

## Study of Recessive Deafness Locus (DFNB18/USH1C) by Linkage Analysis in Local Population

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**Abstract:** The objective of this study was to search for known syndromic recessive loci of deafness in local population of Balochistan. Hearing impairment is an extremely heterogeneous disorder that affects about 1 in 2000 newborns. Genetic hearing loss is most often monogenic. Hereditary hearing loss can be classified as syndromic (SHL) or non-syndromic (NSHL). Usher syndrome type IC (*USH1C*), is an autosomal recessive disorder characterized by profound hearing impairment, early onset of retinitis pigmentosa, and vestibular dysfunction, caused by mutations in *USH1C*. The *USH1C* locus mapped to chromosome 11p15.1. During this study, six families were enrolled with at least three affected individuals for linkage analysis studies. The families are collected from different areas of Balochistan province, i.e. Quetta, Sibi, Mastung and Pishin. Detailed history was taken from each family member for confirmation of consanguineous marriages and pedigree drawing. Blood samples from all participants were obtained for genetic analysis by genotyping; haplotype analysis was constructed. Out of six families, results of linkage analysis studies showed that haplotype of family BUIITEMS-17 were found linked with *DFNB18/USH1C* locus with D11S902, D11S4138 and D11S921 marker. Gene *USH1C/DFNB18* responsible for Harmonin was sequenced and sequencing of all the exons and adjacent splice sites, identified already known homozygous frame shift mutation c238\_239insC in affected members of the family BUIITEMS-17. Our results showed present study is the first report of a family with Usher IC linkage and mutation in Balochistan; further study is needed to explore the mutations and Usher loci prevalent in Balochistan population.

**Key words:** Consanguineous marriages, genetic analysis, pedigree, syndromic deafness

### INTRODUCTION

Hearing loss refers to impairment of hearing ability, while deafness refers to inability to hear with or without amplification (Katbamna and Patel, 2001). Hearing impairment is an extremely heterogeneous disorder that affects about 1 in 2000 newborns (Kahrizi *et al.*, 2008; Xia *et al.*, 2002). Human hearing impairment can be caused by environmental or genetic factors, including exposure to ototoxic drugs, infectious disease like rubella during pregnancy, trauma, excessive noise, or mutations. Hearing loss due to genetic mutations affects approximately 60% of persons with a form of hearing impairment. The hereditary hearing loss may be dominant, recessive, X-linked, Y-linked and mitochondrial (Marazita *et al.*, 1993). Approximately 75% cases of inherited deafness are autosomal recessive, 12-24% autosomal dominant and 1-3% is X-linked.

Genetic hearing loss is most often monogenic. Hereditary hearing loss can be classified as syndromic (SHL) or non syndromic (NSHL) (Xia *et al.*, 2002). Non syndromic NSHL accounts for 70% of genetic hearing

loss while syndromic hearing loss (SHL) account for approximately 30% of genetic hearing loss (Xia *et al.*, 2002; Hertzano and Avraham, 2003; www.hereditaryhearingloss.com).

The *USH1C* gene carries the instructions for the production of a protein called harmonin. Harmonin, linked to inherited forms of deafness in humans, is required for normal mechanotransduction by cochlear hair cells. Harmonin interacts at tip links with CDH23 and F-actin, it might also recruit additional proteins to tip links (Grillet *et al.*, 2009). The *USH1C* locus mapped to chromosome 11p15.1. Usher syndrome (USH) is classified into three clinical subtypes. Usher type I, Usher type II and Usher type III. All these three types of Usher syndrome are differentiated by onset and severity of visual impairment and hearing loss. To date, there are seven USH1 loci (*USH1A*, *USH1B*, *USH1C*, *USH1D*, *USH1E*, *USH1F* and *USH1G*) and five USH1 genes have been identified for type I. Particular mutations of four of these USH1 genes, can also cause non-syndromic hearing loss. Usher type I patients are congenitally deaf newborns, who lose vision within the first 10 years of life. Usher type II patients are

hearing-impaired adolescents with progressive visual impairment starting between the age of 10 and 20 years. Usher type III patients are similar to Usher II, but with progressive hearing loss. (Ahmed *et al.*, 2008; Teschner *et al.*, 2008; Lentz *et al.*, 2005).

*USH1C* is composed of 28 exons, including 20 constitutive exons (exons 1-14, 16-21) and eight alternatively spliced exons (15, A-F, G/G'). Moreover, long isoforms of harmonin, encoded by transcripts containing exons A-F and G/G', have been shown to be expressed in the inner ear, and not in the eye. This interesting finding, along with the existence of a form of non-syndromic recessive deafness (*DFNB18*) that maps to the same chromosomal location as the *USH1C* locus, made *USH1C* a good candidate for *DFNB18* (Ouyang *et al.*, 2002).

*USH1C*, also known as Acadian usher syndrome because this was first identified in French-Acadian population (Lorenz and Preising, 2004). In *USH1C*, 26 mutations have been currently identified, with some of them causing non-syndromic autosomal recessive deafness. Harmonin can bind the cytoplasmic regions of cadherin 23, protocadherin 15, and F actin, and anchors these proteins to the stereocilia actin core. Myosin VIIA and SANS are involved in the process of inclusion of harmonin into the hair cell stereocilia. Molecular analysis of these interactions shows harmonin directly interacts with all four proteins, thereby organizing spatial structure of the hair cell stereocilia (Tazetdinov *et al.*, 2008).

The major objective of the study was the linkage analysis for *DFNB18/USH1C* locus involved with deafness in local population of Balochistan. To achieve this objective, six families were enrolled through the schools for deaf children in Quetta, Pishin, and Sibi. The present study is the first step to explore Usher syndrome/*DFNB18* causing genes and mutations in the population of Balochistan and hence will not only enhance our knowledge about Usher syndrome but also make a contribution to reduce Usher syndrome/*DFNB18* in the population of Balochistan.

## MATERIALS AND METHODS

**Enrollment of families:** The present study was performed at Balochistan University of Information Technology, Engineering and Management Sciences Quetta from August 2008 to November 2009. A total of six families with multiple affected individuals were identified and enrolled through the schools for deaf children from different cities of Balochistan. These families were agreed to participate in the study and to donate blood samples. After approval from our Institutional Review Board (IRB) written informed consent was obtained from all participants. Detailed history was taken from each family to minimize the presence of other abnormalities and environmental causes for deafness. 5ml blood samples were collected from

each of the affected individuals, normal siblings, and parents. During collection of blood samples family members were interviewed to find out the symptoms of usher syndrome in affected individuals. 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. Clinical evaluation (Fundoscopy, Romberg test, tandem gait) for all the families was performed to find out whether the affected patients have history of Usher syndrome.

The pedigree structures were based upon interviews with multiple family members. Pedigrees of the six families were drawn, using the Cyrillic v2.1 and macromedia freehand software.

**DNA extraction:** DNA was extracted from blood samples using inorganic method (Grimberg *et al.*, 1989). After DNA extraction, linkage analysis studies were performed. Three STR markers D11S902, D11S921 and D11S4138 were genotyped to determine if a family was linked or unlinked to *DFNB18/USH1C* locus. Haplotypes were constructed to determine the pattern of inheritance among the affected and normal individuals of each family under study.

**Typing STR markers by PCR:** For *USH1C* deafness locus, three (03) microsatellite markers were typed. Genotyping was performed with un-labeled primers. The markers used for linkage analysis encompassed the chromosomal locations as mentioned by (<http://dnalab-www.uia.ac.be/dnalab/hhh>). For genotyping of the DNA, samples of a particular family, Each of marker was amplified separately, PCR reactions were performed with this 50 ng of template DNA in 10 mL reaction mixture containing 0.8-2.4 uM primers, 1 mL of 10X PCR buffer (100 mM Tris-HCl, pH 8.4, 500 mM KCl, 20 mM MgCl<sub>2</sub> and 1% Triton), 0.8 mL of 1.25 mM dNTPs, and 2 unit of Taq DNA polymerase.

**Amplified product checked through agarose gel:** Mix 3 µL PCR product and 3 µL of 10x loading dye on perafilm. 6 µL mixture was loaded into the well of Agarose gel and run the gel for 15 min and after this visualized the gel to trans illuminator to check whether product is amplified or not.

**Preparation of gel for electrophoresis:** 40% acrylamide (38 g poly acrylamide and 2 g Biss acrylamide dissolved in 100 mL of distilled water), 8M urea, 5X TBE (27 g Trizma base, 13.75 g Boric acid and 10 mL of 0.5 M EDTA PH 8, and adjust the final volume to 500 mL with distil water), and little amount of deionized autoclaved water was added and then put the solution in microwave oven for 30 to 40 sec. Solution was then filtered and then level of the solution was raised up to the required volume. 1.0 % APS (Ammonium per sulphate;

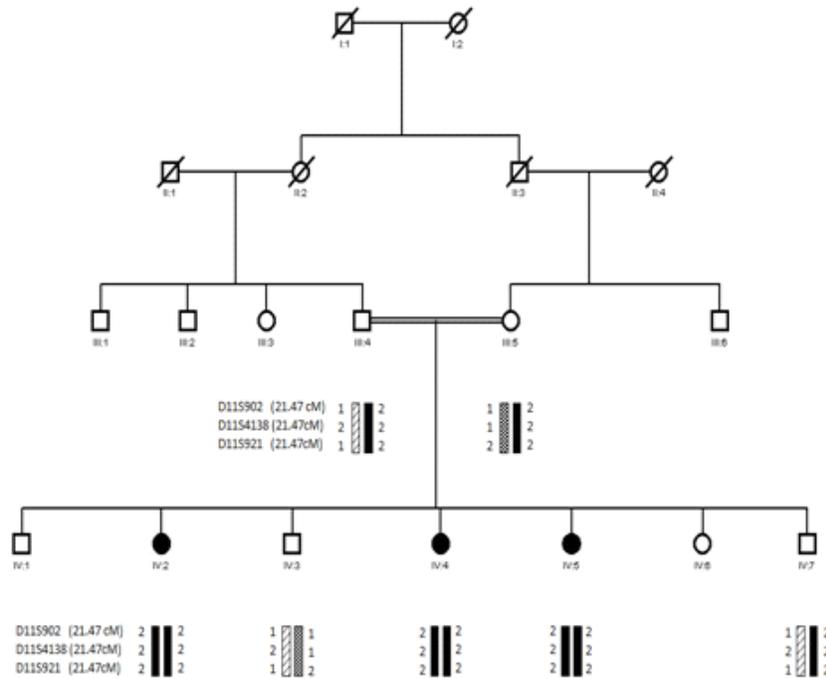


Fig. 1: Pedigree of family BUITMS-17. Three STR markers D11S902, D11S4138 and D11S921 in candidate region of *USH1C* showed homozygosity in the candidate region. Deafness phenotype in the family BUITMS-17 was linked to *DFNB18/USH1C* locus

0.1 g in 1 mL of de-ionized autoclaved water and stored in freezer) and TEMED were added in the solution and mixed it well and then immediately poured it with the help of syringe in between the plates. Comb was inserted and then Plates were left for about an hour at room temperature for polymerization.

**Loading the PCR products into the wells:**

**2X loading dye:** 8 µL of 2X gel loading dye solution(0.05% Bromophenol Blue, 0.05% Xylene Cyanol, 95% Formamide, 20 mM EDTA.PH = 8) was added to the 8 µL of completed PCR reaction The sample were denatured at 95°Cfor 5 min. and immediatually place on ice.10 µL of the sample were loaded on denaturing gel.

**Running the gel and haplotype analysis:** Gel was run at the 100 volts for 3-4 h. After this the plates were separated with the help of scale. Gel was attached with one plate, polyacrylamide gel was stained with Ethidium Bromide Stain and haplotype type was constructed. Haplotype refers to a complete set of genotyped alleles for a chromosomal segment arranged according to cM distance. Haplotype analysis was performed by arranging alleles in a way confirming the disease inheritance pattern in the family. A family was confirmed to be linked to a particular locus when the homozygous data of affected individuals correlated with the disease pattern in the family tree.

**RESULTS**

**Enrolment of families:** A total of six families with multiple affected individuals were identified and enrolled through the schools for deaf children from different cities of Balochistan including Quetta, Pishin, Sibi and Mustang. These families were agreed to participate in the study and to denote blood samples. Written informed consent was obtaining from all participating subjects, 5ml blood samples were collected from each of the affected individuals, normal siblings, and parents. During collection of blood samples family members were interviewed to find out the symptoms of usher syndrome in affected individuals. Pedigrees of the six families were drawn, using the Cyrillic v2.1 and macromedia freehand software.

DNA was extracted from blood samples using inorganic method (Grimberg *et al.*, 1989). After DNA extraction, linkage analysis studies were performed. Three STR markers D11S902, D11S921 and D11S4138 were genotyped to determine if a family was linked or unlinked to *DFNB18/USH1C* locus. Haplotypes were constructed to determine the pattern of inheritance among the affected and normal individuals of each family under study.

Out of six families, single family BUITMS-17, showed in Fig. 1 was linked to *USH1C* locus, and the other five families remained unlinked to *DFNB18/USH1C* locus. Sequencing of Harmonin gene identified an already

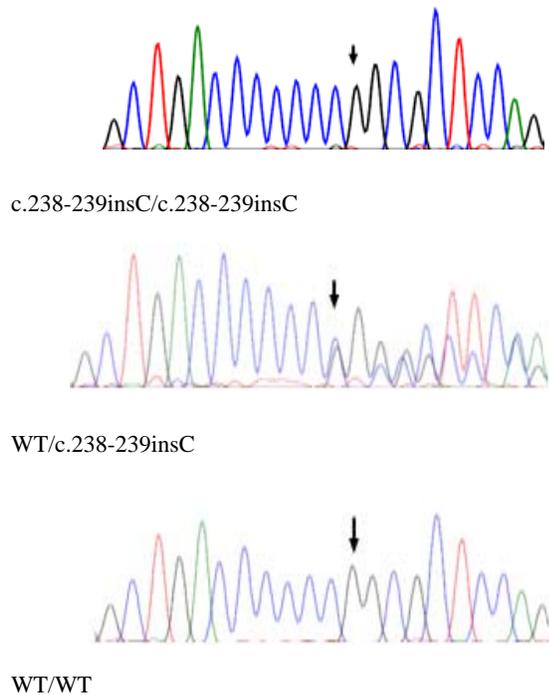


Fig. 2: Sequence analysis of Harmonin: (Upper) affected individual (IV:4) with insertion C (c.238-239insC), (middle) heterozygote (III:5) and (lower) normal wild type IV:3 in family BUITMS-17

known homozygous frame shift mutation c238\_239insC in family BUITMS-17, showed in Fig. 2.

#### Enrolment of family BUITMS-17 linked to USH1C:

This family was collected from Quetta. Blood samples of seven individuals were collected; three affected (IV:2, IV:4, IV:5), two normal siblings (IV:3, IV:7) and parents (III:4, III:5). The affected individuals range in the age from 8 to 30 years. All affected individuals were appeared in 4th generation.

**Medical history:** Physical and clinical evaluation of all the affected individual were ruled out for nonsyndromic as well as environmental cause of deafness. The affected individuals of family BUITMS-17 range in the age of 8 to 30 years. The affected individual were feeling difficulty to see during dim light. Romberg test and tandem gait test remained positive for all the affected individuals of the family BUITMS-17.

**Linkage and haplotype analysis:** After DNA purification, three STR markers D11S902, D11S4138 and D11S921 spanning in the region of *DFNB18/USH1C* were amplified. PCR product was electrophoresis on 6% Polyacrylamide gel at 90 volts for 2 to 3 hours. Polyacrylamide gel was ethidium bromide stained. Alleles

were read manually with larger allele donated by 1 and smaller with 2 and 3. Haplotypes of this family showed linkage to USH1C locus, showed in Fig. 1. The affected individuals were homozygous for STR markers D11S902, D11S4138 and D11S921, while normal siblings and parents were heterozygous. Sequencing of Harmonin gene identified an already known homozygous frame shift mutation c238\_239insC in family BUITMS-17, showed in Fig. 2.

#### DISCUSSION

Hearing loss refers to impairment of hearing ability, while deafness refers to inability to hear with or without amplification (Katbamna and Patel, 2001). Hearing impairment is an extremely heterogeneous disorder that affects about 1 in 2000 newborns (Kahrizi *et al.*, 2008; Xia *et al.*, 2002). Human hearing impairment can be caused by environmental or genetic factors, including exposure to ototoxic, drugs, infectious disease like rubella during pregnancy, trauma, excessive noise, or mutations. Hearing loss due to genetic mutations affects approximately 60% of persons with a form of hearing impairment. The hereditary hearing loss may be dominant, recessive, X-linked, Y-linked and mitochondrial (Marazita *et al.*, 1993). Approximately 75% cases of inherited deafness are autosomal recessive, 12-24% autosomal dominant and 1-3% is X-linked.

Genetic hearing loss is most often monogenic. Hereditary hearing loss can be classified as syndromic (SHL) or nonsyndromic (NSHL) (Xia *et al.*, 2002). Nonsyndromic hearing loss (NSHL) often associated with vestibular dysfunction. NSHL accounts for 70% of genetic hearing loss while Syndromic Hearing Loss (SHL) account for approximately 30% of genetic hearing loss is syndromic in nature. Nonsyndromic hearing loss is a common sensory deafness and can be divided into two types: prelingual and post lingual deafness. Within the prelingual NSHL category, 75-80% are autosomal recessive, 20-25% are dominant, and 1-1.5% are X-linked.

The USH1C gene carries the instructions for the production of a protein called harmonin. Harmonin has the ability to bind to many other proteins in cell membrane and coordinates their activities. In a search for a harmonin (USH1C) interacting partner. USH type 1C is caused by defects in the harmonin USH1C gene (Reiners *et al.*, 2006).

During this study, six families were selected for linkage analysis studies. The families are collected from different areas of Balochistan province, Quetta, Sibi, Mastung and Pishin. The families have at least three affected individuals. Out of six families, single family BUITMS-17 linked to *USH1C* locus. The *USH1C* locus mapped to chromosome 11p15.1 (Tazetdinov *et al.*, 2008; Jan *et al.*, 2005; Fields *et al.*, 2002).

Families were collected from Quetta, Sibi, Mastung and Pishin. The family BUITMS-17 has three affected individuals, two normal individual along with father and mother. Results of linkage analysis studies showed that haplotype of family BUITMS-17 were found linked with *USH1C* locus.

The genotyping results of family BUITMS-17 showed that father, mother (III:4, III:5) and normal individuals (IV:7) were heterozygous, 3 affected individuals (IV:2, IV:4, IV:5) and single normal individual (IV:3) were homozygous with marker D11S902, and data of marker D11S4138 showed that mother (III:5) and normal individual (IV:3) were also heterozygous and 3 affected individual (IV:2, IV:4, IV:5) were again homozygous while father (III:4) and a normal individual (IV:7) were also homozygous. Third marker D11S921 also showed that father (III:4) and 2 normal individuals (IV:3, IV:7) were heterozygous and 3 affected individuals (IV:2, IV:4, IV:5) and mother (III:5) were homozygous. The normal individual (IV:7) of family BUITMS-17 was heterozygous, apparently normal but having one mutated allele from mother and this individual carrying a disease.

Harmonin, linked to inherited forms of deafness in humans, is required for normal mechanotransduction by cochlear hair cells. Harmonin interacts at tip links with *CDH23* and F-actin, it might also recruit additional proteins to tip links (Grillet *et al.*, 2009). The mechanotransduction machinery of cochlear hair cells undergoes a stepwise morphological and functional maturation, where sensitivity to mechanical stimuli develops before adaptation (Waguespack *et al.*, 2007). Harmonin binds *PCDH15* and *CDH23*, both of which are also required for hair bundle morphogenesis, suggesting that harmonin is required for cadherin function during development (Lefevre *et al.*, 2008).

The affected individuals of family BUITMS-17 range in the age of 8 to 30 years. The affected individual (IV: 2) was 10 years old, feeling difficulty to see during dim light. Romberg test and tandem gait test remained positive for all the affected individuals of the family BUITMS-17.

Results of linkage analysis studies showed that haplotype of family BUITMS-17 were found linked with *USH1C* locus. As several previous studies have confirmed that gene responsible for Usher 1C is Harmonin and sequencing of all the exons and adjacent splice sites, identified already known homozygous frameshift mutation c238\_239insC in affected members of the family BUITMS-17. Positional cloning of the *USH1C* gene is 11p15.1, coding for harmonin protein. The harmonin-encoding gene consists of 28 coding exons, alternative splicing of which results in generation of 10 protein isoforms. In turn, are subdivided into three subclasses and include two out of three PDZ domains, one or two coiled-coil domains and, in some splice variants, the PST (proline, serine, and threonine) domain. PDZ domains are the motifs that specifically interact with C-terminal

peptides, or internal peptides that fold into a -fingers. The most general function of PDZ domains may be to localize specific ligands to the appropriate plasma membrane domains. In *USH1C*, 16 mutations have been currently identified, with some of them causing non-syndromic autosomal recessive deafness (Tazetdinov *et al.*, 2008).

Our results showed present study is the first report of a family with Usher1C linkage and mutation in Balochistan; further study is needed to explore the mutations and Usher loci prevalent in Balochistan population.

## CONCLUSION

Considering the very common practice of cousin marriages in Balochistan highly favors the heterogenic nature of deafness and provides a strong basis for the molecular dissection of an array of novel genes/ mutation involved in causing the syndromic/non syndromic deafness. Our results showed present study is the first report of a family with Usher1C linkage and mutation in Balochistan; further study is needed to explore the mutations and Usher loci prevalent in Balochistan population.

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