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Identification and computational analysis of *USH1C*, and *SLC26A4* variants in Pakistani families with prelingual hearing loss

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Abstract

Background: Hearing loss (HL) is clinically and genetically heterogeneous disorder and is the most frequent occurring sensory deficit in humans. This study was conducted to decipher the genetic cause of HL segregating in two large consanguineous Pakistani families (GCNF-01, GCNF-03).

Methods and Results: Family history and pure tone audiometry of both families suggested prelingual HL, while the affected individuals of GCNF-01 also had low vision and balance problems, consistent with cardinal features of Usher syndrome type I (USH1). Exome sequencing followed by segregating analysis revealed a novel splice site variant (c.877–1G>A) of *USH1C* segregating with USH1 phenotype in family GCNF01. While the affected individual of family GCNF-03 were homozygous for the c.716T>A, p.(Val239Asp) previously reported pathogenic variant of *SLC26A4*. Both variants have very low frequencies in control database. *In silico* mutagenesis and 3-dimensional simulation analyses revealed that both variants have deleterious impact on the proteins folding and secondary structures.

Conclusion: Our study expands the mutation spectrum of the HL genes and emphasizes the utility of exome sequencing coupled with bioinformatics tools for clinical genetic diagnosis, prognosis, and family counseling.

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Author's contribution

Experiment design S.A.B., M.A., Z.M.A. S.R.; Methodology, M.N.; Software's, S.A.B., Z.M.A., M.Q., S.R.; Confirmation, M.N. and Z.M.A.; Prescribed analysis, S.A.B., Z.M.A.; Resources, S.A.B, M.A., Z.M.A. S.R.; Data organizing M.N., Z.M.A. M.Q.; Writing-Original draft preparation M.N., S.A.B., M.A., M.Q., Z.M.A.; Writing-review and editing Z.M.A., S.A.B., S.R. and M.Q.; Supervision, S.A.B., M.A., M.Q., Z.M.A.; Funding acquisition S.R., Z.M.A.

Conflicts of interest

The writers announce no conflict of interest. The funders had no role in the experimental strategy, in the collection, analysis and clarification of data, in the writing of the manuscript, or in the verdict to publish the data.

Keywords

Sensorineural hearing loss; USH1C; Usher syndrome; Exome sequencing; genetic heterogeneity; SLC26A4; prelingual hearing impairment

1. Introduction

Hearing play a vital role in our cognitive, speech and social development. Hearing loss (HL) is the most recurrent sensory disability in humans with frequency rate of 1–2:1000 babies, and targeting 360 million people of different ages worldwide [1–2]. Perception of sound signal requires specialized complex inner ear structures, including support cells, hair cells, stria vascularis, and neurons of spiral ganglion [3]. Several factors including genetic and environmental exposure contribute to the clinical heterogeneity of HL. Genetically, HL can be inherited through several patterns, however, non-syndromic autosomal recessive (NSAR) HL accounts 80%, while the dominant and X-linked HL are only 10–20% of all HL [4].

Populations with high rate of consanguineous marriages and isolated based on geographically, religiously, cultural and social aspects, recessive disorders are abundantly found. It is predicted that more than 10% of the congenital or genetic disorders worldwide are connected with expected consanguineous marriages, in majority of the Middle East its ratio is 30% and in Pakistan it is 40% [5]. In Pakistani population, the predicted frequency rate of profound HL is 1.6:1000 individuals, among this 70% of deafness cases are reported in consanguineous families [5–7]. Therefore, Pakistani populations provides predictable and appreciated hereditary reserves for investigating Mendelian disorders. Remembering all these present facts it is planned to be identifying the molecular basis of prelingual HL.

Exome sequencing is a very fast and high throughput technique and it is a reliable method to identify mutations in protein-coding genes. In present study, we enrolled two large consanguineous families segregating prelingual HL from Punjab of Pakistan, and whole exome sequencing (WES) is being performed as a modernized approach on the participating individuals. Exome data of family GCNF-01 and GCNF-03 revealed two pathogenic variants (c.877–1C>T), and (c.716T>A) in *USH1C* and *SLC26A4*, respectively, segregating with the phenotype. We also identified a rare, but likely benign variant (c.148C>G) of *OTOG* also co-segregating with HL in family GCNF-01. Mutations *USH1C*, *SLC26A4* and *OTOG* are common cause of HL in various populations, and their proteins products play pivotal role either in the hair cells development, function, tectorial membrane structure or ionic homeostasis in the inner ear [8–9]. Our study expands the allelic spectrum of HL-associated genes in Pakistani population and explore genotype-phenotype correlation. Findings of the current study will help to better understand the pathophysiology of the disease, the molecular basis of auditory transduction, improve molecular diagnostics, genetic counseling to the affected families and pave the way for formulating therapeutic strategies against prelingual HL.

2. Methods

2.1 Family recruitment and sample collection

All procedures in this study were approved by the Institutional Review Board Committees (HP-00061036) of the University of Maryland School of Medicine, Baltimore, MD, USA; and the Government College University, Faisalabad, Pakistan. The tenets of the Declaration of Helsinki guidelines for human subjects were followed and informed written consent from adults and parents of the minors assent was obtained from all the participating individuals prior to inclusion in the study. Peripheral blood samples 5–10 ml were drawn in EDTA containing tubes and stored at 4 °C. samples were obtained from all the affected individuals, their normal siblings, parents, and grandparents if available. The genomic DNA was isolated from each sample by applying standard protocol used for DNA extraction with slight modification using red blood cells, lysis buffer, proteinase-K buffer, 5.3M NaCl and 10% SDS [10].

2.2 Clinical phenotyping of families segregating hearing loss

Family histories were taken from multiple members to establish family structure, comorbidities, the onset of disease, and treatment. Phenotype assessment was based on detailed medical history, pure tone air and bone conduction audiometry, Romberg and tandem gait tests, and ophthalmic investigation. Although no clinical reports were available, but family history indicated perlingual HL in all the participating affected individuals (Fig. 1A). Audiometric profiling revealed that the affected individuals of family GCNF-01 had phenotype of profound HL for all the tested frequencies (Fig. 1B), while the affected individuals of family GCNF-03 had moderate to profound HL (Fig. 1B). Romberg and tandem gait, and fundoscopic tests revealed vestibular areflexia and retinitis pigmentosa (RP) only in the affected individuals of family GCNF-01, which is consistent with Usher syndrome type I. Among the affected individuals of GCNF-03 we did not find any evidence of vision loss, corneal opacity, or retinal dystrophies.

2.3 Exome sequencing (WES) and data analysis

For WES, the Agilent Sure Select Human Expanded All Exon V5 kit was used to recover the genomic libraries for exome enrichment. The libraries were sequenced on HiSeq4000 Illumina sequencer with a regular of 100x coverage. Genome Investigation Toolkit was used for data analysis [11]. Burrows-Wheeler Aligner was used to aligned the different reads with Illumina Chastity Filter [12]. The variant sites were baptized with GATK Unified Genotyper module. Single nucleotide (SN) variant calls were riddled through variant superiority score recalibration scheme [11]. Primer3 web resource (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design primers for the selected variants Sanger sequencing and segregation analysis.

2.4 Bioinformatics analysis

Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) multiple sequence alignment was used for the analysis of mutated residues across homologs. Several online prediction algorithms, including Mutation Taster (http://www.mutationtaster.org/), Mutation Accessor (http://mutationassessor.org/r3/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT

(https://sift.bii.a-star.edu.sg/), and Combined Annotation Dependent Depletion score (https://cadd.gs.washington.edu/score) were used to evaluate the functional impact of the identified variants. The Varsome (https://varsome.com) online tool was used for the classification of the variants according to the American College of Medical Genetics and Genomics (ACMG) guidelines.

2.5 Molecular Modeling

The three-Dimensional (3-D) structures of wild type and mutant proteins (OTOG and SLC26A4) were generated by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) by using intense mode option. We used ERRAT tool (https://servicesn.mbi.ucla.edu/ERRAT/) to evaluate the stereochemical quality of Phyre2 generated PDB structures. Ramachandran plots are used to visualize amino acid's energetically allowed region distribution in peptide backbone by dihedral phi (x-axis) versus psi (y-axis) angles (http://molprobity.biochem.duke.edu/). Chimera online tool(https://www.cgl.ucsf.edu/chimera/) is used to visualize protein 3-D structures.

3. Results

We investigated two Pakistani families after study approval by the institutional review board Committees (HP-00061036) at the Government College University, Faisalabad, Pakistan and University of Maryland School of Medicine, Baltimore, MD, USA, and written informed consents. WES was carried out to scan the coding regions of the genome of the probands of each family. Bioinformatics analysis of WES data revealed two different homozygous mutations segregating in patients of families GCNF-01 and GCNF-03. The identified pathogenic variants (c.877–1C>T), and (c.716T>A) in *USH1C* and *SLC26A4*, respectively, segregating with the phenotype in families GCNF-01 and GCNF-03. We also identified a rare, but likely benign variant (c.148C>G) of *OTOG* also co-segregating with HL in family GCNF-01.

3.1 Molecular genetic diagnosis of family GCNF-01

In family GCNF-01 we identified two different homozygous variants segregated with prelingual HL, including a novel splice site variant (c.877–1C>T) of *USH1C* and a missense variant [c.148C>G; p.(Gln50Glu)] of *OTOG* (Fig. 1). Consistent with the USH1 phenotype segregating in the affected individuals of family GCNF-01, the c.877–1C>T variant of *USH1C* is predicted to cause loss of canonical acceptor site, leading to cryptic splicing and premature truncation of encoded protein. While the bioinformatics analysis of missense variant p.(Gln50Glu) of OTOG revealed non-evolutionary conservation (Fig. 1C) as well as no significant impact on the protein secondary structure (Fig. 2A), indicating the likely benign nature of this variant (Table 1). Ramachandran plot analysis (Fig. 2B) and comparison of wild type versus p.(Gln50Glu) variant harboring OTOG indicated no significant impact (94% and 93% of total 1497 residues lie in the allowed region, respectively). Considering the close proximity of both genes on human chromosome 5, the *OTOG* variant is likely in linkage disequilibrium with the disease-causing variant of *USH1C*.

3.2 Molecular genetic diagnosis of family GCNF-03

Intriguingly, we observed inter-familial genetic heterogeneity of HL segregating in family GCNF-03. WES followed by Sanger sequencing analysis revealed the presence of a known missense variant [c.716T>A, p.(Val239Asp)] of *SLC26A4*, homozygous in five of the eight tested affected individuals, while the remaining three affecteds were homozygous for wild type allele (Fig. 1A). Clustal-Omega alignment confers that Valine residue at position 239 is highly conserved in SLC26A4 orthologs (Fig. 1C). Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) based modeling of SLC26A4 variant (p.Val239Asp), revealed that valine present at this position is very close to p.Gln235 and both residues are bound to each other through hydrogen bonding (Fig. 2C). Although this bond may remain intact due to p.(Val239Asp), but introduction of negatively charged and less hydrophobic aspartate to replace non-charged valine, is likely impacting the protein folding (Fig. 2C). Ramachandran plots revealed that both for wild type and p.(Val239Asp) variant harboring SLC26A4, 95% of the total residues were present in the allowed region (Fig. 2D). Rama distribution Z-scores for both wild type (−1.17 ± 0.28) and mutant (−1.63 ± 0.28) proteins were also comparable. However, the bad angles percentage for p.(Val239Asp) variant harboring SLC26A4 (2.13%) was slightly higher value than wild type (1.9%).

DISCUSSION

Whole-exome sequencing (WES) has been considered as a streamline approach for identifying pathogenic mutations in Mendelian disorders including HL. Here, we applied WES and identified genetic players of HL segregating in multiple generations of two large consanguineous Pakistani families GCNF-01 and GCNF-03. A novel splice acceptor site variant of *USH1C* was segregating with USH1 phenotype in family GCNF-01. Genetically, USH is an assembly of heterogeneous disorders that are inherited recessively and causes dual sensory dysfunction, vestibular dysfunction and vision loss [13]. *USH1C* is a common cause of USH1 in the populations of Pakistan, France, Israel, Finland, Northern Sweden and the Acadian population of Louisiana, United States [9, 13–15]. In the inner ear, harmonin, a PDZ domains containing protein encoded by *USH1C*, is primarily localized in the mechanosensory stereocilia bundles and is involved in the formation of stable anchorage structure complex, at the upper end of tip-link with cadherin 23 through multivalent connections [8]. In the retinal photoreceptors, harmonin is contained at the synaptic terminals and is involved in both structural and functional unity of this synaptic junction [8, 9, 11, 12, 14–16]. Thus, genetic variations in USH1C with significant impact on the encoded protein organization, are likely going to perturb the synaptic junctions of retinal photoreceptors and also impact the assembly of tip-link mechanotransduction complex in the hair cells stereocilia bundles of inner ear.

Co-segregating with HL in GCNF-03 family was a missense variant [c.716T>A, p.(Val239Asp)] in *SLC26A4*, which encodes a solute carrier protein pendrin. Consistent with prior studies [17], the affected individuals of GCNF-03 also had severe to profound hearing loss (Fig. 1B). Pendrin in the inner ear and thyroid gland functions as a transmembrane ion transporter, which interchange chloride for iodide and bicarbonate, and is intricate in

ion regulation and maintained of pH in cochlear cells [13, 18–19]. The p.(Val239Asp) is a frequently observed allele of *SLC26A4*-associated with HL in Pakistani population [20].

Intriguingly, we identified genetic inter- and intra-familial heterogeneity of HL segregating in both families, which further support the need of comprehensive clinical and genetic screening for accurate diagnosis and future personalized medicine initiatives. In family GCNF-01 we identified co-segregation of a novel missense variant (p.Gln50Glu) of *OTOG* with HL. Previously, five pathogenic variants of *OTOG* have been reported in humans suffering with HL [21–24]. Similarly, *Otog* mutant mice displayed bilateral progressive mild-moderate HL that developed immediately after birth [22–25]. In inner ear, *OTOG* is expressed in sensory epithelium, including the tectorial membrane, otoconial membrane in the utricle and saccule, and cupula that covers the crista ampullary of the semicircular canal in the vestibular organ [22], and function in establishing the fibrillar network of these membranes. Despite the high CADD score, our detailed bioinformatics analysis including 3D modeling, indicate likely benign impact of p.Gln50Glu variant on the encoded protein (Table 1, Fig. 2). In family GCNF-01, this variant of *OTOG* is co-inherited with the likely disease-causing variant of *USH1C* (Fig. 1A), and thus precluding the assessment of any subtle impact of this variant on inner ear structure and hearing function. These findings also highlight the significance of thorough genetic screening to rule out rare coding variants that may co-segregate with the phenotype likely due to linkage disequilibrium with the causal variants.

Inter-familial genetic heterogeneity of HL segregating in family GCNF-03 was apparent after the identification of p.(Val239Asp) variant of *SLC26A4* (Fig. 1A). Although the family history indicated prelingual HL in the affected individuals of family GCNF-03, yet the phenotype in all of them could be attributed to the *SLC26A4* allele. These findings further concur the previous recommendations of performing a) linkage analysis to determine locus heterogeneity; b) WES on all the available family members [26] and advised against pooling the samples of multiple affected individuals even if they present with similar phenotype [27]. To ease genomic verdict of intricate consanguineous pedigrees, widespread clinical phenotyping, informative subdivisions of affected individuals preferentially through linkage analysis, and perform WES on at least one sample/subgroup might be pivotal for complete genetic diagnosis.

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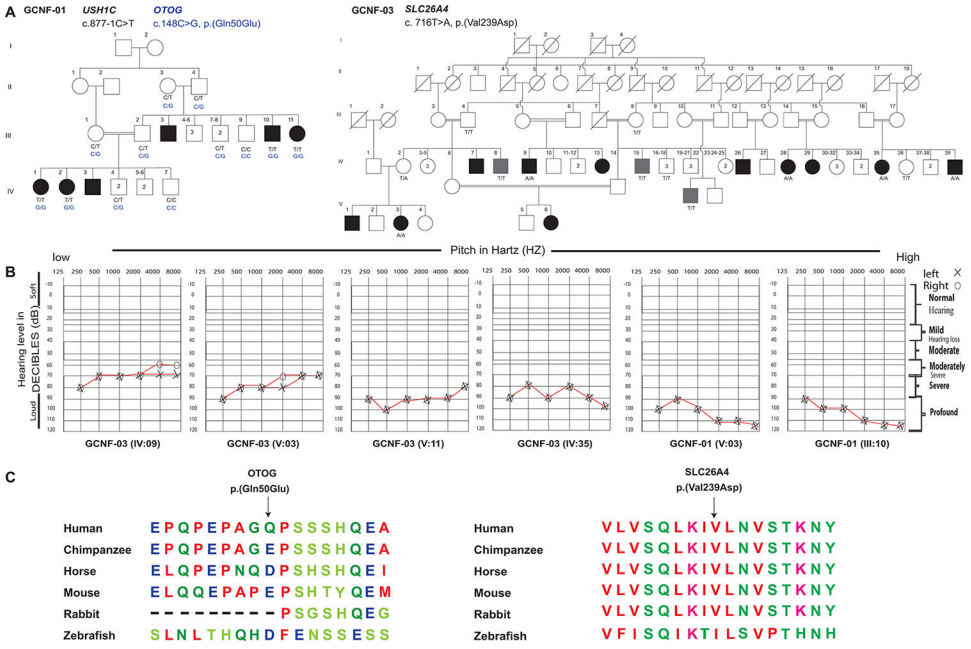

Fig. 1. Pedigree structures and identified variants segregating with HL.

A. Known HL genes variants segregating with HL phenotype in two large consanguineous Pakistani families. Filled and empty squares represent the affected and normal males, respectively, while circles denote females. Double line defines the consanguinity. Genotypes of each participating individuals for the candidate variants are shown below the symbols.

B. Air conduction bone audiometry data of two families GCNF-01 and GCNF-03 show severe to profound HL.

C. Clustal-Omega multiple sequence-alignment of orthologous proteins shown in evolutionary conserved mutated residues across the species.

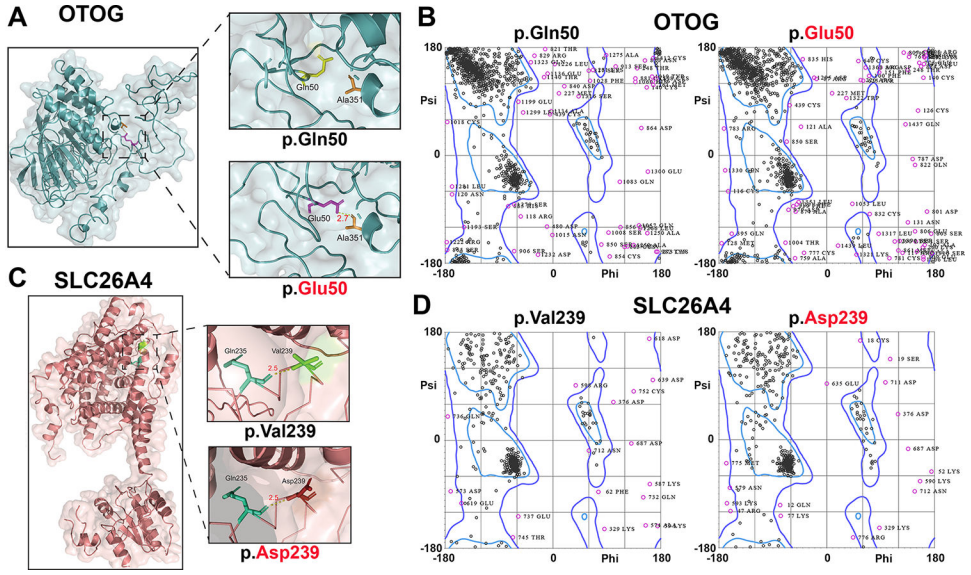

Fig. 2. 3-D molecular modeling and Ramachandran plots for OTOG and SLC26A4 proteins.

A. Wildtype and mutated residues of OTOG at position 50 are shown in orange and magenta, respectively. Protein strands, helix and coils are represented in green.

B. Ramachandran plot analysis of OTOG with p.Gln50 and p.Glu50 variants indicate 94 and 93% of total 1497 analyzed residues lie in allowed region. However, as compare to wild type protein, in which 93 residues (labeled amino acids) are outliers, the p.Glu50 change predicted 100 residues in outlier region.

C. Wild type and mutant residues of SLC26A4 at position 239 are shown in green and red, respectively. Yellow dotted line represents hydrogen bond interacting with neighboring residue shown in turquoise. Protein strands, helix and coils are represented in pink.

D. Ramachandran plots of SLC26A4$^{p.Val239}$ and SLC26A4$^{p.Asp239}$ residues reveal that 96% of the analyzed amino acids are present in the allowed region.

Table 1

Hearing loss causing genes, identified variants and their ACMG classification.

Family	Gene	cDNA change	Protein change	CADD	ExAC	Mutation Taster	Polyphen2	SIFT	ACMG classification (criteria used)	Reference
GCNF01	*USH1C*	c.877-1C>T	Splice site variant	25.7	0.00003	Disease Causing	N/A	N/A	Pathogenic (PVS1, PM2, PP3, PP5)	This study
	OTOG	c.148C>G	p.(Gln50Glu)	11.36	N/A	Polymorphism	Benign	Tolerated	Benign (BA1, BP1, BP4)	This study
GCNF03	*SLC26A4*	c.716T>A	p.(Val239Asp)	23	0.0002	Disease causing	Damaging	Damaging	Likely Pathogenic (PS3, PP2, PP3)	[17]

N/A: Not available; CADD: Combined Annotation Dependent Depletion, https://cadd.gs.washington.edu/; ExAC: Exome Aggregation Consortium, http://exac.broadinstitute.org/; PVS1: Pathogenic very strong [null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease)]; PM2: Pathogenic moderate 2 [Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium]; PP3: Pathogenic supporting 3 [Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)]; PP5: Pathogenic supporting 5 [Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation]; BP1: Benign supporting 1 [Missense variant in a gene for which primarily truncating variants are known to cause disease]; BP4: Benign supporting 4 [Benign computational verdict because 1 benign prediction from GERP vs no pathogenic predictions]