Original Article

Linkage study of DFNB3 responsible for hearing loss in human

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BACKGROUND: Hearing disorders represent a significant health problem worldwide. Recessive inherited cases of the deafness are more prevalent in Pakistan due to consanguineous marriages. Deafness caused by DFNB3 is due to mutation in the gene MYO XVA and its prevalence among Pakistani population is about 5%.
MATERIALS AND METHODS: Families with at least two or more individual affected with deafness were selected from different areas of District Okara of Pakistan. Six consanguineous families of different ethnic groups having deaf individuals were studied. All these families had three or more deaf individuals in either two or more sib ships. Family history was taken to minimize the chances of other abnormalities. Pedigrees drawn by using Cyrillic software (version 2.1) showed that all the marriages were consanguineous and the families have recessive mode of inheritance. Three STR markers were selected and amplified on all the samples of six families through PCR. The PCR products were then genotyped on non denaturing polyacrylamide gel electrophoresis (PAGE). Haplotypes were constructed to determine the pattern of inheritance and also to determine whether a family was linked or unlinked with known DFNB3 locus.
RESULTS: One out of six families showed linkage to the DFNB3 while rest of the families remained unlinked. Carriers of deafness genes were identified and information was provided to the families on request.
CONCLUSION: Knowledge about the genetic causes of deafness provide insight into the variable expression of genes involved in this hereditary problem and may allow the prediction and prevention of associated health problems.

Key words: Consanguineous, deafness, linkage analysis, microsatellite markers, polyacrylamide gel electrophoresis

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Introduction

Deafness is partial or complete loss of hearing. Hearing loss is among the most prevalent sensory defects in humans that severely compromise the quality of life and may results in the socially isolated individuals.[1] 60% of people older than 70 years have hearing loss of at least 25dB.[2] It is a multifactorial disorder caused by either genetic or environmental factors or a combination of both. It is estimated that at least 60% of deafness is due to genetic disorders.[3] Most inherited forms of deafness segregate as monogenic traits but digenic inheritance is also reported.[4] Hearing loss segregates as an Autosomal dominant in monogenic cases, Autosomal recessive, X-linked, Y-linked or mitochondrial mode of inheritance. These monogenic forms of deafness may be Syndromic (characterized by hearing loss in combination with other abnormalities) or non-syndromic (with only hearing loss).[5] Identification of causative genes of hearing loss yet has been a challenge, mainly due to extreme genetic heterogeneity and limited clinical manifestation.[6] Mutations in different genes can cause the same clinical phenotype in hearing-impaired individuals, even within the same family. On the other hand, extreme phenotypic variations between different families (or even in individuals of the same family) can be due to different mutations in the same gene.[7] The study is further complicated by the fact that environmental as well as genetic factors can independently or in combination, cause deafness. Consequently, families with multiple affected individuals showing clear segregation are helpful in these studies.[8] Usually each locus has to be

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mapped on one family with sufficient number of affected individuals by linkage analysis or on small families by homozygosity mapping.[9]

In Pakistan cousin marriages or marriages within the same ethnic groups are common,[10] thus families with hereditary hearing loss are not uncommon. These families offer a useful resource for mapping deafness genes.

The mutation in the gene Myosin XVA is responsible for DFNB3. Protein Myosin XVA encodes MYO XVA gene.[11] MYO XVA was first characterized by a genome-wide homozygosity mapping strategy and localized DFNB3 to 17p11.2. Two percent of the residents of Bengkala, Bali, have profound, congenital, neurosensory and non-syndromic deafness due to an autosomal recessive mutation at the DFNB3 locus.[12] Scanning electron microscopy shows that stereocilia are malformed when the, MYO XVA gene is defective, suggesting that this myosin is essential for maintaining the structure of the stereocilia.[13]

Linkage analysis is a relationship between the loci; i.e., two loci on the same chromosome are said to be linked if the phenomenon of crossing over does not separate them. Actually at the stage of meiosis homologous chromosomes exchange segments as a foundation for the process of recombination or crossing over. If two loci are physically close to each other on the same chromosome then there are rare chances that they will be separated by recombination event. As for this, a crossover will have to occur in small distance between the two loci, which is very rare; the two loci will tend to be inherited together. Sets of alleles for different markers or genes on the same chromosome are termed as haplotypes. Alleles on the same haplotypes are passed on in pedigrees as a block. These blocks are only be broken by crossing over. The term linkage refers to the loci, not to specific alleles at these loci. The most common application of linkage analysis is to try and find the location, in the genome, for a gene responsible for a certain mendelianly-inherited disease.[14]

Linkage analysis is a powerful method not only for mapping new locations but also for refining intervals where deafness-causing loci have been previously mapped. This strategy has helped in gene identification studies for some recessive loci. Using linkage analysis, we have screened selected deafness families from Pakistan to determine whether a family is linked to the known loci DFNB3 or not.

Materials and Methods

Enrollment of the families

Families with at least two or more individual affected with deafness were selected from different areas of District Okara of Pakistan. Family history was taken and pedigree was made personally by visiting each family. Detailed history was taken from each family to minimize the presence of other abnormalities and environmental causes for deafness. Other relatives of the affected families with deafness were also included in the study depending on their willingness and availability. Informed consent was obtained for participating in the study.

Pedigree analysis

Pedigree was drawn on Cyrillic software with the help of data taken from each affected families. At least four generations family data as (sibs, cousin marriage, monozygotic twin and sex) was shown by biological symbols.

Collection of blood samples

Blood samples (5 mL) were collected from all the affected individuals, their normal siblings, parents to trace the mode of inheritance. The blood samples were collected in 50 mL falcon tubes already containing 140 μl EDTA (Ethylene diamine tetra acetate) which works as an anticoagulant, the blood samples were stored in ice boxes immediately after their collection and then the blood samples were stored in -20°C freezer before DNA extraction.

DNA extraction

Genomic DNA was extracted from blood samples by inorganic method.[15] This method was consisted of three main steps:

- Lysis of RBCs with the T.E lysis buffer (Tris HCl, EDTA), which was done by washing the blood three or four times

- Lysis of WBCs and digestion proteins with 100 μL of 10% SDS, 45 μL proteinase-K and 3 mL T.N.E Buffer (Tris HCL, EDTA and NaCl) by adding to the pellet, and incubated 12-18 hours in shaking water bath at 45°C for complete digestion of cellular proteins and WBCs.
- After the period of incubation, salting out of proteins by adding 1 mL of 6 M NaCl to the digested samples. In this way all the proteins were salted out leaving behind the supernatant which contained the DNA, which was then precipitated by adding equal volume of isopropanol. DNA was obtained in the form of pellet by centrifugation.

DNA quantification

The DNA quantification was done on 0.8% agarose gel stained with ethidium bromide (10 mg/mL).

Primer selection and amplification by PCR

The primers were selected from the UCSC Genome browser.[16] All the primers were amplified by a touchdown PCR in which a range of annealing temperature (52°C-62°C) was used [Table 1]. All primer (D17S2196, D17S1794 and D17S2187) were amplified on each genomic DNA sample of selected 6 deafness families. PCR was carried out in five steps: (a) initial denaturing at 95°C for 4 minutes. (b) 10 cycle each for 30 sec. at 94°C for denaturation, 45 sec. at 62°C (there is a decrease of 1°C in temperature after every cycle) for primer annealing and 45 sec. at 72°C for extension. (c) 25 cycle each for 30 sec. at 94°C for denaturation, 45 sec. at 52°C for primer annealing and 45 sec. at 72°C for extension. (d) 10 min at 72°C and (e) final step was held at 4°C until all the PCR products were collected from thermocycler.

PCR products of each of the primer were run on 1.2% agarose gel along with 50 base pair ladder to visualize the bands of amplified products at 110 volts for 40 minutes. Amplified bands of all the 3 primers were visualized and compared with standard ladder and pictures were saved by the gel documentation system.

Polyacrylamide gel electrophoresis

PCR products of each of the primer were run on non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) to examine amplified product of the primers for linkage analysis.[17] For preparation of gel; 44 mL water was added in the beaker, 14 mL Acrylamide + Bisacrylamide (30%) Solution was added. The TAE buffer (1.2 mL), Ammonium per sulphate (APS) (300 μL), Tetramethylethylenediamine (TEMED) (60 μL) were added respectively. 6 μL of each PCR product of each primer was mixed with 3 μL loading dye and was loaded in the wells of the gel. A standard DNA ladder of 50 base pair was also run along with the PCR products of each primer for every sample of deafness families for reference. The gel was run for approximately 3 hours at 150 volts. After the complete running of PCR products, the gel was separated from the glass plates and dipped into Ethidium bromide (10 mg/ml) solution for 10 minutes for staining of amplified DNA bands. The gels were visualized in UV light chamber of gel documentation system of BIO-RAD Model Gel.Doc XR.

Results

Three STR markers, D17S1794, D17S2196 and D17S2186 were genotyped to locus DFNB3 on six families affected with deafness Haplotypes (set of alleles) were constructed using the Cyrillic software to determine the pattern of inheritance among the affected and normal individuals of each family under study. Out of six families, one family (Family PK-DF06) was linked to deafness locus DFNB3. Other five families remained unlinked [Table 2].

Family PK-DF06

This family was collected from Tehsil Renala khurd, district Okara. The family belongs to Arain cast. Blood

Table 1: Primers selected for the DBNF3 locus

Primer ID	Sequence 5-3	Sequence 5-3	Product size	Genetic Position
D17S2196	CCAACATCTAGAATTAATCAGAATC	ATATTTCAATATTGTAACCAGTCCC	139-163	44.62 cM
D17S1794	GGTAGAGATGGGTTTCACCA	GTGTGTCCAGACTTTGACGA	179-189	47.00 cM
D17S2187	CCAGGGGCTAATTTTGAAAT	GCAAGACTCTGTCTCAAAAAA	292-310	48.07 cM

DBNF: A gene for congenital, recessive deafness DFNB

samples of 6 individual were collected containing 4 affected (IV: 1, IV: 2, IV: 3, IV4) and father and mother (III: 1, III: 2). Age of the affected individuals ranges from 11-27 years. After DNA extraction these samples were amplified by using three STR markers (D17S1794, D17S 2196 and D17S 2187) spanning in the region of DFNB3 locus/gene MYO15. After amplification the PCR product was checked on agarose gel first and then these samples were run on a non denaturing Polyacrylamide gel electrophoresis (PAGE). The gel was run for about 2-3 hours at 150 volts. When the run was completed, the gel was put in the gel documentation system to visualize the alleles for the confirmation of homozygosity and heterozygosity. The alleles were then read manually to determine the pattern of inheritance. The larger allele was donated by 2 and the smaller by 1. The family showed linkage to DFNB3 locus. The STR marker D17S1794 showed heterozygousity in both parents while homozygousity in their entire affected individuals. For the STR marker D17S1794 father showed heterozygousity while mother and their entire affected individuals were homozygous. Both parents and their entire affected child were homozygous for the STR marker D17S 2187 [Figure 1].

Discussion

Linkage analysis was done on the selected six families belonging to different areas of district Okara. Out of six families screened for the non-syndromic autosomal recessive locus DFNB3, only one family PK-DF06 was linked. The prevalence of deafness among Pakistani population due to DFNB3 is about 5%. The mutation in the gene Myosin15A is responsible for DFNB3. Protein Myosin15A encodes Myo15A gene.[11]

Brazilian inbred pedigree with 26 subjects affected by prelingual deafness showed that the most probable pattern of inheritance was autosomal recessive. However, their linkage and mutational analysis revealed, among the 26 affected subjects, 15 were homozygous for the novel c. 10573delA mutation in the *MYO15A* gene, 5 were compound heterozygous for the mutation c. 10573delA and the novel deletion c. 9957_9960delTGAC and one inherited only a single c. 10573delA mutant allele, while the other one could not be identified. Given the extensive consanguinity of the pedigree, there might be at least one more deafness locus segregating to explain the condition in some of the subjects whose deafness is not clearly associated with *MYO15A* mutations, although overlooked environmental causes could not be ruled out.[18]

600 consanguineous families segregating hereditary hearing loss as a recessive trait and found evidence of linkage of markers at the DFNB3 locus to hearing loss in 38 of these families ascertained in Pakistan (*n* = 30), India (*n* = 6), and Turkey (*n* = 2). 16 novel recessive mutations of MYO15A associated with severe to profound hearing loss segregating in 20 of these DFNB3-linked families. Importantly, two homozygous mutant alleles c. 3313G > T (p.E1105X) and c. 3334delG (p.G1112fsX1124) of

Table 2: Status of the pedigree analyzed by the linkage analysis for deafness locus DFNB3

S/No	Family No.	Status of Pedigree
01	PK-DF01	Unlinked to the deafness locus DFNB3
02	PK-DF02	Unlinked to the deafness locus DFNB3
03	PK-DF03	Unlinked to the deafness locus DFNB3
04	PK-DF04	Unlinked to the deafness locus DFNB3
05	PK-DF05	Unlinked to the deafness locus DFNB3
06	PK-DF06	Linked to the deafness locus DFNB3

DBNF: A gene for congenital, recessive deafness DFNB

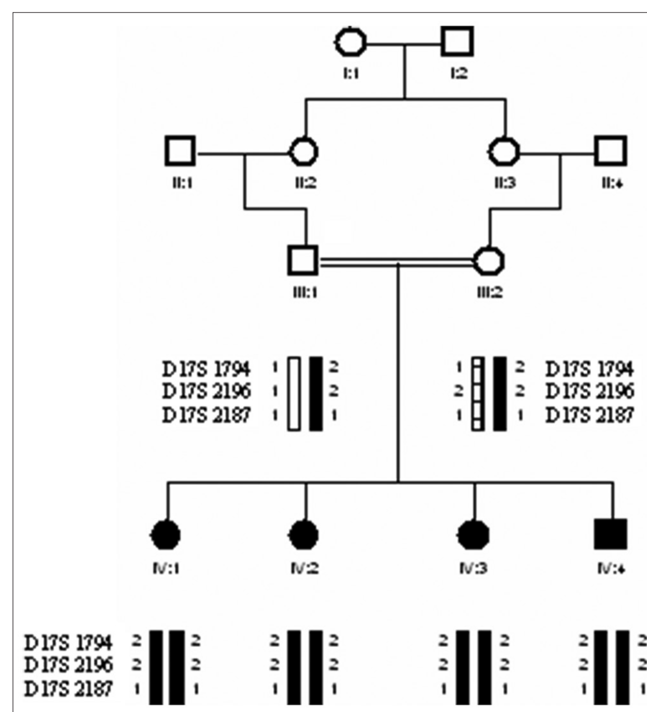

Figure 1: Pedigree of familyPK-DF06. The three STR markers D17S2196, D17S1794 and D17S2187 in the candidate region of DFNB3. Deafness phenotype in this family was linked to DFNB3 locus

MYO15A-located in exon 2 were associated with severe to profound hearing loss segregating in two families.[19]

From 77 consanguineous families, four families were linked to the DFNB3 locus. Sequencing of MYO XV showed that there two novel homozygous mutations: a nonsense (c. 4998C > A (p.C1666X) in exon 17 and a splice site mutation in intron 54 (c. 9229 + 1G > A). A novel mutation of unknown significance, c. 7395 + 3G > C, was identified in the third family, and no mutation was found in the fourth family.[20] Linkage mapping using Affymetri × 50K GeneChips and short tandem repeat (STRP) analysis localized the hearing loss in two families to the DFNB3 locus. Sequencing of the MYO15A gene showed a novel homozygous missense mutation (c. 6371G > A) that results in a p.R2124Q amino acid substitution in the myosin XVa protein in the effected members of one family, while in the other family homozygous missense (c. 6555C > T) mutation resulting in a p.P2073S amino acid change.[21]

The five families (PK-DF01, PK-DF02, PK-DF03, PK-DF04 and PK-DF05) remained unlinked to DFNB3 may be linked to the other known loci, and there is a high probability of reporting new loci/genes due to molecular heterogeneity in our population.

Family size and structure, the number of family members who agree to participate in the linkage study and accuracy of clinical data from each participant all play a major role in the success of linkage analysis. In addition, for the analysis to be successful, it is also necessary to have accurate clinical information about each participant.

The unlinked families show that deafness is a complex genetic disorder and there are the chances that many other known loci are involved in the deafness in these families or some novel locus/gene may be involved, which mean that it need further study to determine the locus and the gene in which mutation caused deafness in these population, which will help to prevent this genetic disorder in the population of Okara and Pakistan as well.

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