·Basic Research·

In silico analysis of a disease-causing mutation in *PCDH15* gene in a consanguineous Pakistani family with Usher phenotype

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Abstract

• AIM: To map Usher phenotype in a consanguineous Pakistani family and identify disease-associated mutation in a causative gene to establish phenotype-genotype correlation.

• METHODS: A consanguineous Pakistani family in which Usher phenotype was segregating as an autosomal recessive trait was ascertained. On the basis of results of clinical investigations of affected members of this family disease was diagnosed as Usher syndrome (USH). To identify the locus responsible for the Usher phenotype in this family, genomic DNA from blood sample of each individual was genotyped using microsatellite Short Tandem Repeat (STR) markers for the known Usher syndrome loci. Then direct sequencing was performed to find out disease associated mutations in the candidate gene.

• RESULTS: By genetic linkage analysis, the USH phenotype of this family was mapped to *PCDH15* locus on chromosome 10q21.1. Three different point mutations in exon 11 of *PCDH15* were identified and one of them, c.1304A>C was found to be segregating with the disease phenotype in Pakistani family with Usher phenotype. This, c.1304A >C transversion mutation predicts an amino-acid substitution of aspartic acid with an alanine at residue number 435 (p.D435A) of its protein product. Moreover, in silico analysis revealed conservation of aspartic acid at position 435 and predicated this change as pathogenic.

• CONCLUSION: The identification of c.1304A>C pathogenic mutation in *PCDH15* gene and its association with Usher syndrome in a consanguineous Pakistani family is the first example of a missense mutation of *PCDH15* causing *USH1* phenotype. In previous reports, it was hypothesized that severe mutations such as truncated protein of *PCDH15* led to the Usher I phenotype and that missense variants are mainly responsible for non-syndromic hearing impairment.

• **KEYWORDS:** deafness and blindness; Usher syndrome; causative gene; missense mutation; Pakistani family
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INTRODUCTION

Usher syndrome (USH) is inherited as an autosomal recessive trait and is characterized by a loss of vision due to retinitis pigmentosa (RP) and bilateral sensorineural deafness[1-3]. USH represents the most common genetic cause of deafness and blindness among children. Affected children are born deaf and progressively develop pigmentary retinopathy leading to blindness[4].

USH is classified into three clinical subtypes, designated as types I, type II, and type III. These types are distinguished by their severity and the age of onset of the disease[5]. USH type I (USH1) is the most severe and is characterized by severe to profound congenital hearing impairment, vestibular dysfunction, and pre-pubertal onset of RP; type II (USH2) is the most frequent form and is characterized by moderate to severe hearing impairment, normal vestibular function, and teenage onset of RP. USH type III (USH3) presents with progressive hearing loss and variable onset of RP and vestibular function[6].

USH1 is an autosomal recessive disorder and its genetic heterogeneity is well established as different mutant genes, causing USH, have been identified. To date, seven different loci USH1B (11q13.5), USH1C (11p15.1), USH1D (10q21-q22), USH1E (21q21), USH1F (10q21-q22), USH1G (17q24-q25), and USH1H (15q22-q23) have been reported to cause USH1. Genes at five of these loci, *MYO7A* (USH1B), *USH1C* (USH1C), *CDH23* (USH1D), *PCDH15* (USH1F), and *USH1G* (USH1G) have been identified[7].

Table 1 A summary of clinical findings of affected individuals with USH

Pedigree code	Age (a)	Clinical findings		
		Hearing loss	Vestibular balance	Blindness
USHR506	15	Congenital, bilateral and profound	Disturb	Profound
USHR507	13	Congenital, bilateral and profound	Disturb	Profound
USHR508	11	Congenital, bilateral and profound	Disturb	Progressive
USHR512	10	Congenital, bilateral and profound	Disturb	Progressive

Table 2 Known USH loci and genetic markers used in this study

Locus I.D.	Chromosomal region	Gene	STR markers used for exclusion studies
USH2A	1q41	Usherin	D1S2141, D1S549
USH1F	10q21.1	*PCDH15*	D10S1220, D10S1225, D10S1221, D10S1208, GATA121A08
USH1C	11q15.1	Harmonin	D11S1981, ATA34E08
USH1B	11q13.5	Myosin 1B	D11S2371, D11S2002, D11S2000
USH1G	17q24-q25	Scaffold protein	ATA43A10, D17S784, D17S949

Mutations of four USH1 genes *MYO7A*, *USH1C*, *CDH23*, and *PCDH15* are also reported to cause nonsydromic deafness, DFNB2, DFNB18, DFNB12 and DFNB23, respectively[8-9].

In this study, the USH1F locus on chromosome 10q21.1 was mapped by genetics linkage analysis in a consanguineous family with Usher phenotype from Khyber Pakhtunkhwa, Pakistan. This region of chromosome 10 harbors *PCDH15* gene. It was reported previously that protein truncating mutations of *PCDH15* cause USH1F and missense mutation of *PCDH15* were associated with isolated deafness (DFNB23) only [10]. On sequencing of *PCDH15* gene, we identified three sequence variants in exon 11 of *PCDH15* gene. One of the sequence variant, c.1304A>C was observed to be segregating with the disease trait and was not present in ethnically matched controls. This pathogenic c.1304A>C mutation predicts the substitution of an amino acid residue aspartic acid to alanine at codon 435 (p.435D>A). The association of a substitution mutation of *PCDH15* to USH trait in a Pakistani family is the first report of an association of substitution mutation with the USH trait.

MATERIALS AND METHODS

Sample Collection and Genomic DNA Isolation This study was approved by Advance Studies and Research Board, Kohat University of Science and Technology, Khyber Pakhtunkhwa, Pakistan and Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan. We studied an autosomal recessive consanguineous three generation family with USH. This family was collected from the Khyber Pakhtunkhwa province of Pakistan, where cousin marriages are commonly practiced. On the basis of clinical history and the results of ophthalmologic, audiometric, and vestibular tests, disease was diagnosed as USH. The clinical information of the affected individuals is presented in Table 1. Blood samples from affected individuals, their parents and clinically normal siblings of the family members were collected with informed consent. Blood samples were also collected from 100 ethnically matched unrelated normal Pakistani individuals and were used as controls for allele frequencies and confirmation of disease associated mutation. Genomic DNA was extracted from peripheral blood by the standard phenol chloroform extraction procedure [11].

Microsatellite and Linkage Analysis To identify the locus responsible for the disease in this family, genomic DNA from each individual was genotyped using microsatellite Short Tandem Repeat (STR) markers for the known USH loci (Table 2). Each STR marker was amplified by polymerase chain reaction (PCR). PCR reactions were performed in a 10 μL volume, each containing 1.5 mmol/L $MgCl_2$, 0.6 μmol/L of each primer, 0.2 mmol/L dNTPs, 1 U *Taq* DNA polymerase and PCR buffer [16 mmol/L $(NH_4)_2SO_4$, 67 mmol/LTris-HCl (pH 8.8), and 0.01% of the nonionic detergent Tween-20] (Bio-line, London, UK). Amplification was performed with an initial denaturation for 4min at 94℃, followed by 35 cycles of denaturation at 94℃ for 45s, annealing at 55℃ for 45s, extension at 72℃ for 45s and a final extension at 72℃ for 5min. The PCR products were separated on 10% non-denaturing polyacrylamide gels (Protogel; National Diagnostics, Edinburgh, Scotland, UK). The gel was stained with ethidium bromide and photographed under ultraviolet (UV) illumination. Alleles were assigned to individuals and genotypic data was used to calculate the LOD scores using the Cyrillic and MLINK software programme (Version 5.2, ftp://linkage.rockefeller.edu/software/linkage/).

Polymerse Chain Reaction Amplification of Genomic DNA and Mutation Screening by Direct DNA Sequencing PCR amplification of the *PCDH15* gene was performed with primers spanning all 35 exons [12-13]. PCR amplification was performed in a 50 μL reaction volume

Table 3 Mutations identified in exon 11 of *PCDH15* gene

Gene name	Accession number	Variant nomenclature (coding)	Exon number	Protein change	Classification
PCDH15	NM_033056	c.1138G>A	11	p.380Gly>Ser	Polymorphism
		c.1263T>C	11	p.421Thr>Thr	Polymorphism
		c.1304A>C	11	p.435Asp>Ala	Pathogenic

Table 4 In silico analysis of mutations identified in exon 11 of *PCDH15* gene

Gene	Nucleotide change	Residual change	Location	PolyPhen		SIFT		PMut	
				Prediction	PSIC score	Prediction	Score	Prediction	RI
PCDH15	c.1138G>A	p.380G>S	Exon 11	Benign	1.860	Tolerated	0.93	Neutral	5
	c.1263T>C	p.421T>T	Exon 11	Silent	0.000	Tolerated	NA	Neutral	NA
	c.1304A>C	p.435D>A	Exon 11	Possibly damaging	0.949	Tolerated	0.07	Pathogenic	0

containing 250 ng of genomic DNA, amplification buffer containing 600 nmol/L of each primer, 1.5 mmol/L $MgCl_2$, 200 mmol/L of dNTPs and 2.5 U Taq polymerase (Applied Biosystems, Warrington, UK) in an PxE thermal cycler (Hybaid, Basingstoke, UK). The amplification conditions were 95℃ for 5min, followed by 35 cycles of 95℃ for 45s, primer specific annealing temperature (55℃-65℃) for 45s, 72℃ for 45s. Aliquots (5 μL) of the PCR products were analyzed by 2.5% agarose gel electrophoresis. PCR products were then purified using GeneJet™ PCR purification kit (Fermentas Life Sciences, Hanover, MD, USA) and sequenced directly using Big Dye® Terminator v3.1 cycle sequencing kit in an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Potential mutations were confirmed by bi-directional sequencing and assessing 100 control samples having ethnic backgrounds matching to patients.

In Silico Analysis of the *PCDH15* Sequencing Variants

To find the influence of mutations identified in the *PCDH15* on its protein structure that may have an important role in disease susceptibility, in silico analysis was performed. Each DNA sequence was blasted using nucleotide blast (Blastn) tools of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn & BLAST). The DNA sequences were further aligned and analyzed to predict the effect of mutation on the protein product with CLC workbench 6. The mutations identified in *PCDH15* were evaluated using online available programs to predict whether variants are deleterious. PolyPhen classifies an amino acid substitution as probably damaging, possibly damaging, benign, or unknown. Provean predicts whether an amino acid substitution affects protein function. Provean prediction tool was used, which is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through PSI-BLAST.

RESULTS

An evidence of linkage and the region of homozygosity for STR markers at USH1F locus on chromosome 10q21.1 were observed for USH phenotype in a consanguineous Pakistani family (Figure 1). On mutation screening of the candidate gene, *PCDH15* in this region, three sequence variants c.1138G>A, c.1263T>C and c.1304A>C in exon 11 were identified (Table 3). However, among the three identified mutations, a c.1304A>C mutation was found to be disease causative as it was segregating with the disease phenotype and was also not present in 100 ethnically matched controls. This c.1304A>C mutation predicts the substitution of an amino acid residue aspartic acid to alanine at codon 435 (p.435D>A).

The multiple sequence alignment of amino acids showed that aspartic acid at position 435 is phylogenetically conserved in different species, and PolyPhen predicted the mutation to be possibly damaging (Figure 2). These results suggest that aspartic acid may be functionally important and the mutation may lead to damaging interference with conformation and function of *PCDH15*. Provean prediction analysis results for p.435D>A yield a score of 3.084 and predicted this change as deleterious. Whereas the other *PCDH15* variations, p.380G>S and p.421T>T were predicted to be neutral with Provean score of 1.860 and 0.000 respectively (Table 4).

DISCUSSION

USH1 genetic subtypes cannot be differentiated on the basis of clinical signs and symptoms, only investigations of linkage analysis in linkage informative consanguineous families [8] or mutational analysis of the genes involved, have been considered useful. Roux *et al* [14] investigated a cohort of patients in France and reported that mutations in USH1 genes cause USH in more than 90% of patients. However in some ethnic groups, a few mutations have a significant carrier frequency. As an example, a mutation c.216G>A in USH1C gene was reported in French Canadians of Acadian origin that accounted for almost all USH1 cases in Acadian population [15], but this mutation has not been found in any other population. In another example, the mutation c.733C>T in the *PCDH15* gene was identified by Ben-Yosef *et al* [16], that accounted for 58% of families of Ashkenazi with USH1. In some USH genes mutations were not found in some sporadic and familial cases of USH from Pakistan, France and Spain, suggested the search for additional novel USH genes[8,14,17].

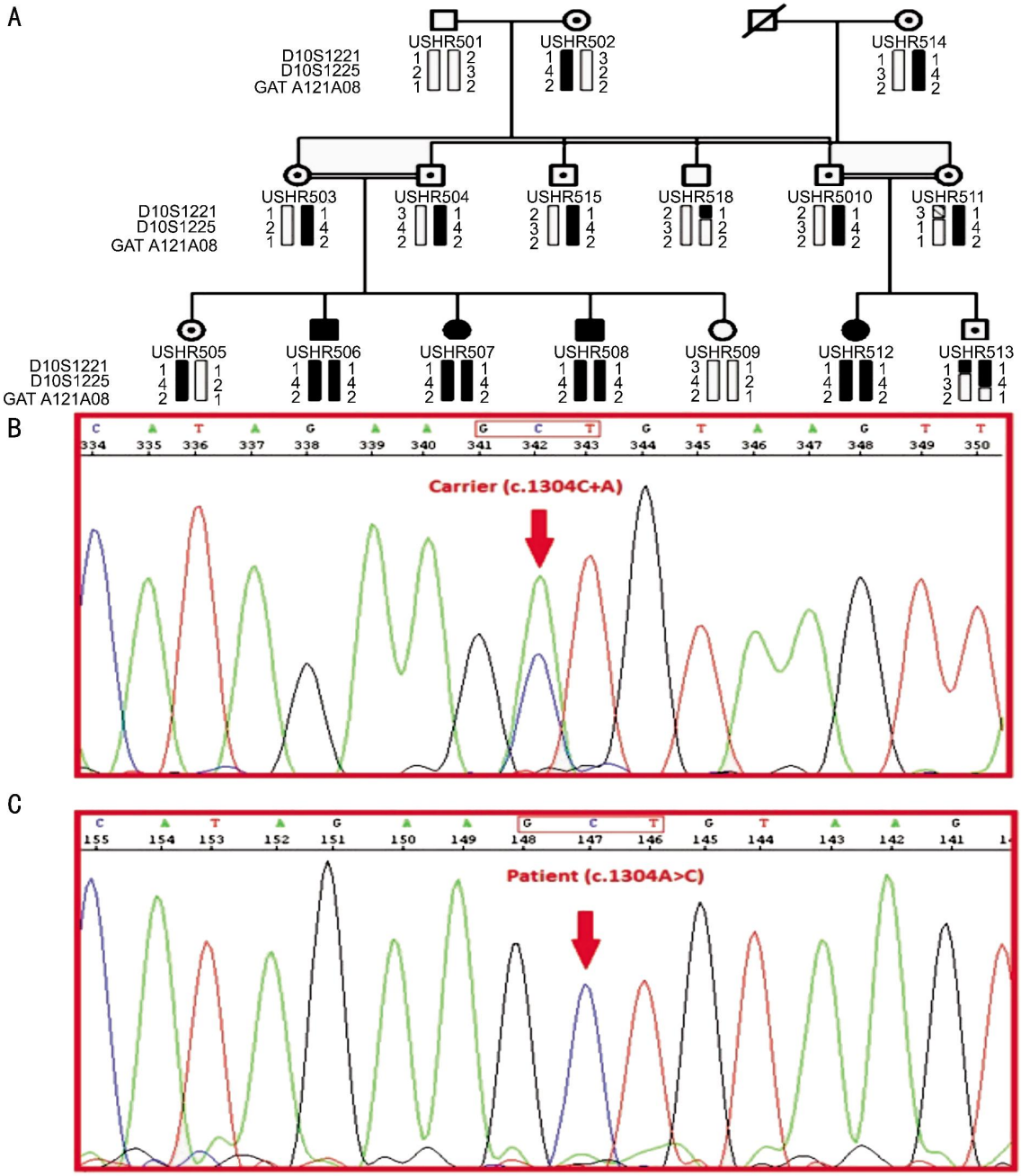

Figure 1 Pedigree of a consanguineous Pakistani family with STR genotyping data mapped to a locus on chromosome 10q21.1 A: In pedigree individual identification numbers are listed below the pedigree symbols. Genotyped markers from the chromosome 10q21.1 region are shown to the left, and individuals' allele numbers for each marker are given next to the bar. Black bars represent the haplotype segregating with the *PCDH15* gene. B: Electropherograms of *PCDH15* exon 11 in a carrier individual C: Electropherograms of *PCDH15* exon 11 in an affected patient; DNA sequence analysis revealed a homozygous A to C substitution at nucleotide 1304 (from the translation start site) in the affected patient, causing the Asp435Ala mutation.

The *PCDH15* gene is a member of the cadherin superfamily. Family members encode integral membrane proteins that mediate calcium-dependent cell-cell adhesion. It plays an essential role in maintenance of normal retinal and cochlear function. The *PCDH15* gene has been mapped by Alagramam *et al*[18] to chromosome 10q21-q22b. Ahmed *et al*[12] identified 33 exons in the *PCDH15* gene and spans about 1.6 Mb of human genomic DNA. Ahmed *et al*[13] identified four additional exons in the *PCDH15* gene, which encode two other cytoplasmic domains. In a Pakistani family, the first 2 exons of the *PCDH15* gene were found critical defined regions to cause USH type IF. Within the promoter region of the *PCDH15* gene instead of TATAA or CAAT sequences, a CpG island, suppressor and enhancer elements have been identified by Alagramam *et al*[19]. The intron sizes in *PCDH15* are variable and three additional genes have been reported by Ahmed *et al*[13]. Within the *PCDH15* gene, large genomic rearrangements have been found that are a significant cause of USH1F syndrome[20].

Mutations in the *PCDH15* gene are responsible for both combined hearing and vision impairment (USH1F) and non-syndromic deafness (DFNB23). To date, more than 30

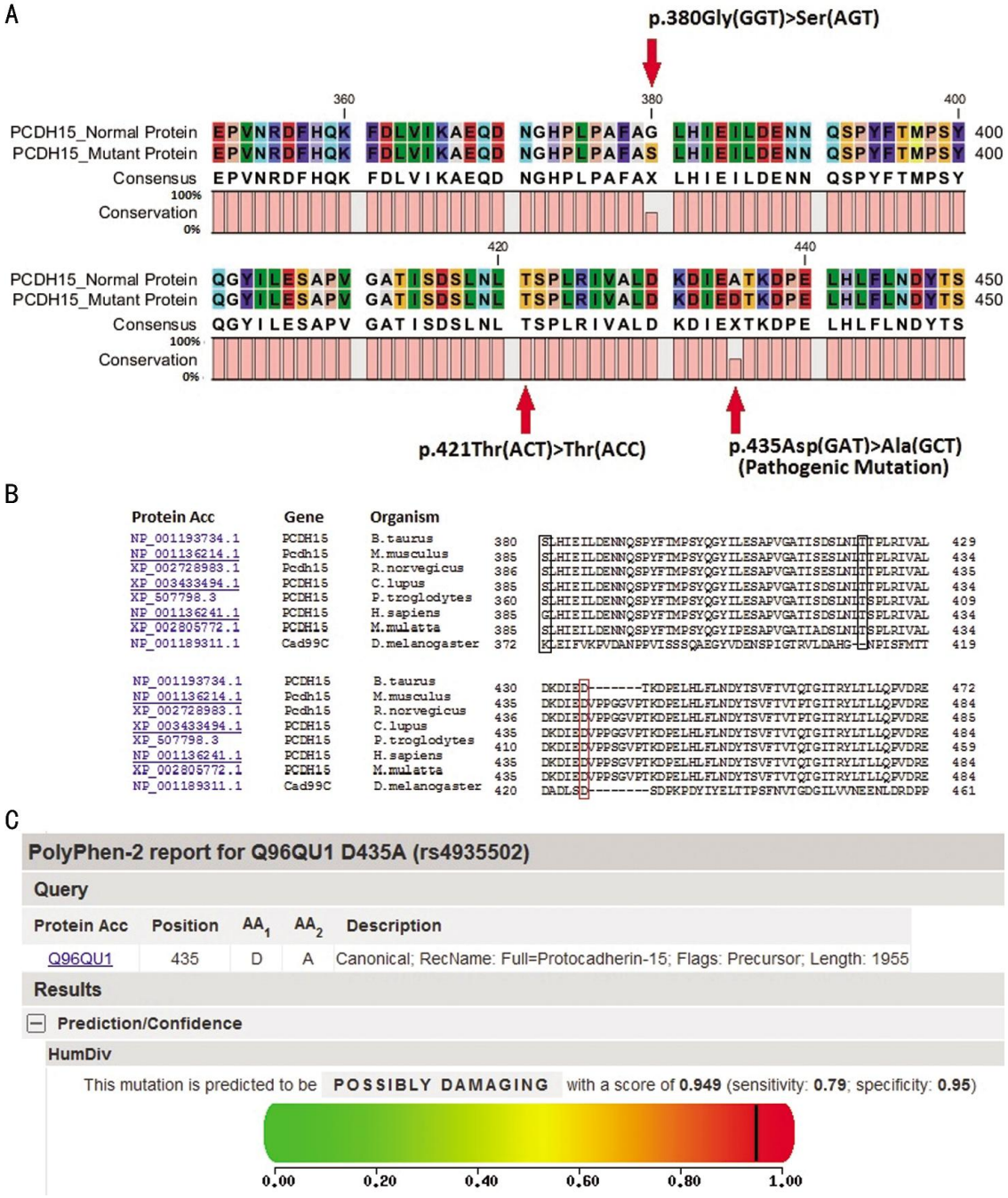

Figure 2 In silico analysis of the PCDH15 sequencing variants A: The mutant and normal *PCDH15* protein sequences were aligned using CLC Workbench V.6 to find the difference between them. The mutant amino acid residues, p.380Gly>Ser, p.421Thr>Thr and p.435Asp>Ala are indicated by red arrows; B: In order to check the phylogenetic conservation analysis of the mutated amino acid residue, amino acid sequences of *PCDH15* from human and different species were downloaded from the NCBI and automatically aligned by Lasergene Meg Align (DNASTAR, Madison, WI, USA). Multiple sequence alignment indicates that asparagine (D) at position 435 (red bar) is highly conserved; C: This diagram showing the PolyPhen analysis results of p.435D>A mutation. The results of PolyPhen analysis classified p.435D>A mutation as possibly damaging with a score of 0.949.

different point mutations have been identified as well as large rearrangements, including deletions and duplications have also been reported.

In previous reports, the only protein truncating mutations in *PCDH15* were found associated with USH, and other substitutions were reported to be responsible for causing DFNB23 phenotype. Ahmed *et al* [12] in a consanguineous Pakistani family with USH1F, investigated the *PCHD15* gene for mutations in affected members and found a homozygous 1940C-G transversion that resulted in a ser647-to-ter (S647X) substitution and predicted to truncate the protein in the EC6 domain. Doucette *et al* [21] reported a novel homozygous 1583T-A transversion that resulted an amino-acid substitution of a valine with an aspartic acid at codon 528 (p.V528D) of *PCDH15* in a consanguineous family from the island of Newfoundland. Ouyang *et al* [22] reported the heterozygosity for a mutation of 3-bp deletion (5601-5603delAAC) in exon 33 of *PCDH15* gene as this

resulted in subsequent deletion of threonine at 1867 and this also caused a missense mutation in patient with USH1F at *PCDH15* locus. Rebibo-Sabbah *et al* [23] reported nonsense mutations in patients with USH1F subsequently in translation of a variable length protein that resulted from partial read-through of this nonsense mutations. Zheng *et al* [24] also reported patients of USH1 who carried mutations of a 1-bp deletion in the *PCDH15* gene (16delT) in compound heterozygosity with a mutation in the *CDH23* gene. The *PCDH15* deletion (16delT) mutation causes a frameshift leading to an altered amino acid sequence from codon 6, followed by a premature termination at codon 11 in the predicted signal peptide of the protein.

In a study, the mutation c.1304A>C (p.435D>A) identified in the *PCDH15* gene by Jaijo *et al* [17] in a random unrelated pool of samples and was presumed to be non-pathogenic alteration. However the change is clearly disease associated in our family (Figure 1). In silico analysis also supports our findings that the change is pathogenic and conserved in different species (Figure 2 and Table 4), which is contrary to the report published by Jaijo *et al* [17].

The mutation identified in this study occurs in a highly conserved extracellular cadherin (EC1) domain of *PCDH15* and is predicted to be more deleterious than the previously identified missense mutations (p.R134G and p.G262D). Physical assessment, vestibular and visual function testing in deaf adults ruled out syndromic deafness because of USH. This study validates the DFNB23 designation and supports the hypothesis that missense mutations in conserved motifs of *PCDH15* cause nonsyndromic hearing loss. This emerging genotype-phenotype correlation in USH1F is similar to that in several other USH1 genes and cautions against a prognosis of a dual sensory loss in deaf children found to be homozygous for hypomorphic mutations at the USH1F locus.

The identification of a missense substitution mutation and establishing its association with USH1F phenotype in a consanguineous Pakistani family is the first example of an association of any missense mutation with USH1F. In exon 11 of *PCDH15* gene, a frameshift mutation, c.1304_1305insC (p.T436YfsX12) causing recessive USH1F was also reported by Ahmed and his colleagues [12]. It indicated that the mutations of *PCDH15*, affecting EC1 domain of its protein product is responsible for causing severe phenotype, *i.e.* USH1F. Recently, a novel transversion pathogenic mutation for nonsyndromic deafness in the USH1F gene *PCDH15* in a consanguineous family has been reported from the island of Newfoundland [21]. The association of a missense mutation and evaluation of its pathogenicity in a Pakistani consanguineous family further support the previous studies and emphasizes the need to know the genetic basis of recessively inherited neurological diseases in Pakistan. As a consequence of the unique socio-cultural practices in the population of Pakistan, approximately 60% of marriages are consanguineous, of which more than 80% are between first cousins[25]. Recessively inherited diseases are more prevalent in population of Pakistan as cousin marriages are common. These large consanguineous families are a powerful resource for genetic linkage studies of recessively neurological inherited disorders. In Khyber Pakhtunkhwa there are several factors contribute to the wide prevalence of genetic disorders in the region including the high rate of consanguinity, social trend to have more children until menopause, selective factors favoring inherited disease, and the lack of public awareness towards the early recognition and prevention of inherited disease. Different tribes of Pashtoons living here are very intimate about their marriages of inter tribal partners resulting in the mixing of blood and thus impurification of tribes. Many people do not agree with medical explanations of a genetic mode of disease inheritance, even in cases where there is an affected child. Complex neurological disorders like USH are frequent in the population of Pakistan and particularly in Khyber Pakhtunkhwa due to consanguinity and have a substantial impact on health care, socio-economic level and quality of life. Finding genetic risk factors involved in these disorders may boost knowledge about the disorder and possible treatment therefore provide a strong background for convincing or changing the Pakistani public's view regarding cousin marriages.

In conclusion, the evaluation of pathogenic role of mutation identified in exon 11 of *PCDH15* gene and its association with USH phenotype in a consanguineous Pakistani family will enable us to further characterize *PCDH15* gene variations and establish genotype/phenotype correlation. It will also help us to understand genetic mechanism of disease progression. A better understanding of *PCDH15* mutations and their effect on protein product and the resulting outcome that how some mutations results into less severe phenotype (DFNB23) or more severe phenotype (USH1). This knowledge will also be helpful in developing diagnostic and therapeutic strategies and also help in reducing the burden of genetic diseases by extending genetic counseling to individuals having strong history of genetic disease/ disorders.

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