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Original Article

Association of Patterns of Otoferlin variant A1090E with Ethnicity, Developmental Milestones and Onset of Disease Course in Children with Non-Syndromic Hearing Loss

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ABSTRACT

Objective: To assess the association of variant genotypes with ethnicity, developmental milestones and onset of disease course in children with non-syndromic hereditary hearing loss.

Study Design: Case-control study.

Place and Duration of Study: ENT Department, Combined Military Hospital, Rawalpindi and CREAM Lab-I, Army Medical College, Rawalpindi, Pakistan from Jan to Dec 2022.

Methodology: The study enrolled 200 subjects, aged 06 months to 10 years: 100 subjects with non-syndromic hearing loss (NSHL) were placed in Group-A, and 100 healthy individuals in Group-B. Blood samples were collected from each enrolled subject. DNA was extracted followed by polymerase chain reaction, and restriction fragment length polymorphism. Chi-square test was applied to check for association.

Results: Analysis of the results indicated ethnicity regions as follows: Punjab 71% (NSHL) and 72% (controls); Khyber Pakhtunkhwa (KP) 19% (NSHL) and 15% (controls); Islamabad (Capital region) 06% (NSHL) and 05% (controls); Sindh 03% each (NSHL & controls) and Baluchistan 01% (NSHL) and 05% (controls). The wild genotype GG was the most common amongst subjects of all regions with highest frequency in Awan caste, both in case and control subjects. Statistically significant (p-value<0.005) difference was observed in NSHL subjects as compared to controls. There was no statistical significance observed between ethnicity and the wild genotype GG, heterozygous genotype GA and mutant genotype AA.

Conclusion: In non-syndromic hearing loss (NSHL), the Otoferlin variant A1090E was found to be significantly associated with regional backgrounds among cases. The variant may play a protective role in subjects against NSHL.

Keywords: Deafness, Developmental Milestones, Non-Syndromic Hearing Loss, Otoferlin.

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INTRODUCTION

Hearing loss (HL) is commonly observed in children, adversely affecting the child's language, speech, and cognitive development. It can be classified as conductive or sensorineural.[1] Sensorineural hearing loss (SNHL) accounts for more than 90% of HL in adults.[2] The susceptibility to permanent SNHL has been witnessed in preterm infants due to their longer stay in neonatal intensive care unit.[3] In Pakistan, nearly 14.5 million individuals suffer from hearing loss with approximately half of these cases being due to genetic causes, and consanguineous marriages in being responsible for a 70% rise in HL cases.[4,5]

Numerous genes involved in the onset of non-syndromic hearing loss have been identified.[6] Otoferlin is a transmembrane presynaptic vesicle protein encoded by the OTOF gene. It is a protein that enables an individual to perceive sound and maintain balance, and mutation of OTOF gene isone of the most common contributing factors responsible for the development of non-syndromic hearing loss (NSHL).[7,8] Various populations have shown association of OTOF to development of NSHL.[9]

One of the missense Otoferlin variants, A1090E, has been found to be associated with cases of hereditary hearing loss in a large consanguineous Pakistani cohort.[10] Since no research has been conducted on the variant of interest recently, the purpose of the current study was to investigate the genetic association between ethnicity and the A1090E variant of OTOF.

METHODOLOGY

This case-control study was conducted at the CREAM Laboratory, Department of Biochemistry, Army Medical College Rawalpindi, Pakistan, in collaboration with ENT Department, Combined Military Hospital Rawalpindi, Pakistan, between Jan

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and Dec 2022. Approval was obtained from Ethical Review Committee of Army Medical College, Rawalpindi (ERC/ ID/ 148, dated January 17, 2022).

Inclusion Criteria: Children of either gender aged between 6 months and 10 years old, with severe to profound NSHL, along with age-matched healthy individuals were included.

Exclusion Criteria: Children with syndromic, non-genetic congenital, and acquired hearing loss, and those with any other genetic disease were excluded.

Sample size was calculated using WHO sample size calculator, with prevalence of hearing loss 0.12%, which generated a total sample size of 200.[11] Non-probability purposive sampling was used to collect data after taking informant consent from parents or guardians. We included 100 children with non-syndromic hearing loss, and 100 healthy, age-matched controls Figure.

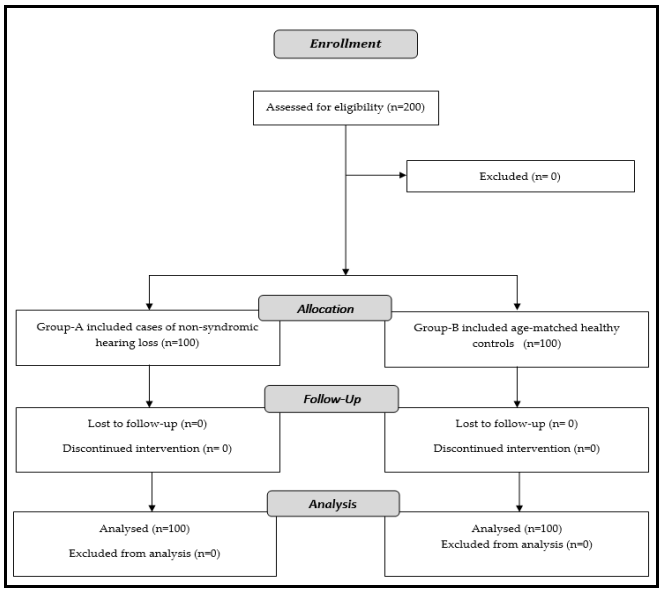

Figure: Patient Flow Diagram (n=200)

Blood samples were taken under aseptic conditions from each participant and processed for molecular analysis followed by DNA extraction, polymerase chain reaction, and restriction fragment length polymorphism.

Molecular Analysis: DNA extraction kit, (Favorgen biotech corporation, Cat. No.: FABGK; 001) was used to extract DNA. The DNA bands were visible on 0.8% agarose gel. The https://www.omim.org/entry/603681 resource was used for gene information. The gene sequence was obtained from the https://asia.ensembl.org/index.html.

Primers for Otoferlin A1090E variant were constructed by using the Primer3Plus, a bioinformatics tool at https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. The sequence of primers were as follows.

Forward primer sequence 5′-CTGAAGGGCCAGCAGGA-3′

Reverse primer sequence 5′-GAAGTCGTCAGCGGAGAAGT-3′

The size of primer product was 234bp.The target gene fragment (234bp) was amplified through PCR following; 95°C for 5 minutes, wit 35 cycles of denaturation at 95°C for 30 seconds, annealing at 61.1°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for seven minutes. Amplified polymerase chain reaction products were visualized on gel electrophoresis. Fast digest Hin6I (Thermo Fisher Scientific, US) restriction enzyme was used to digest the amplified PCR products.

Data analysis was carried out using Statistical Package for Social Sciences (SPSS) version 22. Frequencies and percentages were determined to analyze the quantitative variables. SNPStats, an online SNP analysis tool, was used to analyze the genotypes frequency distribution (https://www.snpstats.net/start.htm). Chi-square test was used to analyze the association between the variant A1090E of OTOF and ethnicity in the NSHL patients and controls, with a p-value<0.05 being significant.

RESULTS

The OTOF variant A1090E is biallelic, with major allele G and minor allele A.[12] The genotype associations of the A1090E variant of OTOF with ethnic region in NSHL children and controls were detailed in Table-I.

Statistically significant (p=0.006) difference of ethnic regions was observed in NSHL subjects as compared to controls. However, in control subjects, Chi-square test was statistically insignificant (p=0.05). Wild genotype GG was the most common genotype among all the sub-ethnic groups. The wild genotypes GG, heterozygous genotype GA and mutant genotype AA and any sub-ethnic group were not found to be significantly associated (Table-II). The wild genotype GG was found to be the most common in Awan caste,

both in cases of non-syndromic hearing loss and control subjects.

Table-I: Association of Genotypes of Variant A1090E of OTOF with Ethnic Region (n=200)

Ethnic regions	Genotypes							
	Group-A (n= 100)				Group-B (n= 100)			
	GG n=85	GA n=7	AA n=8	*p*-value	GG n= 85	GA n=5	AA n=10	*p*-value
Punjab	61(61%)	04(4%)	06(6%)	0.006	60(60%)	05(5%)	07(7%)	0.615
Khyber Pakhtunkhwa	17(17%)	02(2%)	00(0%)		14(14%)	00(0%)	01(1%)	
Islamabad	04(4%)	00(0%)	02(2%)		04(4%)	00(0%)	01(1%)	
Baluchistan	00(0%)	01(1%)	00(0%)		05(5%)	00(0%)	00(0%)	
Sindh	03(3%)	00(0%)	00(0%)		02(2%)	00(0%)	01(1%)	

Table-II: Frequency of Genotypes OTOF Variant (A1090E) with caste (n=200)

Cast	Group-A n (%) (n = 100)			Group-B n (%) (n=100)		
	GG (n= 79)	GA (n= 08)	AA (n= 13)	GG (n= 88)	GA (n=05)	AA (n=07)
Rajput	08(10.0%)	--	01(7.5%)	06(07.0%)	--	--
Bugti	02(03.0%)	--	01(7.5%)	01(1.2%)	--	01(14.3%)
Awan	18(23.0%)	01 (12.5%)	03(24.0%)	14(16.3%)	--	01(14.3%)
Baloch	01(1.5%)	--	--	03(04.0%)	--	01(14.3%)
Gujjar	02(03.0%)	--	01(7.5%)	03(04.0%)	--	01(14.3%)
Sayed	02(03.0%)	--	--	05(06.0%)	--	01(14.3%)
Qureshi	01(1.5%)	--	01(7.5%)	01(1.2%)	--	--
Arain	04(05.0%)	02(25.0%)	02(16.0%)	--	02(40.0%)	02(28.6%)
Kamboh	--	--	01(7.5%)	02(2.4%)	--	--
Sheikh	01(1.5%)	--	01(7.5%)	02(2.4%)	--	--
Mughal	--	--	01(7.5%)	--	--	--
Abbasi	--	01(12.5%)	01(7.5%)	02(2.4%)	01(20.0%)	--
Khan	02(03.0%)	01(12.5%)	--	--	--	--
Tanoli	01(1.5%)	02(25.0%)	--	02(2.4%)	01(20.0%)	--
Pathan	07(09.0%)	01(12.5%)	--	--	--	--
Haral	--	--	--	--	01(20.0%)	--
Pashtun	02(03.0%)	--	--	05(06.0%)	--	--
Bhatti	06(08.0%)	--	--	--	--	--
Qazi	02(03.0%)	--	--	--	--	--
Sawati	01(1.5%)	--	--	--	--	--
Saraiki	01(1.5%)	--	--	03(04.0%)	--	--
Khokhar	02(03.0%)	--	--	04(4.8%)	--	--
Bhutta	01(1.5%)	--	--	--	--	--
Ansari	02(03.0%)	--	--	02 (2.4%)	--	--
Warraich	01(1.5%)	--	--	02 (2.4%)	--	--
Malik	02(03.0%)	--	--	02 (2.4%)	--	--
Somro	01(1.5%)	--	--	08 (09.0%)	--	--
Yousafzai	01(1.5%)	--	--	03 (04.0%)	--	--
Gondal	01(1.5%)	--	--	--	--	--
Maikan	01(1.5%)	--	--	01 (1.2%)	--	--
Mohmand	02 (03.0%)	--	--	02 (2.4%)	--	--
Jutt	02 (03.0%)	--	--	02 (2.4%)	--	--
Phular	02 (03.0%)	--	--	--	--	--
Butt	--	--	--	02 (2.4%)	--	--
Minhas	--	--	--	01 (1.2%)	--	--
Niazi	--	--	--	01 (1.2%)	--	--
Bangash	--	--	--	02 (2.4%)	--	--
Rajpar	--	--	--	01 (1.2%)	--	--
Detho	--	--	--	02 (2.4%)	--	--
Sial	--	--	--	01 (1.2%)	--	--
Janjua	--	--	--	01 (1.2%)	--	--
Paracha	--	--	--	01 (1.2%)	--	--
Sangha	--	--	--	01 (1.2%)	--	--

In wild genotype GG cases, the developmental milestones were normal in 67% of the subjects and delayed in 18% of cases. In the heterozygous genotype GA cases, the developmental milestones were normal in 06% study subjects whereas delayed in 01% of study subjects. In cases with genotype AA, the developmental milestones were normal in 06% of subjects and delayed in 02% of the subjects. In genotype GG controls, developmental milestones were normal in 65% and delayed in 20% of controls. In heterozygous genotype GA controls, the developmental milestones were normal in 03% controls and delayed in 2% controls. In mutant genotype AA controls, developmental milestones were normal in 09% of controls and delayed in 01% of controls (Table III).

Table III: Association of Variant of A1090E of Otoferlin with Developmental Milestones (n=200)

	Developmental milestones			
	Group-A (n=100)		Group-B (n=100)	
	Normal (n=79)	Delayed (n=21)	Normal (n=77)	Delayed (n=23)
Wild homozygous GG	67%	18%	65%	20%
Heterozygous GA	06%	01%	03%	02%
Mutant homozygous AA	06%	02%	09%	01%

There was no significant association (p=0.12, 0.13) found between variant of Otoferlin and developmental milestones in cases and controls respectively.

In genotype GG cases, the disease began prelingual in 80% of cases, while in 05% of cases, it began post lingual. In heterozygous genotype GA subjects, the disease began prelingual in 07% cases and no heterozygous GA was found to have post lingual disease onset. In genotype AA cases, the disease began prelingual in 07% of subjects and post lingual in 01% of subjects. In wild genotype GG controls, the disease began prelingual in 66% controls and post lingual in 19% controls. In heterozygous genotype GA controls, the disease began prelingual in 05% controls and no heterozygous GA control was found to have post lingual disease onset. In genotype AA controls, the disease began prelingual in 09% controls and post lingual in 01% controls (Table-IV).

Table IV: Frequency of variant of A1090E of Otoferlin with onset of disease

Genotypes	Onset of Disease			
	Group-A (n = 100)		Group-B (n=100)	
	Prelingual n=94	Postlingual n=6	Prelingual n=80	Postlingual n=20
Wild homozygous GG	80%	05%	66%	19%
Heterozygous GA	07%	0%	05%	0%
Mutant homozygous AA	07%	01%	09%	01%

Nearly 11% of the cases with wild genotype GG had a progressive disease history, whereas 74% of subjects had a non-progressive disease course. The course of disease in heterozygous genotype GA patients was progressive in 01% and non-progressive in 06% of cases, respectively. The disease course in

persons with mutant genotype AA was non-progressive in 07% of suffering subjects and progressive in 01% of diseased subjects. In subjects of genotype GG controls, a progressive course of disease was observed in 25% of individuals and 60% in non-progressive individuals. In genotype GA subjects the progressive disease course was observed in 02% controls and in 03% subjects it was observed non-progressive, in genotype AA controls, the progressive disease course was observed in 04% and disease course was observed non-progressive in 06% controls.

There was no significant association (p=0.15) found between variant of Otoferlin and disease course in cases.

DISCUSSION

Hearing loss is a prominent defective disorder most observed among children. Nearly 1.57 billion children are affected with this hearing defect. Hearing loss is further divided into many types. Various factors can cause the onset of hearing loss including environmental, social, congenital and genetic. Genetics play a significant role in unraveling the disease basis. Genetics has been found to contribute to hearing loss in about 50% and approximately 70% of them are nonsyndromic hearing loss cases. In Pakistan's racially different communities, nonsyndromic forms of deafness can be passed on by genetic abnormalities in number of genes.[13] However, because of the recessive form of inheritance, custom and tradition that encourage cultural consanguinity avoid impairment.[14] Among the various ethnic groups in Punjab, Pakistan, Rajput families had the highest consanguinity ratio, followed by Jutt, Warayah and Pakhtun.[15] This usually occurs as a simple Mendelian trait of autosomal dominant, recessive, X-linked or mitochondrial inheritance. Among 124 genes reported to be associated with non-syndromic deafness, 77 autosomal genes are inherited recessively.[16]

Our study was aimed to explore the relationship between the Otoferlin gene variant and ethnicity. Our region wise study data shows 71% of the HL cases were from Punjab to be the most prominent region having hearing defect cases. Because of cultural consanguinity, genetic diseases are prevalent among Punjabis.[17] while Baluchistan showed the least (01%). To investigate the insight analysis of wild genotypes GG, heterozygous genotype GA, and mutant genotype AA, non-syndromic hearing loss cases and controls were split based on ethnicity. However, no genotype showed a significant association.

The wild genotype (GG) was found to be more prevalent (61%) in cases, among the residents of Punjab followed by KPK (17%), Capital region (4%), and Sindh (3%). Similarly, the frequency of wild genotype was found to be more prevalent in control subjects from Punjab (60%), followed by KPK (14%), Baluchistan (5%), Islamabad (4%), and Sindh (2%). This indicates that the gene variant plays a protective role among the residents of Punjab.

The heterozygous genotype (GA) was observed to be present higher in Punjab (4%) as compared to KPK (2%) and Baluchistan (1%) among cases. The control group presented the genotype (GA) only in the subjects from Punjab (5%). This shows that the heterozygous genotype is not common among the subjects in both the groups and very few of the participants carried the heterozygous GA. Similarly, the mutant genotype AA was not found to be commonly occurring among the subjects of cases, suggesting that the risk genotype of the studied gene variant is not related to the NSHL. The mutant genotype (AA) was observed in the subjects from Punjab (6%) and capital region (2%) among the cases while, this genotype was found to be in the highest frequency in the subjects from Punjab (7%) in control group. The homozygous wild genotype GG was observed to be the most frequent in both the cases (18%) and the controls (16%), while the heterozygous (AG) and the homozygous (AA) genotypes were very less observed in both the cases and controls. Moderate to severe hearing loss is caused by some mutant genes in 47% of cases and OTOF is one of them.[18] Regarding the wild type and mutant genotypes among the different sub-ethnic groups, there was a mixed trend observed, and no subgroup showed significant number of wild or mutant genotypes. The developmental milestones were observed delayed more in the subjects with wild type genotypes both in case and control groups.

The relationships between socio-demographic parameters and genetic variants provide sound evidence to explore the contribution of such factors with that of genetic data. This further expands the genetic knowledge based on populations and its link to various factors. The study needs to include larger population size to interpret crucial data based on population genetics.

LIMITATIONS OF STUDY

Our main limitations were a limited duration of study and lack of diversity of data.

CONCLUSION

The homozygous GG genotype appeared to be the most prominent genotype in both the cases and controls in the subjects from Punjab, and this genotype is more frequently observed in the Awan caste subjects from both the groups.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

IR & AR: Data acquisition, data analysis, critical review, approval of the final version to be published.

AA & IM: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

ZAB & NA: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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