Linkage analysis of hearing impairment in families of Bannu Distric
Farmanullah¹, Jabbar Khan², Muhammad Ismail³, Muhammad Rafi⁴, Ehsanullah⁵, Abdullah Jalal⁶

Abstract
Objective: To link congenital hearing loss with known loci to establish a platform for future research.
Methods: The cross-sectional study was conducted from February 2016 to March 2017 in Bannu, Khyber Pakhtunkhwa, Pakistan, and comprised families with Pashtun ethnicity having at least 2 individuals suffering from congenital hearing loss. Deoxyribonucleic acid from whole blood samples was extracted by salting-out method. Amplification was done through touchdown polymerase chain reaction to see any possible linkage to already reported deafness loci. Linkage analysis was carried out using microsatellite markers for each locus. Genotyping of the samples was done and haplotypes were accordingly generated to either include or exclude the linked / unlinked regions.
Results: Of the 4 families, family PKDF 1620 showed linkage with DFNB12/CDH23 (D10S1432, D10S606, and D10S1694) and family PKDF 1625 had linkage with DFNB3/MYO15A (D17S2196, D17S2207 and D17S2206). Families PKDF1623 and PKDF1624 showed no linkage with any of the prevalent reported loci in Pakistan.
Conclusion: Linkage to DFNB12 and MYO 15 showed heterogeneity of congenital deafness.
Keywords: Congenital deafness, Linkage, Touch-down PCR, Haplogroup. (JPMA 69: 1632; 2019). doi: 10.5455/JPMA.300796.

Introduction
Being social animals, humans love to be part of a community. Hearing is very critical for them to communicate and participate.¹,² Human ear converts physical vibration into an encoded nerve impulse. It can be considered a biological microphone. The sense of body balance and body position is the main function of the human ear. Hearing impairment or hearing loss is the most common sensory defect, affecting normal communication in 10% people aged 65 years or more.³ Hearing impairment is the most common birth defect. Its prevalence is comparatively higher in developed countries.³,⁴ The disease is extremely heterogeneous with more than 50% pre-lingual hearing impairments being likely genetic. Of these, more than 90% are monogenic autosomal recessive traits.³,⁴ Some forms of genetic deafness are recognised as syndromic or non-syndromic.³⁻⁵ Although hearing loss is common worldwide, identifying its genes is always a challenge for researchers due to extreme genetic heterogeneity and limited clinical differentiation.⁶ At least 1% of the genes that code for human proteins are associated with hearing loss, accounting for more than 300 genes involved in causing the disorder.⁷ Up to 85% of inherited hearing loss is autosomal recessive non-syndromic deafness, which is very heterogeneous.⁸

Consanguineous families from Pakistani population have played an important role in the identification and mapping of genes responsible for hearing loss.⁹ The prevalence of profound bilateral hearing impairment is 1.6 per 1000 individuals in Pakistani population.⁹ Till now, 24 genes and 17 loci of autosomal recessive hereditary hearing loss have been identified.¹⁰⁻¹⁵ The current study was planned to characterise the families having individuals with congenital hearing loss through linkage analysis for the known loci so as to establish a platform for future research work in this field.

Patients and Methods
The cross-sectional study was conducted from February 2016 to March 2017 in Bannu, Khyber Pakhtunkhwa (KP), Pakistan, and comprised families with Pashtun ethnicity having at least 2 individuals suffering from congenital hearing loss. After permission was obtained from the ethics committee of Gomal University, Dera Ismail Khan,
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Pakistan, pedigree of each family was drawn and 8ml blood sample of each individual was collected in 50ml Falcon tubes containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. Detailed information of each family was recorded on a pre-designed proforma. Written consent of both the parents and the patients were obtained from each family. DNA from all blood samples was extracted through salting-out method.

For carrying out linkage analysis, a 96-well master plate map was designed in which each sample was dispensed in triplicate. In addition to the affected individuals, the parents and normal individuals were also included. The deoxyribonucleic acid (DNA) of selected families was amplified through touchdown polymerase chain reaction (PCR) for any possible linkage to already reported deafness loci in Pakistan. Linkage analysis was carried out using microsatellite markers for each locus. Forward primers were labelled with one of the fluorescent dyes (Table).

For PCR reaction to perform, 50ng genomic DNA, 240 M of deoxyribonucleotide triphosphate (dNTP), 0.8 unit of Taq polymerase, 0.4 M of each of forward and reverse primers and 1x Taq reaction buffer were used in 5 µl reaction volume. The reaction was carried out through 40 cycles in 2 consecutive phases of 10 and 30 cycles each. The phase I of 10 cycles comprised denaturation at 95°C for 45 seconds, annealing at 54°C for 45 seconds and extension at 72°C for 60 seconds. In phase II of 30 cycles, denaturation was done at 95°C for 45 seconds, annealing at 54°C for 30 seconds and extension at 65°C for 2 minutes. Denaturation in the first cycle was done at 96°C for 5 minutes while the final extension was done at 72°C for 10 minutes.

Pooling of labelled amplified PCR products was done in a way so as to get different sizes. A 30–40 nucleotides difference was maintained not to have overlapping of the products if labelled with the same flourochrome when analysing the data. One µl of each PCR product was pipetted into 96-well pooling plate using capillary Hamilton syringe possessing sex needles. The mixture of 7.5µl formamide and 0.5µl internal size standard PET (photo-induced electron transfer fluorogenic primers) was put in each well afterwards. The samples were accordingly genotyped through genetic analyser (ABI PRISM® 3730) and the results were analysed through gene-mapper version 4.0 software (ABI).

Haplotypes were generated for including or excluding the linked / unlinked regions. Alleles were arranged in a way that confirmed the inheritance pattern of

<table>
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M: Multiplex polymerase chain reaction (PCR), td : Touchdown PCR, J: (Juli CR), ASR: Allele Specific Region.

Table: Markers used for linkage analysis of known genes of DFNB.
segregating disease. Linkage to a particular DFNB locus was confirmed when haplotypes of affected members correlated with the inheritance pattern of disease in the pedigree.

Loci selected for screening included DFNB12, an autosomal recessive locus identified in a Syrian family to chromosome 10;\(^{17,18}\) cadherin 23 (CDH23) gene which consists of 69 exons, and mutations have been linked to age-related hearing impairment;\(^{19}\) and MYO15A / DFNB3 which is a motor protein present in hair cells in a cap-like structure at the top of the stereocilia, and mutations are a significant cause of deafness globally.\(^{19-21}\)

**Results**

There were 4 families that were named PKDF1620, PKDF1623, PKDF1624 and PKDF1625. PKDF1620 and PKDF1625 showed linkage with DFNB12 / CDH23 and DFNB3 / MYO15A respectively, and were designated as linked families. Pedigrees PKDF1623 and PKDF1624 were screened with DFNB 1, 2, 3, 4, 12 and 39, but the two families showed no linkage, and were designated as unlinked families.

PKDF 1624 was a large consanguineous family (Figure 1) with 5 affected individuals present in two loops. The 1st loop contained 2(40%) affected individuals; 1(50%) female (VI:3) and 1(50%) male (VI:4), 3(60%) normal individuals (VI:1, VI:2, VI:5) and their parents (V:1, V:2). In the second loop, there were 3 affected individuals (VI:6, VI:7, VI:8); 2(66.6%) normal children (VI:9, VI:10) and the parents (IV:5, IV:6). The parents of the 1st loop were age 45-50 years, while the normal were of 18, 21, 23 years of age respectively. The 2 affected individuals were aged 13 and 15 years. The parents of the 2nd loop were aged 55-60 years, their normal children were aged 14, 18 years, and the 3 affected individuals were aged 11, 13 and 17 years.

PKDF1623I was a large consanguineous family with 6 affected individuals in two loops. The 1st loop contained 1(16.6%) affected individual (IV:1), 5(83.3%) normal. The father (III:4) was among the normal, while the mother (III:3) was among the affected individuals. In the 2nd loop, there were 2 affected males (IV:6, IV:7), 2 normal males, 1 normal female (IV:5, IV:8, IV:9), an affected father (III:1) and a normal mother (III:2). The grandfathers (III:1, III:3) of the 2nd loop were affected but grandmothers were normal. Parents of the 1st loop were aged 45-50 years, normal individuals were aged 18-23 years, while the affected individual was aged 22 years. Parents of the 2nd loop were aged 55-60 years, normal children were of aged 5, 8 and 4 years, while the two affected individuals were 6 and 18 years old.

PKDF1620 is a consanguineous family that contained 2 affected individuals (Figure 2); 1(50%) female (IV:2) and 1(50%) male (IV:3), 1 normal individual (IV:1) and their parents (III:1, III:2). Affected individuals had no signs of goitre, diabetes, thyroid, infections (cytomegalovirus [CMV], Rubella), or night blindness. Hearing status of affected individuals was severe to profound. All the affected members were homozygous, while all normal members were heterozygous for D10S1432, D10S606 and D10S1694.

PKDF1625 was a 2-loop consanguineous family. The 1st loop contained 2 affected individuals; 1(50%) female...
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(Iv:1) and 1(50%) male (Iv:3), and 3 normal individuals (Iv:2, Iv:4, Iv:5) and their parents (Iii:1, Iii:2). In the 2nd loop, there were 2 normal individuals (Iv:6, Iv:7) and their parents (Iii:3, Iii:4). Affected individuals had no signs of goitre, diabetes, thyroid, infections (CMV, Rubella) or night blindness but had severe hearing impairment. All the affected individuals were homozygous, while the normal members of this family were heterozygous for D17S2196, D17S2206, and D17S2207 markers.

Discussion
Consanguinity is one the major factors in increased incidence of autosomal recessive hearing impairments.\textsuperscript{9-11} Due to consanguinity, it is possible to identify carriers and, hence, genetic counselling can be offered to reduce the incidence and establish platform for non-syndromic autosomal recessive hereditary hearing loss in KP. We aimed at characterising the families having congenital hearing loss through linkage analysis for known loci as no work has been done in this regard in this area. DFNB12 is a non-syndromic autosomal recessive hereditary hearing loss locus associated with mutations in gene CDH23. Initially this locus was reported in a Syrian family and then in a Cuban family at chromosome 10q21-q22.\textsuperscript{17} It is coupled with missense mutations of CDH23, which are considered as hypomorphic alleles with lasting activity for retinal and vestibular function, but not for auditory cochlear function.\textsuperscript{17-19} DFNB12 allele is phenotypically dominant to the USH1D and can present normal retinal and vestibular function even in the presence of USH1D allele.\textsuperscript{18} Mutations in CDH23 can cause either DFNB12 or the Usher type-1.\textsuperscript{17-19} It has been reported that all the reported DFNB12 cases are because of missense mutations in the highly conserved calcium binding domains of the CDH23.\textsuperscript{18,20} Prevalence of DFNB12 in Pakistani population is 7% and it is among the highly prevalent loci in Pakistani population.\textsuperscript{17,20,22} Similarly, missense and nonsense mutations in DFNB3 / MYO15 gene lead to congenital recessive deafness.\textsuperscript{20,21,23} DFNB3 is responsible for 5% of recessive deafness in Pakistani population. Recessive mutations of MYO15 cause profound hearing impairment.\textsuperscript{17,20,22} During the current study, family PKDF1620 showed linkage with DFNB12, through D10S1432, D10S606, and D10S1694 markers of DFNB12. CDH23 lies within the region of these microsatellite markers D10S606, D10S1432, D10S1694 used for haplotype study. The family PKDF1625 showed linkage with DFNB3 / MYO15 via D17S2196, D17S2207 and D17S2206 markers. Both the families had neither any sign and symptoms, retinitis pigmentosa nor any history of night blindness. Families PKDF1623 and PKDF1624, were also characterised for all the reported loci of DFNB but they did not show any linkage, indicating that these 2 families possessed certain mutations yet to be reported in Pakistan or had some novel mutations that need to be explored.
The current study had only 4 families, but has to some extent made possible the carrier screening that can be very useful to carry out genetic counselling for minimising the incidence of hereditary hearing loss prevailing because of the high rate of consanguinity in our region.

Conclusion
Linkage of only 2 families to DFNB12 and MYO 15 showed heterogeneity of the hearing loss disorder.

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References