 gene to identify mutations responsible for nonsyndromic and syndromic phenotypes of these families.

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#### Introduction

Deafness, partial or complete hearing impairment, is one of the most prevalent sensory defects in humans worldwide (Mckusick, 1992). Approximately one child in a thousand is born with significant hearing impairment. Impact of hearing loss is deeply influenced by the severity of hearing defect and by the age of onset (Marazita *et al.*, 1993; Petit. *et al.*, 2001). Deafness is an etiologically heterogeneous trait with many known genetic and environmental causes, genetic factors account for at least half of all cases of profound congenital deafness (Baldwin *et al.*, 1995), with 70 % cases showing autosomal recessive pattern of inheritance, 12-24 % autosomal dominant, 1-3 % X linked and less than 1 % mitochondrial (Marazita 1993; Petit *et al.*, 1996). There are two main forms of genetic hearing loss, syndromic and non-syndromic; about 30 % of the hereditary hearing loss is syndromic while 70% is nonsyndromic (Kalatzis, *et al.*, 1998, Friedman *et al.*, 2003). USH1F was mapped to chromosome 10q 21-22 (Wayne *et al.* 1997). DFNB23 was also mapped to an interval that overlapped the location of USH1F. DFNB23 / USH1F nonsyndromic as well as syndromic recessive deafness was caused by allelic variants of PCDH15 gene. Most sever mutation such as frame shift and non-sense mutation results in usher phenotype while less sever mutation such as missense mutation manifest only hearing loss (Ahmed Z. *et al.*, 2003).

Mutations in different genes can cause the same clinical phenotype in hearing impaired individuals, even in the same family; while on the other end extreme phenotypic variation between different families or even in the individuals in the same family can be due to mutations in the same gene (Masmoudi *et.al.*, 2000). The recent rapid development of molecular biology techniques applied to the genetics of normal and defective hearing result in mapping of 67 non-syndromic recessive deafness loci (Friedman *et al.*, 2003) and twenty two defective genes. Non-syndromic deafness may accounts for 70% of all the genetically determined cases of deafness (Bergstrom *et al.*, 1971). Non-syndromic hearing loss may be autosomal dominant (DFNA), autosomal recessive (DFNB) and X-linked (DFN). In general, recessive inheritance shows prelingual onset of hearing loss, are almost sensorineural and are fully penetrant and bilateral. The severity is severe to profound with all frequencies affected. The autosomal dominant form of deafness seems to be progressive, postlingual and is often unilateral or mild bilateral associated with conductive and sensorineural deafness. The severity is ranging from moderate to severe. Hearing loss is seen in middle, high or all frequencies with only three loci having hearing loss in the low frequency range (Fraser *et al.*, 1976; Petit *et al.*, 1996). In syndromic cases of deafness the affected individuals have a specific pattern of additional clinical features, which are not related to audition. Nearly 400 forms of deafness have

been identified in which the presence of associated clinical findings permits the diagnosis of a specific form of syndromic deafness (Gorlin, 1995). It may account for 30% of all genetically determined cases. Syndromic deafness can be either dominant, recessive, X-linked or mitochondrial. Linkage analysis is a powerful tool not only for identification of new gene loci but also for refining intervals where deafness causing loci has been previously mapped. This strategy has helped in gene identification studies of deafness loci. (Van Camp and Smith 1997).

## Methods and Materials

### *Enrolment of families*

Families with three or more deafness-affected individuals were enrolled through the schools and centers for special education. If a family had other affected relatives with deafness then they were also included in the study depending on their willingness and availability. Informed consents were obtained from all family members who participated in the 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 pigmentation, hair pigmentation and problems related to balance, vision, night blindness, thyroid, kidneys, heart and infectious diseases like meningitis, antibiotic usage, injury and typhoid. The pedigree structures were based upon interviews with multiple family members and pedigrees of the enrolled families were drawn, using Cyrillic program (Cyrillic 2.1) Figure (1-3). The enrolled families provided convincing evidence for an autosomal recessive mode of inheritance. Family members rarely marry outside the kindred and consequently consanguineous reunions were common. Audiometric testing was performed for all deaf individuals, where- possible.

### **Blood collection and DNA extraction:**

5-10 ml venous blood was collected in 50 ml falcon tubes containing 100-400 µl (0.5 M) EDTA from all participating individuals. DNA extraction was carried out by inorganic method (Grimberg et al., 1989). Blood was frozen at -70 °C for 20-30 min or at -20 °C for 24 hrs before DNA extraction. Before starting DNA extraction, the frozen blood samples were allowed to thaw for lyses of red blood cells. 30-35 ml Tris EDTA buffer (Tris HCl-10mM, EDTA-2mM, pH=8.0) was added in 10ml blood for washing. The samples were centrifuged at 2900 rpm for 20 min at 25°C, the supernatant was discarded up to 15-20 ml and the pellet was broken by gentle tapping. TE buffer was added up to 45 ml and shaked vigorously and centrifuged at 2900 rpm for 20 minutes at 25°C. This process was repeated until pellet become light pink

then supernatant was discarded and pellet was resuspended in 6 ml TNE buffer (Tris HCl-10 mM, EDTA-2 mM, NaCl-400mM) for 10 ml initial blood volume. 200 µl of 10 % SDS and 50 µl of proteinase-K were added for protein digestion and incubate in incubator shaker at 37°C overnight.

1 ml saturated NaCl (6M) was added and samples were shaked vigorously to precipitate out proteins and placed on ice to enhance precipitation by chilling effect for 10-15 min then centrifuged at 2900 rpm for 15 min at 25°C, to pellet down the salts and proteins and supernatant was taken in a 15 ml falcon tube and the pellet was discarded. Again centrifuged the supernatant at 2900 rpm for 15 min at 25°C and the supernatant were taken in another 50 ml falcon tube.

The DNA precipitation was carried out by adding equal volume of isopropanol and inverting the tubes gently and left at room temperature for 10 minutes to concentrate DNA. Centrifuge at 2900 rpm for 10 min at 25°C and DNA pellet was washed with 10 ml 70% ethanol to remove salts. The DNA pellet was dried at 37°C in an incubator.

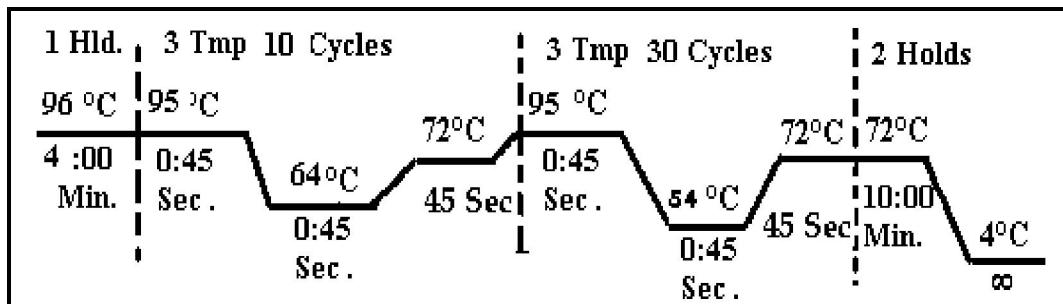
1.5 ml low TE buffer (Tris HCl-10 mM, EDTA-0.2 mM) was added per 10 ml of blood and the tubes were placed in an incubator shaker at 37°C overnight to dissolve the DNA. The DNA was heat shocked at 70°C in shaking water bath for 1 hour to inactivate any remaining nucleases and stored at -20°C.

DNA concentrations were estimated by agarose gel (0.8 %) electrophoresis. Working concentrations were kept at 25 ng/µl and 50 ng was used for 10 µl PCR reaction.

### **PCR For Microsatellites**

Gene Amp PCR system 9700 & 2700 (Perkin Elmer) were used for the amplification. In PCR amplification of microsatellites, 10µl reaction volume was used containing 3 fluorescently labeled primers with forward primer labeled with FAM, was used for linkage analysis of the locus DFNB23, 1µl of 10X PCR Buffer (50 mM KCl, 0.1X Triton, 1.5mM or 2.0mM MgCl<sub>2</sub>), 0.8µl of 1.25 mM dNTPs (Pharmacia), 0.5 units of Taq DNA polymerase and 0.4 µl of each primer (8 µM) and 2µl of 50 ng template DNA. The samples were amplified using touchdown PCR. The initial denaturation step at the beginning of the PCR was for 4 minutes at 96°C. A post PCR step for 10 minutes at 72°C was added to extend all unfinished products.

The markers used for each linkage encompassed the chromosomal locations reported for deafness locus DFNB23. The markers were mostly dinucleotide repeats and were chosen from the Marshfield Comprehensive Human Genetic Maps or Genthon Human Genetic Map for chromosome 10q.



### Preparation of Samples for ABI 3100 Genetic Analyzer:

Fluorescently labeled markers were used for screening DFNB23; PCR products of different sizes labeled with FAM were pooled by adding 1 $\mu$ l of each PCR product in 11.4 $\mu$ l of deionized formamide and 0.6  $\mu$ l of size standard (Perkin Elmer). The samples were denatured at 95°C for 5 minute before running in the ABI Prism 3100 genetic analyzer.

### Genotyping

Alleles in base pairs for each marker were recorded. For initial screening few members from each family were genotyped for three markers for this locus. Additional markers from the corresponding regions were also typed if some of these markers were uninformative. Markers were run to define the region of homozygosity and all family members were genotyped and haplotypes generated to either include or exclude the linkage region (Table 1).

### Lod Score calculations:

Lod Scores were calculated using different utility programs of the FASTLINK computer package. MLINK and LINKMAP were used for two-point lod and Multipoint lod scores respectively. A fully penetrant recessive model with no phenocopies and disease allele frequency of 0.001 was assumed. Marker order and map distances were chosen from the Marshfield genetic map. Meiotic recombination frequencies were considered to be equal for males and females. Allele frequencies for microsatellite markers were calculated by genotyping 90 randomly collected unaffected individuals from the same population (Table 2).

### Results and Discussion

Large consanguineous families are a powerful resource for mapping and identifying deafness loci and genes that modify deafness phenotype (Guilford al. 1994a; Baldwin *et al.* 1995, Fukushima *et al.* 1995). Pakistan has diverse ethnic groups and marriages are usually done within families, castes and ethnic groups, hence these families have been

instrumental in mapping recessive deafness loci and genes.

Among the families screened for DFNB23/USH1F locus, three families were found linked to this locus. DFNB23 present on chromosome 10 is an allelic form of USH1F and resides the gene PCDH15 (Protocadherin15), that encodes protein protocadherin is responsible for DFNB23/USH1F.

Families with autosomal recessive deafness were collected from different cities of Pakistan. All affected individuals of family had prelingual, mostly severe to profound sensorineural deafness. All the families were non syndromic and did not have any other phenotype associated with deafness, except PKDFD 608 who had the history of balance problem. Detailed history was taken from the families to rule out the environmental deafness. The cast of each family was also noted to see that if there are any other particular loci / mutation more prevalent in that ethnic group. All the enrolled families were screened for linkage to the selected recessive deafness loci. Three families were linked to DENB23/USH1F.

In the present study as mentioned earlier three families (PKDF338, PKDF608 and PKDF627) were found linked to DFNB23/USH1F, Lod score for all families are given in table 2. The haplotypes comparison of these three families showed no similarity. This suggests that these families might have different mutations. In families PKDF338 and PKDF627 deafness was profound without any history of vestibular dysfunction or retinitis pigmentosa. The ages of the affected individuals were from 5 - 17 years. Therefore these two families were linked to DFNB23 which is non syndromic locus. The mutation responsible for non syndromic deafness DFNB23 as mentioned earlier is missense mutation and there is probability of this type of mutation in these families. Family PKDF608 had the history of vestibular dysfunction. The ages of the affected individual's ranges from 12 - 30 years. Funduscopic evaluation was not there but due to the presence of balance problem this family will be categorized as syndromic and therefore linked to USH1F. While mutation responsible for syndromic deafness USH1F is non sense and frame shift mutation. Therefore there is

probability of non sense and frame shift mutation in this family. It is suggested that further genetic analysis should be done by sequencing the PCDH15 gene to identify mutations responsible for nonsyndromic and syndromic phenotypes of these families.

Linkage analysis is done for the screening of carrier's states and to offer to the genetic counseling as consanguineous marriages are common in Pakistan. By keeping in view the above data in mind it is clear

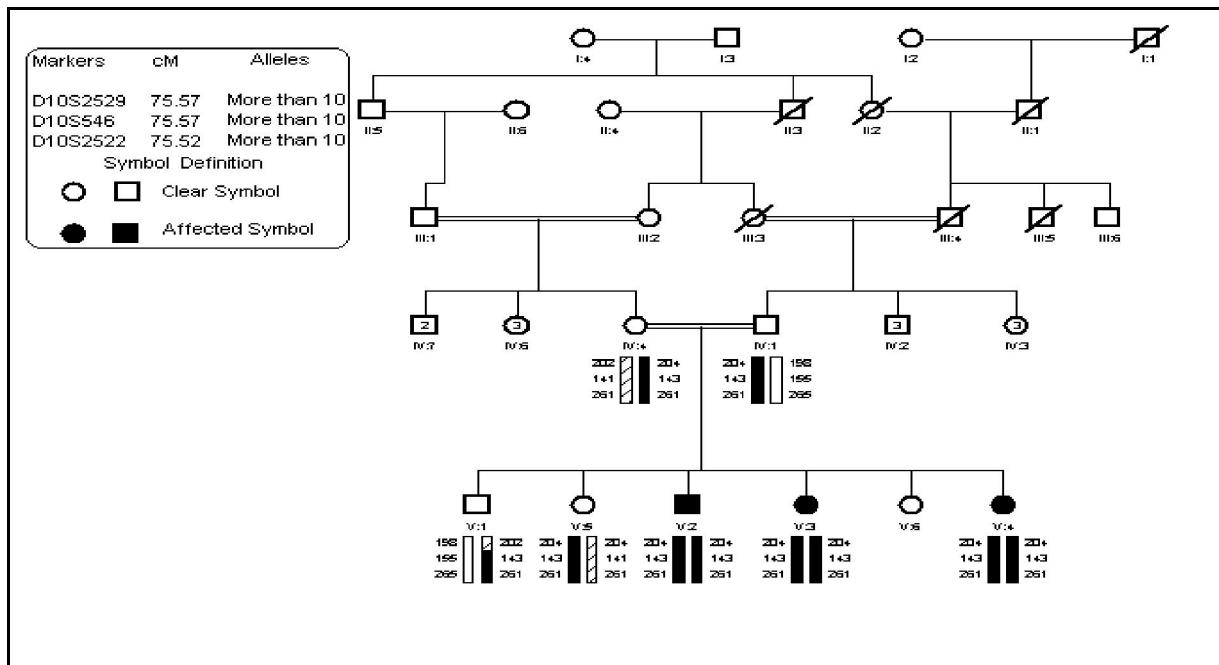
that, those individuals who are phenotypically normal but genetically they are carriers. These information are important to keep because we can inform the families about their carrier states and can guide them in the sense to avoid their marriages within the families. In this way we can decrease deaf individuals by preventing carriers' marriages and hence the burden on the families and also on the society.

**TABLE 1: STS Markers Used For Linkage Analysis of DFNB23/USH1F Locus.**

DFNB23	Marker	cM	Dye	PCR Program	Conditions	ASR
1	D10S2522	75.57	FAM	TD 64-54	2.5mM	243bp
2	D10S2529	75.57	FAM	TD 64-54	1.5mM	200bP
3	D10S546	75.57	FAM	TD 64-54	1.5mM	148bP

**Table 2: Two Point LOD Score.**

Families	Markers	Marshfield Map Position (cM)	Two-point LOD Score at $\theta=0$
PKDF338	D10S2529	75.57	2.28
	D10S546	75.57	2.28
	D10S2522	75.57	0.96
PKDF608	D10S2529	75.57	0.79
	D10S546	75.57	3.86
	D10S2522	75.57	1.31
PKDF627	D10S2529	75.57	2.69
	D10S546	75.57	2.69
	D10S2522	75.57	0.75



**Fig 1. Pedigree drawing of family PKDF 338.**

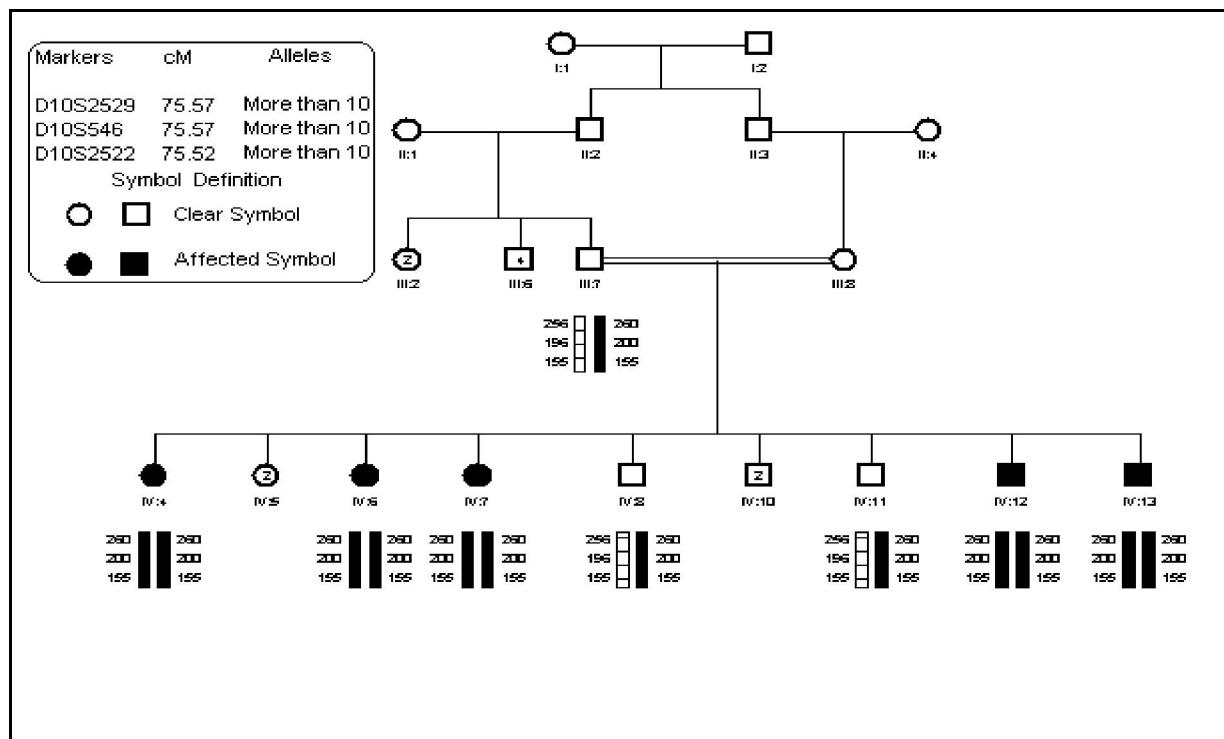


Fig 2. Pedigree drawing of family PKDF 608.

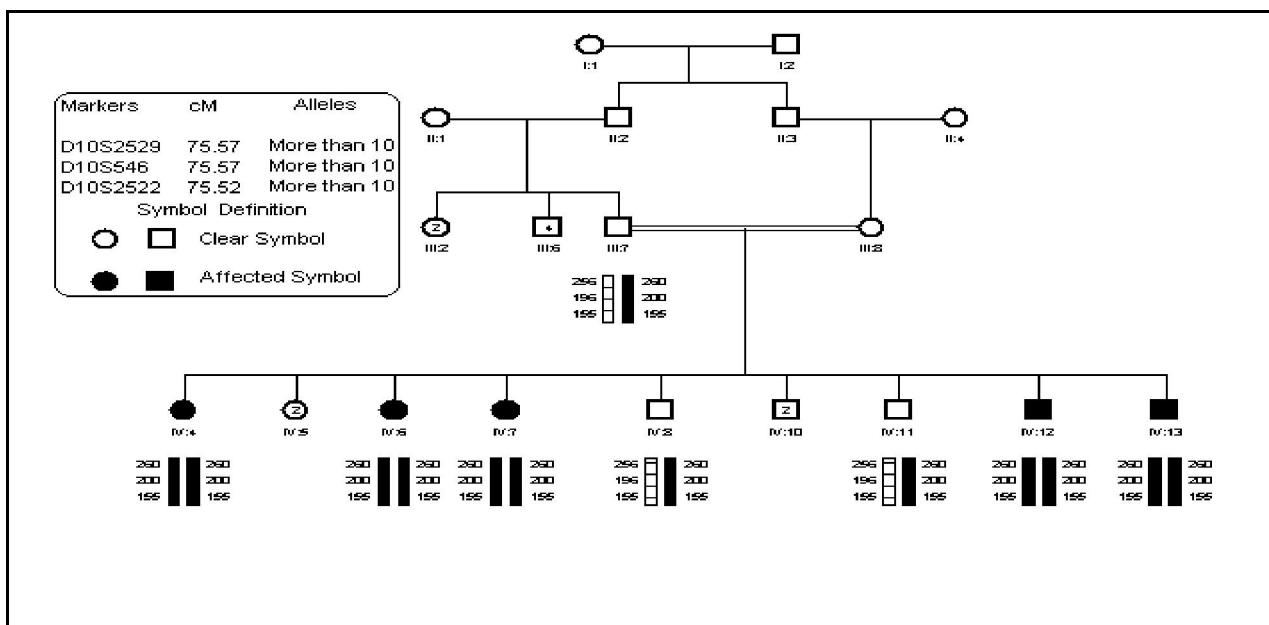


Fig 3. Pedigree drawing of family 627.

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