Original Article

Association of the c.75C>A Variant in CLCC1 with Autosomal Recessive Retinitis Pigmentosa in Pakistan

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ABSTRACT

OBJECTIVE: To identify the disease-causing allele of retinitis pigmentosa, a heterogeneous genetic disorder in a single affected family.

METHODOLOGY: A cross-sectional descriptive study was conducted at the Sindh Institute of Ophthalmology & Visual Sciences Hyderabad from December 2022 to December 2023, with approval from the SIOVS ethical committee. A consanguineous pedigree with multiple affected members was included, while pedigrees with only one affected member or secondary causes of vision loss were excluded. After getting informed consent, each enrolled participant's blood samples (10 cc) were collected, and DNA was extracted. The family was subjected to Sanger sequencing for the CLCC1 gene.

RESULTS: In this study, one reported c.C75A, p.Asp25Glu allele in the CLCC1 gene was identified from an endogamous pedigree in Sindh, Pakistan. The identified c.C75A, p.Asp25Glu allele is a common cause of autosomal recessive retinitis pigmentosa (arRP) in Pakistani-affected individuals. This allele is estimated to have occurred 2000-5000 years ago and has been transmitted to affected individuals of Pakistani origin and global descent across various geographical regions. All the affected patients underwent detailed clinical investigations, including fundus photography and optical coherence tomography, to confirm the retinitis pigmentosa symptoms. The Sanger sequencing method was used to detect pathogenic variants, and bioinformatics tools were utilized to investigate the pathogenesis of identified alleles and compare phenotype-genotype correlations.

CONCLUSION: The finding of frequent disease-causing alleles from Pakistani-affected patients will significantly improve existing genetic databases and facilitate more accurately the affected diagnosis of gene testing.

KEYWORDS: CLCC1 gene, night blindness, endogamous pedigree, genetic counseling, Sanger sequencing, phenotype-genotype correlation

INTRODUCTION

Retinitis pigmentosa (RP) is a rare set of clinically and genetically heterogeneous disorders; RP impairs the light-sensitive rod cells in the retina, causing progressive rod cell degeneration that results in low light and peripheral vision. Retinitis pigmentosa is expressed differently in isolated individuals[1]; some lose eyesight more quickly, while others experience gradual vision loss. RP can also be linked to additional syndromic disorders such as developmental delays or hearing loss[2].

Retinitis Pigmentosa (RP) is a primary cause of hereditary blindness that varies frequently among different demographic groups. According to data from the United States and Europe, the prevalence of RP is approximately 1 in 4,000 people worldwide[3]; the highest frequency was found in rural South India, where it was reported to be 1 in 372[4]. In Pakistan, the prevalence of RP is not reported, but according to a hospital-based study, 20% of students attending blind schools have this condition. Numerous factors, such as geographic location, ethnic background, and the specific genetic modifications at work, can affect these figures[5]. Variable populations may have variable incidence rates for RP, a genetically heterogeneous disorder with diverse inheritance patterns, and certain populations may have a higher frequency of specific genetic variations associated with RP, making them more susceptible to the disorder.

At least 150 mutations were identified in nineteen genes with phenotypes manifesting RP-associated symptoms[6]. Non-syndromic RP can be inherited in

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three different ways: autosomal dominant in roughly 30–40% of instances, autosomal recessive in 50%–60% of cases (the most common hereditary RP globally), or X-linked in nearly 5%–15% of cases[7]. There have also been isolated reports of the digenic or mitochondrial modes of inheritance. The most frequent forms of syndromic RP are Usher syndrome, which is the most common cause of collective blindness, deafness and Bardet-Biedl syndrome, which RP characterizes along with polydactyly, obesity, renal abnormalities, hypogenitalism, and mental retardation.

Retinitis pigementosa is a heterogeneous disorder, and c.75C > A allele is a moderately common cause of arRP in the Pakistani population. Identifying c.75C > A allele associated with RP helps better understand the underlying genetic mechanisms driving the disease. Information provides insights into disease progression, potential treatment targets, and personalized medicine approaches.

METHODOLOGY

Ascertainment and clinical evaluation of families

A cross-sectional descriptive study was carried out after approval from the ethical review committee of the Sindh Institute of Ophthalmology & Visual Sciences from December 2022 to December 2023. The consanguineous families manifesting the symptoms of night blindness and consisting of more than one affected were included, and the pedigree consists only one affected or secondary cause of blindness was excluded. This research identified over twenty affected families from different Eye Hospital in Sindh. Each affected patient underwent a clinical examination held by relevant supporting ophthalmology staff; the detailed history and clinical examination, including fundus examination and optical coherence tomography, were performed. After informed consent was obtained, 10cc of Venous blood sample was taken and placed in a 50ml Falcon tube containing 200ul of 0.5M EDTA. DNA of all available participants in the pedigree was extracted through an optimized organic method[8], and 25ng dilution was prepared for PCR amplification.

Mutation Screening and Bioinformatics Analysis

Using the open-source Primer3 tool program, the sequencing primers for the CLCC1 gene were developed and optimized according to different annealing temperatures. The forward and reverse boundaries of the CLCC1 gene were amplified on all individuals available in pedigree using previously described techniques[9]. Chromas v.2.5 was used to retrieve and evaluate Sanger sequencing results. Phyre2 was used to generate the 3D structures of the CLCC1 protein to distinguish it from the mutant protein, Ramachandran plots were created to determine the quality of the protein's three-dimensional structure by observing torsion angle values, and Clustal omega W was used to observe amino acid conservation alignment in different homologue species. These bioinformatics tools helped to determine the pathogenicity of the identified variants and to understand the potential impact on health.

RESULTS

The RP-01 family was registered from a remote location in Sindh, Pakistan and enrolled from tertiary care facility Layton Rahmatulla Benevolent Trust (LRBT) Rashid Abad. The clinical examinations identified the retinal degeneration condition in the family. Each affected individual displayed retinal pigmentosa symptoms, and the family did not exhibit any association with any other anomaly. Sanger sequencing was performed on one affected member of this family; the c.C75A, p.Asp25Glu mutation was found in RP-21 (**Figure 1B**), and segregation of the identified mutation was confirmed on remaining individuals (**Figure 1A**). Several bioinformatics tools were used to assess the potential impact of variation. The Clustal-W tool was used to analyze the conservation of amino acids; aspartate is a highly conserved residue across various homologous species (**Figure 1C**). The c.C75A alteration was predicted using bioinformatics proficiency tools; all tools supported this transitory change. Polypehn2 scores a 1 for this change, indicating it is likely harmful, with a Provean score of -3.00 and mutation evidence supporting variation c.C75A as a disease-causing mutation. The phrye2 tool results show the wild and mutant protein changes in the CLCC1 gene (**Figure 1D**)

After a comprehensive clinical examination, the fundus examination of patients III–5 showed a mildly pigmented free zone in the distant periphery of both eyes and a minor spread of pigmentation in that same area (**Figure 2A**). The fundus of individuals III–6 showed large pigmentations in the far peripheral region of the right eye, whereas little pigmentations are present in the mild area; in the left eyesight of individuals III–6, the fundus contains mild pigmentation in the periphery region, a macular clear from pigmentation and a slight pigmentation in the vast periphery region were seen (**Figure 2B**). In individuals III-7, the pigmentation is apparent in the far peripheral area, and a mild left eye region was transparent from pigments. In contrast, in the right eye of individual III-7, the mild region of the fundus was clear from pigmentation, and the far peripheral region of the eye was foggy and condensed (**Figure 2C**). Optical coherence tomography (OCT), a diagnostic test, was performed to analyze the retinal thickness and the condition of the retinal pigment epithelial layer among the individuals. The OCT result of individual III-5 reveals that both eyes' retinal pigment epithelial layer was smoothly settled with retinal thickness measurements of both eyes 213 µm and 220 µm, and the standard average measurement was 269 µm (**Figure 2A**). The OCT result for individuals III-6 indicated that both eyes were abnormally thick; the

thickness values obtained for each eye were 245 µm and 235 µm, respectively, and the average thickness value was 228 µm and retinal pigment epithelial layers were scattered in both eyes (**Figure 2B**). In individuals III-7, the retinal pigment layer was lightly settled. The OCT thickness value was recorded at 197µm and 214µm in both eyes of individuals III-7, and the typical average value of both eyes was 219µm (**Figure 2C**).

Figure I: (1A) showing the pedigree of RP-01 (1B) chromatogram results of c.C75A, p.Asp25Glu, (1C) Clustal-W results of different homologues species, (1D) Phyre2 results of wild and mutant protein of Asp25Glu

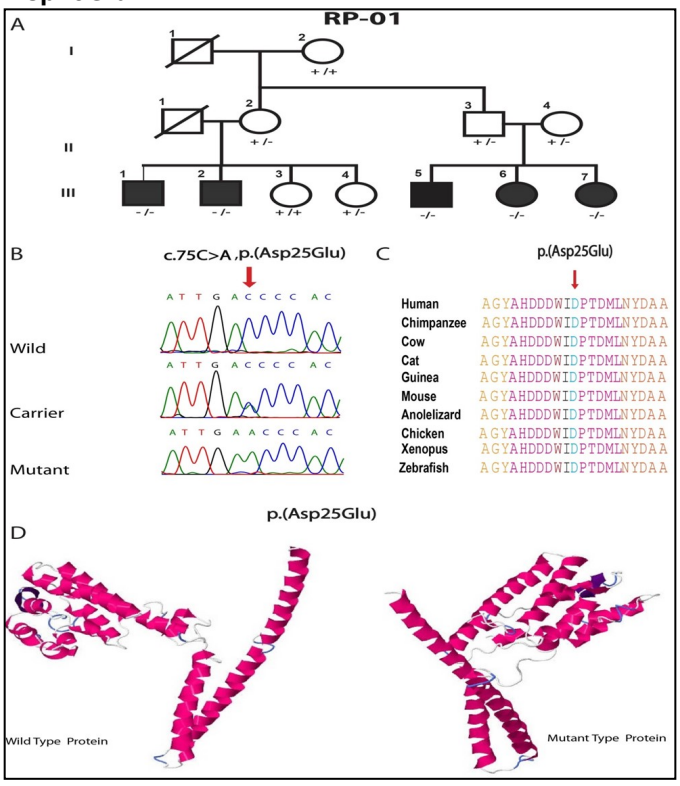
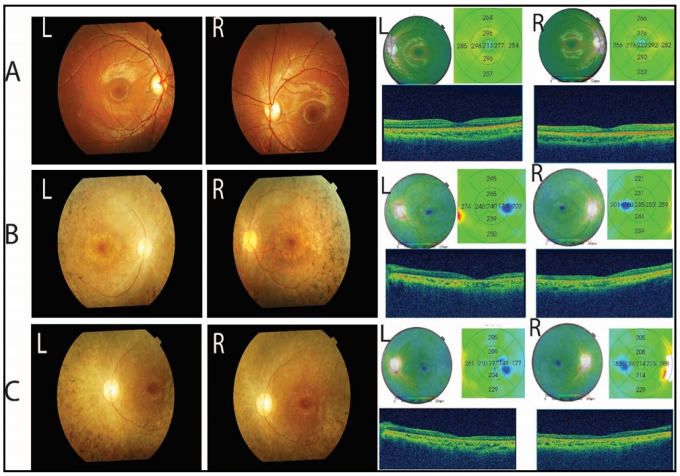


Figure II: (2A) fundus and OCT photographs of III-5, (2B) fundus and OCT photographs of III-6, (2C) fundus and OCT photographs of III-7



DISCUSSION

Chloride channel CLIC-like protein 1 regulates cellular trafficking signals and plays an essential role in retinal structure. In addition, blockage of CLC Cl- channels in mice results in retinal degeneration[10]. Cl-channels promote aberrant lysosomal trafficking function, which causes retinal degeneration by stimulating the ions inside the endosomal-lysosomal pathway's medium[10].

The association of CLCC1 to cause retinal degeneration disorder was first reported in Pakistan. A homozygous missense mutation c.75C>A, p.D25E of CLCC1 was found in seven consanguineous families of the Pakistani population and one British-Bangladeshi family. This mutation was associated with arRP; the p.Asp25Glu mutation reduced CLCC1 channel function; additionally, mutant CLCC1 protein accumulated in granules within the endoplasmic reticulum (ER) lumen, suggesting a disruption in protein processing or trafficking[11].

The Knockdown of CLCC1 mRNA in cultured retinal pigment epithelium (RPE) cells induced apoptosis, identified the crucial role for CLCC1 in cell survival and TALEN knockout (KO) of CLCC1 in zebrafish resulted in lethality by eleven days post-fertilization. Zebrafish KO showed no satisfactory electroretinogram (ERG) cone and cone spectral sensitivity response, reduced eye size, retinal thickness, and expression of rod and cone opsins and Injection of wild-type CLCC1 mRNA rescued the phenotypic defects in zebrafish KO. Clcc1+/- KO mice showed abnormal ERG results and photoreceptor numbers; the CLCC1 is crucial for maintaining retinal integrity and function. These findings indicate that intracellular chloride transport mediated by CLCC1 maintains retinal integrity and function. CLCC1 is essential for the survival and function of normal retinal cells, highlighting its potential as a therapeutic target for arRP and possibly other retinal degenerative disorders[11].

The exon 1 of the CLCC1 gene contains a substitution variation, c.C75A, which causes the missense variation p.Asp25Glu. The encoded protein of the CLCC1 gene is 551 amino acids long and comprises ten exons totalling 4.83 kilobases in size. According to data from the gnomAD database (http://gnomad.broadinstitute.org/), the allele frequency of the variant c.C75A, p.Asp25Glu, is 0.000056, indicating a shallow frequency of the abovementioned variant (https://gnomad.broadinstitute.org/gene/ENSG00000121940?dataset=gnomad_r4)[12]. Additionally, the CLCC1 gene's probability of LOF intolerance (PLI) was determined using the method, and the PLI score for CLCC1 was found to be 0.03. Furthermore, neither the HGMD nor the ExAC databases (https://gnomad.broadinstitute.org/gene/ENSG00000121940?dataset=exac) database. According to bioinformatics tools, the variation is likely

pathogenic and responsible for protein abnormalities. Additionally, it was utterly segregated from the familial phenotype. For a better understanding of the activity of the CLCC1 gene in human eyes and the impact of its variations on the development of RP, in vitro and animal-based functional investigations are crucial.

The c.75C > A CLCC1 mutation is a moderately common cause of autosomal recessive retinitis pigmentosa (arRP) in the Pakistani population; it is essential to note that this figure reflects only a part of the genetic causes contributing to this condition in the Pakistan population. A thorough clinical examination revealed the typical symptoms of retinitis pigmentosa, typically present in people with RP with mutations in other genes. The condition usually manifests itself in the second decade of life. The youngest patient, III–5, had good vision and no complaints, but a fundus examination revealed mild pigmentation, which may indicate the beginning stages of RP.

According to the earlier study, sharing an identical single nucleotide polymorphism (SNP) haplotype among affected individuals from seven consanguineous families of Pakistan and one British-Bangladeshi family indicated a common ancestral mutation. The frequency estimation of 0.03 is derived from the EM algorithm within the Golden Helix SVS program. A previous study further supports that the British-Bangladeshi family shares an identical haplotype, which indicates that the family is probably of a Punjabi Pakistani ancestor. The findings regarding the CLCC1 mutation in the Pakistani population offer valuable insights into its origin and transmission. The estimated timeframe of 2000–5,000 years ago suggests that this pathogenic variation emerged relatively recently in human history, possibly coinciding with significant historical background or population movements in the region[13]. The fact that affected families of Pakistani descent are found in various areas globally highlights the dispersal of this genetic variant over time, likely through migration and intermarriage.

Furthermore, the absence of further pathogenic variation in the CLCC1 gene shows a high degree of genetic conservation; this indicates that the CLCC1 gene is highly involved in biological processes, such as embryonic development, where small changes may have caused significant effects. Deciphering the significance of this gene variation for human development and health necessitates an understanding of its functional requirements.

CONCLUSION

This finding of a pathogenic allele in this family provides information to better understand the disease pattern at a molecular level, which can significantly aid in genetic counseling and the development of targeted treatments for affected patients. By identifying the specific mutations and their phenotypic effects, the consultant professionals can better guide the affected families in Pakistan. Additionally, this data contributes to understanding the allele frequency of the identified mutation, which is crucial for epidemiological studies and future research endeavors.

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AUTHOR CONTRIBUTION

Waryah YM: Study design, collection of data and drafting,
Khidiri FF: Critical review
Ansari A: Help in re-identification of family and review of article
Mehmood S: Data analysis
Abbasi S: Help in clinical chemistry investigations
Memon S: Help in clinical analysis

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