• Clinical Research •

Novel *ATOH7* mutation and structural characterization in families with optic nerve hypoplasia

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Abstract

● **AIM:** To detect and segregate causative mutations in congenital families with optic nerve hypoplasia (ONH).

● **METHODS:** Two unrelated consanguineous Pakistani families with severe ONH, showing features of micropthalmia, nystagmus, corneal opacity, and keratopathy were included. Genetic analysis was carried out by Target Panel Sequencing, and the nucleotide variant was confirmed by Sanger sequencing. In silico analyses were carried out to study the protein order-disorder functions and their effects on messenger ribonucleic acid (mRNA).

● **RESULTS:** Target panel sequencing revealed that the afflicted family members carried a novel frameshift mutation (NM_145178.4; c.91del G; p.Gly31Glyfs*55) that ensued in the conservation of an amino acid residue in the bHLH domain of ATOH7 protein. *In silico* studies predicted that the activity of the *ATOH7* gene is probably affected by this mutation, which results in a shortened and non-functional protein. Three-dimensional structural analysis shows that DNA binding may be impacted by amino acid changes from non-polar to positively charged and *vice versa* (Arg42Pro and Pro18Arg), as well as from positively charged (Arg) to a small polar amino acid (Gly).

● **CONCLUSION:** A novel *ATOH7* mutation is harmful. This study also emphasizes the potential effects of modified ATOH7 configurations on the stability and functionality of proteins.

● **KEYWORDS:** optic nerve hypoplasia; congenital families; *ATOH7* gene; novel mutation; disorder protein; structural analysis

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INTRODUCTION

Optic nerve hypoplasia (ONH) is characterized as a prominent cause of childhood blindness, occurring at a rate of 2.4% to 17.3% cases per 100 000 population, and leading to congenital vision impairment[1-2]. ONH has emerged as a frequent and severe congenital optic nerve abnormality, marked by optic nerves that are small; they may appear normal in size but are often grey or pale. Approximately 15% to 25% of infants with severe vision loss are diagnosed with congenital ONH, a non-progressive underdevelopment of the optic nerve that affects one or both eyes[3]. On fundoscopy, these optic nerves display the characteristic “double-ring sign”, linked with discrepancies in the size and distribution of the retinal veins[4]. Although the phenotypic presentation of bilateral ONH is highly flexible, ranging from almost normal vision to complete blindness, bilateral ONH remains a common cause of legal blindness, with about 80% of affected individuals meeting this criterion[5]. ONH can also arise as a sporadic condition, mostly linked with developmental abnormalities, including autism spectrum disorders, hemispheric or pituitary irregularities,

malformations of the anterior eye segment, and septo-optic dysplasia[6-7].

Typically, ONH manifests as sporadic cases, many of which are still undiagnosed at the molecular level: rare cases of familial ONH have also been reported[8-9]. Over the past ten years, breakthroughs in high-throughput sequencing technology have led to the identification of many ONH-associated mutations, most of which are found in genes related to brain or retinal transcription factors[10].

Atonal basic helix-loop-helix (bHLH) transcription factor 7 (ATOH7) is a protein comprised of 152 amino acids with two key domains: a DNA-binding basic domain inside the first helix and another helix-loop-helix domain for dimerization of protein[11]. Retinal development depends on the mouse ortholog *Math5*, which controls the differentiation of retinal progenitor cells (RPCs) into retinal ganglion cells (RGCs)[12]. *Math5*$^{-/-}$ mice have eyes of normal size but with more cone photoreceptors[13] and no optic nerves, chiasms, or RGCs. It has also been shown that *ATOH7* transgene expression promotes RGC development in human induced pluripotent stem cells (hiPSCs)[14]. A genome-wide association study indicated that many polymorphic variants in the 5'-UTR of *ATOH7* were related to the size of the optic disc[9].

ATOH7 mutations in humans have resulted in congenital retinal non-attachment, also known as congenital retinal detachment (partial detachment due to underdevelopment) with intraocular proliferation, and autosomal recessive ONH[15]. In the DNA-binding basic domain of *ATOH7*, a nucleotide variant (p.Asn46His) has been associated with the development of persistent hyperplasia of the primary vitreous (PHPV)[16]. This confirms a prior observation in *Math5*$^{-/-}$ mice, wherein aberrant retinal vascular formation and chronic hyaloid vasculature were caused by a lack of RGCs[11]. A previous study on Turkish siblings found that a homozygous frameshift mutation in the *ATOH7* was linked to a number of developmental eye abnormalities, including microphthalmia and persisting fetal vasculature as well as ONH. In another study on a Pakistani consanguineous family, a homozygous *ATOH7* missense mutation (p.Glu49Val) had severe manifestations in multiple affected individuals within a family. The phenotype included severe retinal and vitreous abnormalities, retrolental mass, dense corneal opacities, and microcornea and microphthalmia[17].

Although *ATOH7* has been implicated in a variety of ocular developmental disorders, its exact role in ONH remains unclear. Previous research identified several non-coding nucleotide variants, including one within an intron, but no mutations in the coding region of the *ATOH7* gene, in multiple ONH cases—some of which were associated with septo-optic dysplasia[18-19].

The present study investigated two families with ONH, characterized by corneal clouding, nystagmus, microphthalmia, underdeveloped optic discs, and corneal disease through genetic analysis. The observed phenotype was linked to a novel frameshift mutation in the *ATOH7* gene (NM_145178.3; c.91delG; p.Gly31Glyfs*55) in the affected family members. This study also highlights the potential impacts of altered ATOH7 configurations on protein stability and functionality.

PARTICIPANTS AND METHODS

Ethical Approval The study was conducted in adherence to ethical guidelines and regulations. Before the commencement of the study, ethical approval was obtained from the Institutional Review Board of the University of Health Sciences, Lahore (UHS), Pakistan (2/14/2017). The study strictly followed the Helsinki guidelines (modified 2013) for participant recruitment and data collection. To ensure full transparency and respect for individual autonomy, written informed consent was taken from every family member who participated in this study. Also, consent for minors was gained from their legal guardians to ensure their welfare and protection throughout the research process.

Genetic Analysis of ONH Families Genetic analyses were carried out for two unrelated consanguineous families (geographical and caste-distinct; family 1: Baloch and family 2: Syyed) that were recruited from the remote areas of the Sindh province of Pakistan. Targeted sequencing focuses on capturing specific nucleotide regions of the genome, particularly the exons of genes associated with certain phenotypes[20]. At the University of Exeter's Human Genetics laboratory in the UK, panel analysis was performed on two families. One affected individual from families with ONH underwent next-generation sequencing (NGS) using the TruSight One sequencing panel, which targets about 120 genes linked to nystagmus. The genomic DNA library was prepared by using an enzyme-based method and enriched for the targets with the Illumina TruSight One Sequencing Panel, followed by sequencing on the HiSeq 2500 (Illumina, Inc., San Diego, CA, USA). This kit enriches 62 000 exons from 4813 genes, covering a total target region of 12 Mb. A genetic counselor (molecular biologist) curated the TruSight gene list to include approximately 2462 genes related to Mendelian disease phenotypes, all validated for clinical testing. Pre-defined gene panels were used for clinical testing. Genomic DNA was extracted from the whole blood (Qiagen, Valencia, CA, USA), and about 50 ng was utilized for library preparation according to the protocols of the manufacturer (Illumina). Four clinical samples and one HapMap control were pooled and sequenced across two lanes on a HiSeq 2500, averaging five samples per lane[21].

ATOH7 Structural Analysis

Protein order-disorder prediction The Meta Disorder web-based tool was applied to predict whether ATOH7 is an

Table 1 Clinical summary of the affected individuals with ONH

Parameters	Family 1		Family 2 Affected IV:1
	Affected IV:3	Affected IV:5	
Age	15y	8y	5y
Problem	Congenital glaucoma, AC shallow (Pthisical eye)	Band keratopathy (soft eye)	Congenital glaucoma, advanced AC shallow
Onset	By birth	By birth	By birth
One eye or both	Both	Both	Both
Still getting worse	Yes	No	Yes
Cataract surgery performed	Glaucoma surgery performed	Yes	Glaucoma surgery performed
Photograph	-	-	-
Visual acuity	No perception of light	3/60 both eyes	Hand movement (both eyes)
Intraocular pressure	Soft	7 mm Hg	6 mm Hg
Anterior segment (cloudy corneas, iridocorneal adhesion, shallow anterior chamber)	Collapsed anterior chamber	Band keratopathy, AC shallow	Cornea cloudy, AC very shallow
Pupil dilation [cataract (nuclear, lamellar, posterior subcapsular), better photograph]	No clear view, cataract lens	IOL in place, decentered	Lens clear, pupil irregular, peripheral iridectomy present
Fundus examination (mass behind lens, optic nerve hypoplasia)	Optic disc hypoplastic, retina flat	Optic disc hypoplastic, retina flat	Glaucomatous optic atrophy, retina flat

ONH: Optic nerve hypoplasia; IOL: Intraocular lens; AC: Anterior chamber.

intrinsically disordered protein or not[22]. This method uses 15 predictions from primary disorder predictors and then weights their output according to method accuracy. The presence of disorder regions in proteins is associated with variations in the composition of hydrophilic as well as flexible amino acids. To determine the specific contribution of amino acid composition to flexibility and hydrophilicity, the Composition Profiler tool (http://www.cprofiler.org/) was utilized.

Domain prediction The important functional domains in the protein were predicted through InterProScan. It offers a comprehensive functional analysis of proteins by categorizing them into families and predicting domains and significant sites. This classification process relies on the utilization of predictive models, also known as signatures, which are sourced from multiple member databases within the InterPro consortium (https://www.ebi.ac.uk/interpro/search/sequence/) and SMART (http://smart.embl-heidelberg.de/).

Conservation analysis The PredictProtein (https://predictprotein.org/) is a web server, which is used to predict the conserved regions in the protein. The conseq program implemented in PredictProtein distinguished a highly conserved functional complex of the protein query.

Tertiary structure prediction After running a basic local alignment search tool (BLAST) search on the ATOH7 amino acid sequence, we were unable to find any homology with the protein data bank crystal structures. Therefore, an ab-initio modeling technique utilizing trRosetta was used in the lack of a structural homologue[23]. After the creation of five models, the Ramachandran plot was used to evaluate the models. The model that had the highest number of amino acids inside the permitted region was used to examine how mutations affected the structure of the protein.

Structure-based analysis of mutations using dynaMut2 By the use of DynaMut2, the normal mode analysis (NMA) method was utilized to evaluate the mutations affecting protein stability and dynamics. Mutations with values below zero (0) were classified as destabilizing, while those with values above zero were classified as stabilizing, based on the projected values of the Gibbs free energy ($\Delta\Delta G$). The DynaMut2 tool, available at (https://biosig.lab.uq.edu.au/dynamut2/), facilitated this evaluation process.

Nonsense-mediated decay prediction The effect of deletion mutation on mRNA was study through NMDEscPredictor (https://nmdprediction.shinyapps.io/nmdescpredictor/).

RESULTS

Clinical Summary Table 1 presents the clinical characteristics of affected individuals of families with ONH.

Genetic Studies Two unrelated families with congenital ONH characteristics are included in this study, and pedigree analysis suggests an autosomal recessive inheritance pattern. A novel frameshift deletion mutation, *ATOH7* (Chr10: 68231650; NM_145178.4; c.91delG; p.Gly31Glyfs*55), was found to be present in both families through targeted sequencing (Figure 1). This variant is classified as pathogenic by the guidelines of American College of Medical Genetics and Genomics (ACMG) because it causes a premature or truncated protein due to a deletion of the base G. The deletion of base G at position 91 of the coding DNA sequence leads to the formation of Gly at position 31 and a frameshift causing protein termination or truncation at amino acid position 55. Co-segregation analysis revealed that all affected individuals were homozygous for the variant, while phenotypically normal individuals were found to be heterozygous, representing carrier status. As no wild-type individual was available for

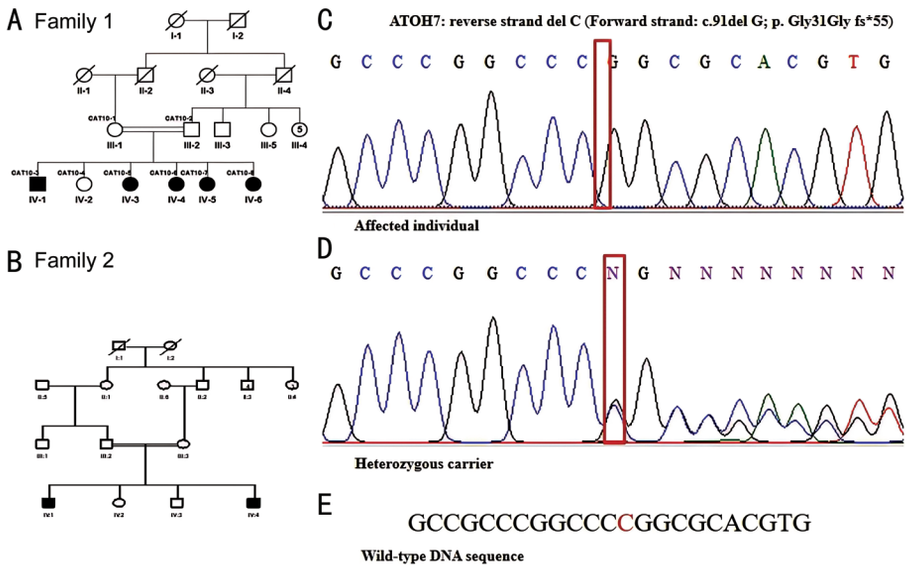

Figure 1 Pedigree analysis of unrelated consanguineous families with optic nerve hypoplasia A, B: Autosomal recessive mode; siblings are represented by horizontal lines, generation lines by vertical lines, and affected members by filled circles and squares. C-E: Sequencing data (reverse strand) of two families reveal that cases are homozygous for variant (C) and carriers (parents) are heterozygous (D) for *ATOH7*; (NM_145178.4; c.91del G; p. Gly31Gly fs*55), while homozygous (CC in reverse strand) wild-type (E).

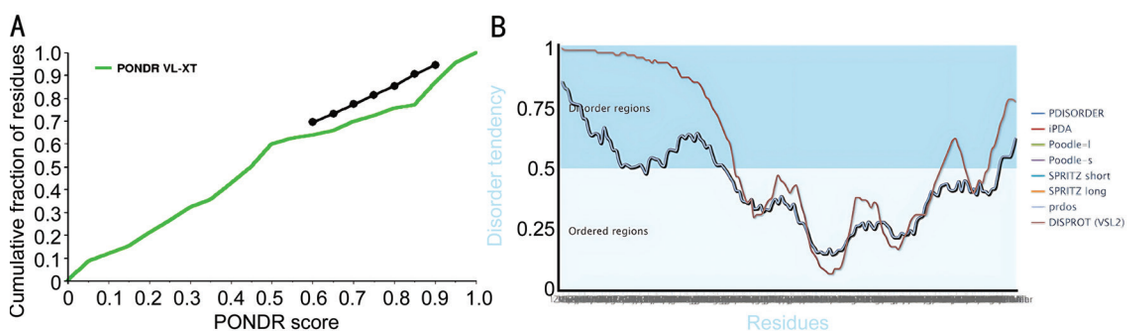

Figure 2 The disorder prediction of ATOH A: Characterization of protein as order or disorder by CDF plot of PONDR server. The black line is the reference line, the protein above the line is predicted to be ordered, while, the protein below the reference line is anticipated to be disordered protein. The results showed that AOTH is a disordered protein as the green line (representing the protein) under the black line (representing the reference). B: Amino acid distribution of ATOH as order or disorder. The results showed that both N- and C-terminal regions are highly disordered with few amino acids in the middle that are ordered. CDF: Cumulative distribution function; ATOH: Atonal homolog.

sequencing, chromatograms representing heterozygous carriers and homozygous mutants were included.

Protein Structural Analysis

Order/disorder prediction of ATOH7 The ATOH7 is a 152 amino acid long protein. The basic motif present in residues 41-52 is required for a certain type of DNA binding. In contrast, the HLH domain, which is present in residues 53-96, interacts with other family residues to form homozygous or heterozygous dimers. Disorder prediction of protein identifies that ATOH7 is a disorder protein (Figure 2A). The majority of proteins are disordered, with just a small number of amino acids discovered to be ordered in the middle, according to the metaDisorder server (Figure 2B). Additionally, the amino acid composition revealed that ATOH is abundant in proline and arginine, two amino acids that promote disorder (Figure 3A). Flexible amino acid content further contributes to the disorderly behavior of proteins. Remarkably, Figure 3B of the data indicated that the amount of rigid and flexible amino acids in protein is similar. Hydrophobic amino acids are also more abundant in the protein (Figure 3C).

Domains of ATOH7 The important domains in ATOH7 were predicted through interproscan and SMART. The SMART revealed the presence of the bHLH domain comprising the amino acids from 46-98 (Figure 4).

Conservation analysis of ATOH7 Conservation analysis was performed using the predictprotein server. The results showed that the bHLH domain is the highly conserved region of the protein (Figure 5).

Three-dimensional structure of ATOH7 Three-dimensional structure of ATOH7 was determined by trRosetta. Five models were generated, and the models which were further subjected to structure evaluation. The model with 99.2% residues in the

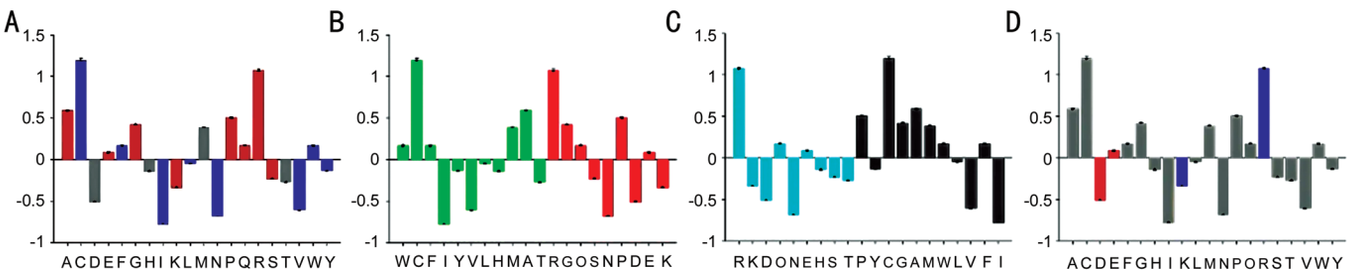

Figure 3 Composition of amino acids in ATOH A: Amino acids that promote disorder in ATOH. Grey represents neutral amino acids, red represents disorder-promoting amino acids, and blue represents order-promoting amino acids. B: The flexibility of the amino acid composition. Red color represents flexible amino acids, and green color represents rigid amino acids. C: ATOH's hydrophobicity. Hydrophilic amino acids are represented by the color cyan, whereas hydrophobic amino acids are represented by the color black. D: Charged amino acid distribution. The color blue denotes positively charged residues, while the color red denotes negatively charged residues. ATOH: Atonal homolog.

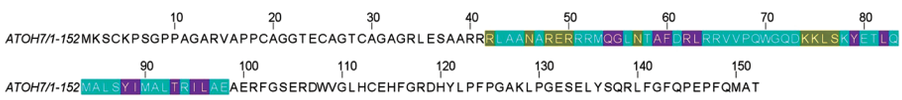

Figure 4 Domains of ATOH7 as predicted by Interproscan and SMART The green highlighted is the bHLH domain. The DNA binding site is highlighted in yellow and the dimer interface residues are highlighted in purple. ATOH7: Atonal basic helix-loop-helix (bHLH) transcription factor 7.

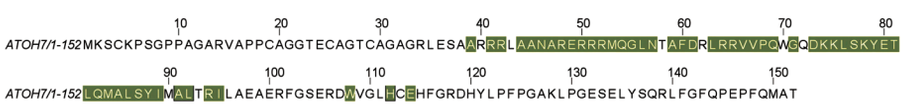

Figure 5 The conservation of ATOH7 The highly conserved residues are highlighted in yellow as predicted by the predictprotein server. ATOH7: Atonal basic helix-loop-helix (bHLH) transcription factor 7.

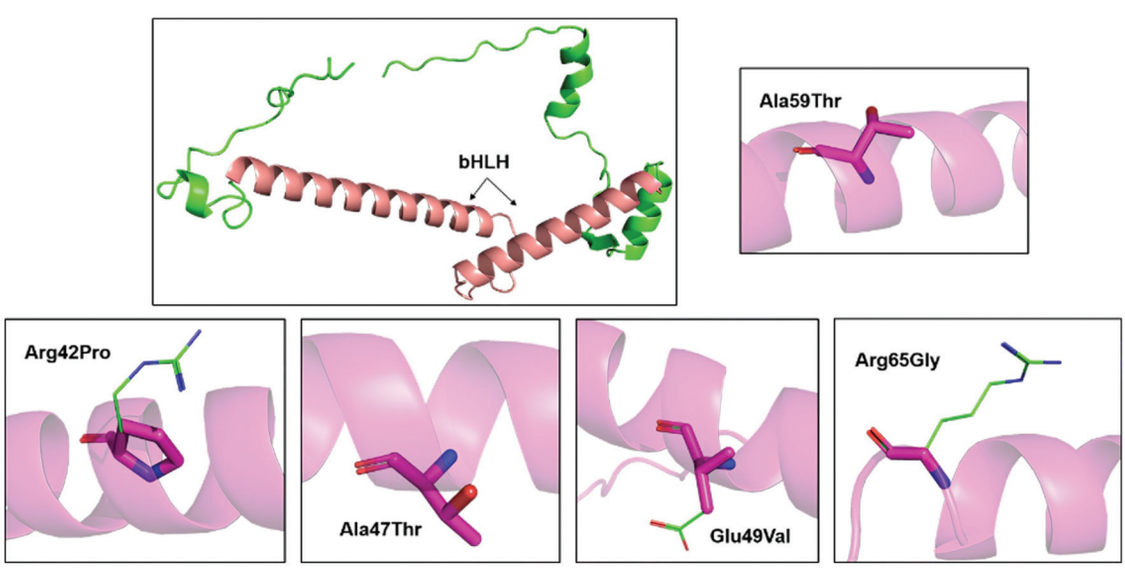

Figure 6 Three-dimensional structure of ATOH determined through trRosetta represented by green Single-nucleotide polymorphisms are represented by purple color. ATOH: Atonal homolog.

allowed region was selected for further analysis. The model suggested that ATOH7 is a disordered protein with an ordered hHLH (basic-helix-loop-helix) domain. There are five single-nucleotide polymorphisms (SNPs, Arg42Pro, Ala59Thr, Ala47Thr, Arg65Gly, and Glu49Val) present in the bHLH domain of the protein (Figure 6). Arg42Pro, Glu49Val is present in the DNA binding domain and may have an impact on the DNA binding of ATOH7. Ala59Thr mutation present at the dimer interface, hence may affect dimerization of ATOH7. The amino acid change from non-polar to positively charged amino acids and *vice versa*, and from positively charged amino acid (Arg) to a small polar amino acid (Gly) may have an impact on DNA binding.

The effect of mutations was further evaluated through the Dynamut web server. The results showed that Arg42Pro, Ala59Thr, Arg65Gly, and Glu49Val are destabilizing the protein structure, whereas the Ala47Thr mutation does not have the destabilizing effect (Table 2).

The effect of c.91delG was observed on the mRNA transcript. The mutation results in nonsense-mediated decay of mRNA as

Table 2 Effect of mutations on protein stability

Mutation	MCSM	SDM	DUET	Delta_stability_encom	Delta_vibrational_entropy	ΔΔG _prediction	Predicted outcome
Arg42Pro	0.031	-1.65	-0.308	-0.036	0.045	-0.126	Destabilizing
Ala59Thr	-1.095	-1.97	-1.117	0	0	-0.563	Destabilizing
Glu49Val	-0.173	-1.48	-0.255	-0.03	0.037	-0.108	Destabilizing
Ala47Thr	-0.955	-1.97	-0.968	0.174	-0.218	0.629	Stabilizing
Arg65Gly	-0.322	-0.36	-0.387	-0.029	0.036	-0.029	Destabilizing

MCSM: Mutation cutoff scanning matrix; SDM: Site directed mutator; DUET: Domain-based uncertainty estimation tool.

predicted through NMDEscPredictor. The c.91delG mutation result in the introduction of a premature stop codon at position 55. The resulting truncated protein no longer has the bHLH domain and hence is not able to perform its function.

DISCUSSION

Our study a novel frameshift deletion/nonsense mutation in *ATOH7* in two unrelated consanguineous families presenting with autosomal recessive ONH, characterized by a broad phenotypic spectrum, including nystagmus, microphthalmia, corneal opacity, and microcornea, keratopathy, partial retinal detachment, andcongenital cataracts.

RGCs are the first-born retinal neurons, and extensive evidence implies that their production of ATOH7 is necessary. RGCs are nearly completely absent in several *in vivo* models due to loss-of-function mutations[8,24]. Patients with mutations in *ATOH7* or its remote regulatory enhancer element exhibit ONH, a phenotype that is also outlined in model animal models harboring analogous genetic disruptions[8]. These abnormalities in the retinal vasculature include retinal neovascularization, insufficient retinal vascularization, and the consequent tractional detachment of the retina[17,19,24]. These alterations, which are believed to follow RGC loss, could be brought about by a reduction in paracrine substances or connections between cells.

Despite the presence of partial retinal detachment in these cases, corneal opacity[25] was more likely attributed to collapse of the anterior chamber rather than direct sequelae of retinal pathology[26]. As a result, the retinal findings in FEVR instances coincide with those in cases of *ATOH7* mutations, both in terms of retinal detachments and vasculature. Furthermore, significant ONH was a persistent feature of eyes with *ATOH7* mutations, which set them apart from eyes with FEVR.

A study by Ghiasvand *et al*[15], firstly investigated the connection of *ATOH7* mutations causative for non-syndromic congenital retinal non-attachment/detachment. Generally, congenital retinal non-attachment, also known as retinal dysplasia, indicates the development of congenital blindness due to congenital retinal detachment[9]. Another study by Khan *et al*[17] also investigated a case with a variable ophthalmological phenotype, including microphthalmia, corneal opacity, severe vitreoretinal dysplasia, nystagmus, microcornea, ONH, persistent fetal vasculature, and congenital cataracts caused by *ATOH7* gene deletion or missense mutations.

In the present study, structural analysis of ATOH7 was carried out by applying different web-based in silico tools. ATOH7 is a 150-amino-acid protein characterized by a basic motif necessary for DNA binding and an HLH domain for dimerization with other family members. Bioinformatic disorder prediction tools reveal that the ATOH7 protein is largely disordered, containing only a short, structured domain within its middle region. The protein is enriched with disorder-promoting amino acids like proline and arginine, as well as flexible amino acids. Structural analysis identified the bHLH domain as highly conserved and suggested that the protein is disordered except for this domain. The presence of specific SNPs within the bHLH domain may affect DNA binding and dimerization. The c.91delG frameshift non-sense mutation causes a premature termination codon, which is characteristically recognized by the nonsense-mediated mRNA decay pathway, resulting in mRNA degradation and reduced protein levels. If nonsense-mediated mRNA decay is incomplete or diverted, translation of the decay mRNA may produce a truncated protein lacking the bHLH domain, thus damaging its function.

Previous studies have reported the unearthing that the ubiquitinated BHLH factors are degraded by proteasomes[25]. It has been demonstrated that dimerization-dependent proteasomal degradation is induced when several human bHLH proteins, including E47, dimerize with the *C. elegans* HLH-2[27]. In another study, it was found that by modifying important helix-1 residues, protein dimerization was undermined and the dimerization-dependent breakdown was removed. It is likely that reduced dimerization results in better stability of bHLH monomer, even if abolished, but reserved dimerization may have the reverse consequence because of deformed dimer shape or just lower chromatin binding. A variety of factors may influence how bHLH dimerization affects protein breakdown.

Another study of the Pakistani population discovered that the two variations may be damaging, as indicated by the HumVar model scores of 0.758 and 0.985. Based on the alignment of orthologous protein sequences, the E49 residue of the *ATOH7*

gene is fully conserved, whereas the S62 residue of the PBLD gene is conserved in mammals and birds but not in zebrafish (Danio rerio) or worms (*C. elegans*). The transcription factor is bound to DNA by a consensus hexanucleotide sequence called the E box at the E49 residue[28], which is located inside the basic motif of the bHLH domain. Computational modeling indicates that the missense mutation is likely to interfere with DNA binding and transcription factor function.

It is assumed that the mutant ATOH7 proteins may behave as dominant-negative proteins similar to the Id family of bHLH factors since they are likely to form heterodimers but remain functionally inactive. As a result, the consequences of these *ATOH7* mutations could extend beyond retinal development, surpassing the scope of this current study. While this paper sheds light on various mechanisms through which transcriptional function loss might manifest in these patient variants, it is crucial to conduct functional assessments within a relevant developmental setting. Utilizing a hiPSC-derived retinal organoid or an animal model would permit a comprehensive exploration of changes in transcriptome, DNA targets, and the morphology of RGCs[19].

In this study, a novel nucleotide variation in *ATOH7*, identified as c.91delG; p. Gly31Glyfs*55, was found in two unrelated consanguineous families exhibiting symptoms of ONH. Computational analyses based on three-dimensional modeling, suggest that this mutation disrupts bHLH domain of the ATOH7 protein, resulting in structural disorder. For affected individuals and their families, a prompt and accurate diagnosis of ONH is crucial, as it forms the basis for genetic counseling. This approach ensures timely identification, enables interdisciplinary groups to support for early childhood development during a critical, developmental window, and provides actionable insights for future management strategies. The study did not explore whether the mutation is a founder effect, suggesting further research in larger datasets may provide a clearer understanding.

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