Association of R1939W and P1987R variants of Otoferlin (OTOF) gene with severe to profound nonsyndromic sensorineural hearing loss in Pakistani subjects

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ABSTRACT

Objective: To find possible association of R1939W and P1987R variants of OTOF gene with severe to profound NSSHL in cochlear implant subjects.

Methods: It was a case control study, conducted from June 2021 to February 2022, comprising 50 cases of severe to profound NSSHL who had received cochlear implant from ENT Department, CMH Rawalpindi and 50 age-matched healthy controls from PEMH Rawalpindi. Blood samples were collected from all the subjects, followed by DNA extraction and allele-specific polymerase chain reaction, performed at Multi-disciplinary Laboratory of Department of Biochemistry and Molecular Biology, Army Medical College Rawalpindi. Statistical analysis was done using ‘SPSS’ and ‘XLSTAT’, followed by genetic analysis using ‘SNPstat’.

Results: Mean age of the cases was 5.96 ± 4.62 years (N=50), comprising 58% males and 42% females. All had bilateral and prelingual HL. Parental consanguinity was 72%, whereas 62% cases had a positive family history of deafness. Alleles of R1939W and P1987R were not associated with NSSHL, as shown by their p values of 0.56 and 0.89 respectively. For R1939W ORs were 0.71 (dominant model) and 0.80 (overdominant model), indicating negative association with NSSHL. Regarding P1987R OR was 0.96 (log-additive model). Genotypes of both variants were not in HW Equilibrium (p <0.0001), whereas their alleles showed high LD (D'=0.92).

Conclusion: High percentage of parental consanguinity was observed among cochlear implant candidates. The OTOF variants R1939W and P1987R were found to have protective roles against NSSHL in study population.

KEYWORDS: Nonsyndromic deafness, Sensorineural hearing loss, Otoferlin gene, R1939W variant, P1987R variant, Consanguinity, Cochlear implant.

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INTRODUCTION

Hearing loss (HL) is a globally prevalent, multifactorial, and polygenic condition. About 124 genes have been identified to be associated with nonsyndromic HL, and 78 of them are inherited in an autosomal recessive manner. Worldwide, 1.5 billion people suffer from hearing impairment, which by the year 2050 is estimated to reach 2.5 billion. According to an estimate, 736,900 cochlear implant surgeries have been performed around the globe, by the year 2019. In Pakistan, 244,254 individuals suffer from deafness.

Certain environmental and genetic factors play their roles in the onset of the disease. Consanguinity increases the susceptibility of HL, owing to reduction in genetic diversity. Pakistan has a high prevalence of consanguineous marriages (70%). Various genetic studies have been carried out to explore their associations with HL. Otoferlin (OTOF) gene located...
on chromosome 2p23.3 (48 exons), is associated with nonsyndromic autosomal recessive deafness, DFNB9. It is mainly involved in the fusion of synaptic vesicles with plasma membrane of the inner hair cells of cochlea and release of neurotransmitter at the ribbon synapse, participating in conduction of sound signals to the brain. Numerous studies have investigated the role of otoferlin gene in hearing impairment. A Chinese study determined 25 pathogenic variants of OTOF gene including p.R1939W in 34 subjects suffering from Auditory Neuropathy Spectrum Disorder. A genetic study involving Muslim families from Indian Jammu and Kashmir sequenced OTOF gene and found c.2122C>T mutation to be prevalent among the cases. In Pakistan, polymorphisms of about 57 deafness associated genes have been identified, where 47% of moderate to severe HL is contributed by six genes i.e., OTOF, GJB2, TMCI, MYO15A, SLC26A4, and TMPRSS3. Mutation analysis of 21 Pakistani Punjabi subjects suffering from nonsyndromic HL, with history of consanguinity, revealed two novel pathogenic variants; c.4990-4991delT and c.2443delCin OTOF gene. Another study conducted on a Pakistani family comprising seven subjects with autosomal recessive HL found pathogenic OTOF variant c.3289-1 G>T as a risk factor for the disease. In view of these significant associations between OTOF gene and hereditary deafness, it is critical to provide evidence-based information on the prevalence of OTOF variants in Pakistan and their association with the disease. So that a national genetic database on hereditary deafness could be established and extensive genetic testing be provided.

**METHODS**

The study was conducted at Multi-Disciplinary Laboratory of Department of Biochemistry and Molecular Biology, Army Medical College Rawalpindi, in collaboration with ENT Department, Combined Military Hospital (CMH) Rawalpindi and Chemical Pathology Laboratory, Pak Emirates Military Hospital (PEMH) Rawalpindi. It was a case control study, from June 2021 to February 2022.

**Ethical Approval:** A formal approval from Ethical Review Committee of Army Medical College was taken before commencement of the study (ID#148, dated 15th June 2021).

Total sample size was 100, calculated by WHO sample size calculator with deafness prevalence of 0.12% (95% CI and 5% error). Participants were selected using nonprobability purposive sampling technique. Case group comprised 50 individuals suffering from severe to profound nonsyndromic sensorineural hearing loss (NSSHL). All cases had received unilateral cochlear implant (CI) from CMH, Rawalpindi. Control group comprised 50 age-matched healthy individuals (both genders). Five ml venous blood samples were collected from all study participants, under aseptic measures, after taking informed written consent.

**Molecular Analysis:** Extraction of DNA was done by organic method followed by gel electrophoresis (1% agarose). OTOF gene information was obtained from Online Mendelian Inheritance in Man (https://www.ncbi.nlm.nih.gov/omim). The sequence of exon 47 of the OTOF gene was downloaded from National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/nuccore/224465243). Primers for the two variants were designed using online bioinformatics tool WASP (Web-based Allele Specific Primer). The reaction mixture was prepared using Taq buffer (1X) 2.0μl, MgCl2 (2mMol) 1.6μl, dNTPs mix (0.2mMol) 0.4μl, forward primer (1pmol/μl) 0.8μl, reverse primer (1pmol/μl) 0.8μl, Taq DNA polymerase (1 unit) 1.0μl, DNA template (200ng) 1.0μl, nuclease free water 12.4μl. AS-PCR parameters for P1987R were: hot start at 95°C for five minutes, denaturation at 95°C for 30 seconds, annealing at 58.8°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for seven minutes. Similar protocol was used for P1987R except for annealing temperature that was set at 61.1°C. Total 35 cycles were run. The end, gel electrophoresis (2% agarose) was performed with the PCR products to detect the wild/mutant alleles.

**Statistical Analysis:** Deafness associated parameters were analyzed using SPSS (version 22). Age was expressed as mean ± standard deviation. Categorical data was exhibited as frequency percentages. The two genetic variants were analyzed using online bioinformatics tool SNPstat (available at: https://www.snpstats.net/start.htm). In order to determine the association of OTOF gene variants with NSSHL, Pearson’s Chi square test and odds ratio (OR) were used. For the correlation analysis, Cochran-Armitage Trend test was applied using another statistical software XLSTAT (version 2022.5.1). To test for Hardy-Weinberg Equilibrium (HWE), Exact test was applied, and Linkage Disequilibrium (LD) was calculated for the two variants using haplotype frequency given by SNPstat. P-value < 0.05 was considered significant.

**RESULTS**

All cases had severe to profound sensorineural HL, diagnosed by Auditory brainstem response (ABR)
and brainstem evoked response audiometry (BERA). Physical examination, tympanometry, CT scan and MRI (brain) were unremarkable for any external/middle ear pathology or associated syndromic malformations. Those cases who had used hearing aids before cochlear implant reported no benefit. Cases visited ENT OPD for activation/mapping of implant device, and/or post-implant speech therapy.

Mean age of the cases was 5.96 ± 4.62 years. There were 29 (58%) males and 21 (42%) females. All cases had bilateral and prelingual onset of HL, 47 (94%) had non-progressive and 3 (6%) had progressive disease course. Milestones’ development was normal in 33 (66%) and delayed in 17 (34%) cases. Fig.1 shows family history of NSSHL cases.

Mean marital age of the cases’ mothers was 22.10 ± 3.14 years and that of the fathers was 26.65 ± 3.73 years. Most of the cases were of Punjabi ethnicity- 34 (72.34%), followed by Pathan-9 (19.15%), Balochi-2 (4.26%), Sindhi-1 (2.13%), and Siraiki-1 (2.13%). Regional distribution showed that 30 (61.22%) cases were from Punjab, 12 (24.49%) from KPK, 2 (4.08%) from Islamabad, 2 (4.08%) from Sindh, 2 (4.08%) from AJK/GB, and 1 (2.04%) from Balochistan.

R1939W and P1987R are biallelic variants, both having major alleles C and minor alleles T and G respectively. Allelic and genotypic frequencies in the two groups are given in Table-I.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Groups</th>
<th>Genotypic Frequency</th>
<th>Allelic Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>C/T</td>
</tr>
<tr>
<td>R1939W</td>
<td>Case</td>
<td>26% (13)</td>
<td>74% (37)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>20% (10)</td>
<td>78% (39)</td>
</tr>
<tr>
<td>P1987R</td>
<td>Case</td>
<td>10% (5)</td>
<td>90% (45)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12% (6)</td>
<td>88% (44)</td>
</tr>
</tbody>
</table>

Correlation analysis of R1939W showed \( Z(\text{observed}) = -0.91 \) with \( p \) (two-tailed) = 0.36. For P1987R, \( Z(\text{observed}) = 0.32 \) with \( p \) (two-tailed) = 0.75. Fig.3 shows scatter plots for the two variants. \( P \)-values for HWE were <0.0001 for both variants in the two groups. LD calculation for major alleles of the two variants showed \( D'=0.92 \) and \( r^2 \) (correlation coefficient) = 0.46, where haplotype frequency of CC was 0.165.

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Table-I: Allelic and genotypic frequencies of R1939W and P1987R variants of OTOF gene (N=100).

Fig.1: Family history of nonsyndromic sensorineural hearing loss (NSSHL) cases, (N=50).

Fig.2(a): Allele-specific PCR result of R1939W variant OTOF gene.

Fig.2(b): Allele-specific PCR result of P1987R variant OTOF gene.
DISCUSSION

All cases had prelingual onset of NSSHL, with increased prevalence in males (58%). Parental consanguinity was 72%, and 62% of the cases had family relatives affected with HL. Mean maternal marriage age was 22.10 years.

There was no significant association of R1939W alleles with NSSHL as given by \( p \) value of 0.56 i.e., \( > 0.05 \). Alleles of P1987R were also not associated with the disease (\( p =0.89 \)). Similar results were obtained using trend test i.e., \( p=0.36 \) for R1939W and \( p=0.75 \) for P1987R, showing absence of significant association with the disease.

For the association analysis of R1939W, three best genetic models were dominant, recessive and overdominant. According to the dominant model, the genotypes C/T-T/T were negatively associated with NSSHL (OR=0.71), indicating their protective roles against the disease. OR could not be calculated for the recessive model as genotype T/T could not be detected in the case group. In the overdominant model, the heterozygote C/T also had negative association with
NSSHL (OR=0.80). Log-additive was the best model for P1987R, that showed decreased risk of disease with the genotype C/G (OR=0.96). These findings suggest that NSSHL is less likely to occur in the presence of these two variants of OTOF gene. The scatter plots (Fig.3) show decreasing trends for both the variants, suggesting negative correlation between the genotypes and their observed proportions.

Allelic frequencies of both the variants were in Hardy-Weinberg proportion of p + q=1. P-value of <0.0001 indicated that the genotypic frequencies were not in Hardy-Weinberg Equilibrium of p^2 2pq q^2. Linkage disequilibrium calculation showed D' =0.92, which is very close to one, indicating a high degree of correlation between major alleles of the two variants. Correlation coefficient r^2 was 0.46, meaning that the genotype of one variant could be predicted in 46/100 individuals if the other variant was known.

There seems to be an increased prevalence of hereditary deafness among CI candidates, as shown by prelingual onset of HL, significant family history and parental consanguinity. The most common inheritance pattern of NSSHL is autosomal recessive, where disease occurs when both parents carry the same recessive alleles at a locus, the probability of which increases with cousin marriages, a prevalent phenomenon in our country. Also, about half of the HL cases have a genetic etiology, that usually presents early in life and tends to run in families, thus emphasizing the need for early detection, management and hence restoration of speech and language skills.

Increased prevalence of HL in males is also confirmed by a US study, where males were affected twice as often as females. About 61% Pakistani females get married by the age 22 years and median age at first birth is 22.8 years, indicating that advanced maternal age as risk factor for genetic disorders is not prevalent in our region.

The results of association analysis are contrary to a Pakistani study in which R1939W was found to be a causative factor for nonsyndromic hereditary HL. An American study found heterozygous mutation of P1987R to be associated with nonsyndromic auditory neuropathy. A high value of D' indicates that the alleles of the two variants are inherited together in our population as a haplotype, suggesting only few recombination events between them.

R1939W and P1987R variants of OTOF gene were found to be protective against NSSHL in our study subjects, suggesting other pathogenic variants of this gene or other genes as probable risk factors for hereditary HL in Pakistani population. This study has added to the national database of single nucleotide polymorphisms associated with HL, that will aid in selection of variants for future genetic testing and screening of hereditary HL. The genetic analysis data can be used as a reference for pharmacogenetic research and future genetic therapies. Furthermore, linkage disequilibrium data can be used in genome wide association studies (GWAS), for identification of risk alleles associated with HL in our population.

As consanguinity is the major underlying contributor to hereditary deafness, sharing results with the clinicians will help in establishing genetic counselling programmes with special focus on cousin marriages and disease risk estimation in future generations. There is a need of pre-marital screening and carrier detection of at-risk family members as is currently being done for thalassemia under Sindh Prevention and Control of Thalassemia Act, 2013.

Limitations of the study:
1. Sample size was small as few cochlear implant centers are present to manage deafness in our country.
2. Family members of deaf children could not be sampled due to financial constraints.
3. Other otoferlin gene variants could not be studied due to limited research budget.

CONCLUSION

NSSHL was more prevalent in males, presenting in early years of life, with bilateral, prelingual and nonprogressive disease course. Increased percentage of cousin marriages and family history of HL were detected in CI subjects. Alleles of R1939W and P1987R variants of OTOF gene were in high LD, however, they had no association with NSSHL. Genotypes of both the variants had protective roles against the disease.

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Conflict of Interest: None.

REFERENCES


Authors’ Contribution:
HN, ZAB did data collection, wet lab work, statistical analysis, manuscript writing, and final approval of manuscript.
AR, AM, provided technical assistance, did study design, data interpretation, data review for intellectual content and final approval of the manuscript.
All authors bear responsibility and accountability for the integrity of the work.