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RESEARCH ARTICLE

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Identification of novel homozygous variants in *FOXE3* and *AP4M1* underlying congenital syndromic anophthalmia and microphthalmia

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Abstract

Background: Anophthalmia and microphthalmia are severe developmental ocular disorders that affect the size of the ocular globe and can be unilateral or bilateral. The disease is found in syndromic as well as non-syndromic forms. It is genetically caused by chromosomal aberrations, copy number variations and single gene mutations, along with non-genetic factors such as viral infections, deficiency of vitamin A and an exposure to alcohol or drugs during pregnancy. To date, more than 30 genes having different modes of inheritance patterns are identified as causing anophthalmia and microphthalmia.

Methods: In the present study, a clinical and genetic analysis was performed of six patients with anophthalmia and microphthalmia and/or additional phenotypes of intellectual disability, developmental delay and cerebral palsy from a large consanguineous Pakistani family. Whole exome sequencing followed by data analysis for variants prioritization and validation through Sanger sequencing was performed to identify the disease causing variant(s). American College of Medical Genetics and Genomics (ACMG) guidelines were applied to classify clinical interpretation of the prioritized variants.

Results: Clinical investigations revealed that the affected individuals are afflicted with anophthalmia. Three of the patients showed additional phenotype of intellectual disability, developmental delays and other neurological symptoms. Whole exome sequencing of the DNA samples of the affected members in the family identified a novel homozygous stop gain mutation (NM_012186: c.106G>T: p.Glu36*) in Forkhead Box E3 (*FOXE3*) gene shared by all affected individuals. Moreover, patients segregating additional phenotypes of spastic paraplegia, intellectual disability, hearing loss and microcephaly showed an additional homozygous sequence variant (NM_004722: c.953G>A: p.Arg318Gln) in *AP4M1*. Sanger sequencing validated the correct segregation of the identified variants in the affected family. ACMG guidelines predicted the variants to be pathogenic.

Warda Akbar and Asmat Ullah contributed equally to this work.

Conclusions: We have investigated first case of syndromic anophthalmia caused by variants in the *FOXE3* and *AP4M1*. The present findings are helpful for understanding pathological role of the mutations of the genes in syndromic forms of anophthalmia. Furthermore, the study signifies searching for the identification of second variant in families with patients exhibiting variable phenotypes. In addition, the findings will help clinical geneticists, genetic counselors and the affected family with respect to prenatal testing, family planning and genetic counseling.

KEYWORDS
anophthalmia, *AP4M1*, *FOXE3*, novel variants, sensory processing disorder, whole exome sequencing

1 | INTRODUCTION

Congenital anophthalmia and microphthalmia are rare developmental eye defects that lead to the absence or reduction of one or both ocular globe, occuring as a result of a mutation in eye development during the first 8 weeks of life.[1] To date, the combined prevalence of anophthalmia and microphthalmia has been reported to be 0.32–0.4 per 10,000 live births.[2] Based on the absence of ocular structures, congenital anophthalmia is classified as primary, secondary and degenerative anophthalmia.[3] Primary anophthalmia occurs because of a defect in optic pit formation and optic outgrowth from the forebrain during the early 4 weeks of gestation. It results in the absence of lens, optic nerve and chiasma. Secondary anophthalmia occurs by orbital growth arrest, which leads to an absence of extraocular muscles and a decrease in the optic foramen size.[4] In degenerative anophthalmia, ocular development occurs initially but subsequently starts to degenerate.[5] Depending upon the physical appearance of the ocular globe and the reduction of axial length, microphthalmia is also categorized as severe, simple and complex.[6]

Anophthalmia and microphthalmia can occur in isolation or associated with other ocular disorders.[7] The most common ocular disorder associated with anophthalmia and microphthalmia is "coloboma", which is an abnormal closure of ocular fissures in the same or contralateral eye.[8] In rare cases, the disorder is also associated with anomalies of organs other than eyes. For example, in Waardenburg anophthalmia syndrome, anophthalmia is associated with osseous synostosis, ectrodactylism, polydactylism and syndactylism.[9] Similarly, in the case of frontonasal dysplasia, the ocular phenotypes of microphthalmia or anophthalmia are associated with an abnormal development of the head and face.[10,11]

Anophthalmia and microphthalmia may be caused either by genetic or non-genetic factors. The genetic factors include chromosomal aberrations, copy number variations and single gene mutation. It may result from a single gene mutation and may be inherited in an autosomal recessive, autosomal dominant or X-linked manner.[12] The non-genetic factors include virus infection, exposure to drugs and deficiency of vitamin A during early pregnancy.[13]

The major reported genes associated with anophthalmia and microphthalmia include *SOX2* (MIM#184429), *OTX2* (MIM#600037), *RAX6* (MIM#601881), *PAX6* (MIM#607108), *STRA6* (MIM#610745), *ALDH1A3* (MIM#600463), *RARB* (MIM#180220), *VSX2* (MIM# 142993), *FOXE3* (MIM#601094), *BMP4* (MIM#112262), *BMP7* (MIM#112267), *GDF3* (MIM#606522), *GDF6* (MIM#601147), *ABCB6* (MIM#605452), *ATOH7* (MIM#609875), *C12orf57* (MIM#615140), *TENM3* (MIM#610083), *VAX1* (MIM#604294), *SALI2* (MIM#602219) and *YAP1* (MIM#606608).[14] The *SOX2* gene mutation is the most common cause of anophthalmia and microphthalmia phenotypes and contributes to approximately 15%–20% of cases in individuals.[15] All other genes are rarely affected. To date, the genetic cause of approximately 50%–60% of the cases remains unknown.[16]

In the present study, we recruited a consanguineous family aiming to determine the genetic cause of the disease using whole exome sequencing.

2 | MATERIALS AND METHODS

2.1 | Family recruitment and ethical statement

The study was approved by Institutional Review Board (IRB) of Quaid-i-Azam University, Islamabad, Pakistan (RB-QAU-177) and Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad, Pakistan (No. F1-1/2015/ERB/SZABMU/808). Before collection of blood samples, all participants or their guardians signed an informed consent form. The consanguineous family (ND-20) under study belongs to Khyber Pakhtunkhwa province of Pakistan. A detailed pedigree was designed after obtaining the information by interviewing the family members, which reveals the mode of inheritance of the disease and the number of affected individuals in the family. Informed consent including presentation of photographs for publication was obtained from all the participants or their parents. Peripheral blood was collected from six affected (IV-2, IV-9, IV-11, IV-12, IV-14 and IV-15) and five unaffected (III-3, III-9, III-10, III-13 and III-16) individuals of the family in ethylenediaminetetraacetic acid-containing vacutainer sets. The diagnosis of the disease was confirmed after obtaining medical reports and family history along with the on-spot examination by optometric physician.

2.2 | Whole exome sequencing and in silico analysis

Two affected members (IV-11 and IV-14) of the family were subjected to whole exome sequencing. For this, genomic DNAs of the affected members of the family was prepared for exome sequencing in accordance with the instructions of the SureSelect Target Enrichment Kit (Agilent, Santa Clara, CA, USA) and libraries were prepared by using biotinylated DNA oligonucleotides (SeqCap EZ Human Exome Library, version 3.0; Roche Nimblegen, Madison, WI, USA). The sequencing was performed on an Illumina platform (Illumina, Inc., San Diego, CA, USA). This method of sequencing used the SureSelect, version 6, capture kit in accordance with the manufacturer's instructions. The analysis and alignment of the sequencing reads were performed using the Burrows–Wheeler Aligner (BWA) tool and a Genomic Analysis Toolkit (GATK)[17,18] was used for variant calling. For the annotation of these called variants, we utilized ANNOVAR.[19]

Based on consanguinity and inheritance pattern of the disease in pedigree, homozygous and compound heterozygous variants were selected during exome data analysis. The variant selection criteria include variants shared by exomes of both the patients, having minor allele frequency < 0.001 in gnomAD, CADD-Phred score > 20, exonic variants and splice sites (±12 bp).

Variants that were previously reported benign or likely benign in ClinVar were excluded. The variants were screened that were deleterious and rare, with their expression in eye tissues or central nervous system having been reported. A list of homozygous variants was analyzed to prioritize homozygous pathogenic variants, whereas the other list of heterozygous variants was searched for compound heterozygous variants. The selected variants were checked for validation and segregation via Sanger sequencing using DNA of all available family members. After the prioritization of sequence variants shared by both the affected individuals, exome data of patient IV-14 was re-analyzed for homozygous pathogenic variants that are either absent or heterozygous in the exome of IV-11 (see Supporting information, Table S1).

For Sanger sequencing, primers were designed using Primer 3 software (https://primer3.ut.ee). PCR amplification and Sanger sequencing were performed using standard protocols. Reference sequences of the genes were downloaded from ensemble genome browser (https://www.ensembl.org/index.html). Sanger sequencing results were aligned to the reference sequence using BIOEDIT sequence alignment editor, version 6.0.7 (Ibis Biosciences, Carlsbad, CA, USA).

2.2.1 | In silico analysis 3D protein structure modelling

Clinical significance of the identified variants was interpreted using American College of Medical Genetics and Genomics (ACMG) (https://wintervar.wglab.org/url.php) guidelines. The selected variants were screened in several databases including gnomAD (https://gnomad.broadinstitute.org), Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) and 130 in-house exomes of healthy control individuals.

The AlphaFold2 algorithm (https://alphafold.ebi.ac.uk) was utilized to predict the in silico structures of both the wild-type and mutant FOXE3 proteins, which were subsequently visualized using PyMol v2.4 (https://pymol.org/2). For the prediction of genetic and physical interactions of FOXE3 with proteins and genes, we utilized the GeneMANIA (https://genemania.org). Evolutionary conservation of the mutated amino acid (arginine) in AP4M1 in orthologs was examined using HomoloGene (https://www.ncbi.nlm.nih.gov/homologene).

3 | RESULTS

3.1 | Clinical characteristics

The pedigree of the present family showed six patients afflicted with bilateral anophthalmia/microphthalmia. Three of these patients segregated additional phenotypes of cerebral palsy, hearing loss and behavioral issues in two different loops (Figures 1 and 2).

Individual IV-9 was a 20-year-old male at the time of recruitment. He was delivered via spontaneous vaginal delivery (SVD) at term with immediate cry. He was vaccinated according to Expanded Programme on Immunization (EPI) schedule. There were no maternal risk factors. He is developmentally normal with no vision sense. His speech and hearing is intact. On examination, there was bilateral anophthalmia with no other positive findings (Figure 2).

Patient IV-11 was a 16-year-old female at the time of recruitment. She was delivered via SVD at term with immediate cry and vaccinated according to EPI schedule. She is developmentally normal but affected with bilateral anophthalmia. She has no vision sense with normal hearing and speech. She had never admitted to hospital before. On examination, she was obeying commands and talking relevantly. She does not have obvious facial dysmorphisms. All her limbs and joints are normal in anthropometry. Systemic examination was unremarkable, and all reflexes were elicitable.

Individual IV-12 was a 13-year-old female at the time of recruitment. She was born with congenital bilateral anophthalmia. She is developmentally normal. She does not have any other facial, dermatological or limbs abnormality.

Patient IV-14 was an 8-year-old male at the time of recruitment. He was born at full term with normal delivery and an immediate cry after birth. He was vaccinated as scheduled by the obstetricians. There were no natal, antenatal or postnatal risk factors for anophthalmia and global development. He was born with bilateral anophthalmia with small palpable fissures. He is globally delayed child with neck-holding achieved at 9 months and sitting without support at 13 months of age. He is afflicted with hearing loss and vision since birth, which is progressively increasing in the form of fits and behavioral problems. He is affected with aphasia and unable to carry out his routine activities and feed himself. He has been admitted to our ward multiple times because of intractable fits and nutritional anemia. On

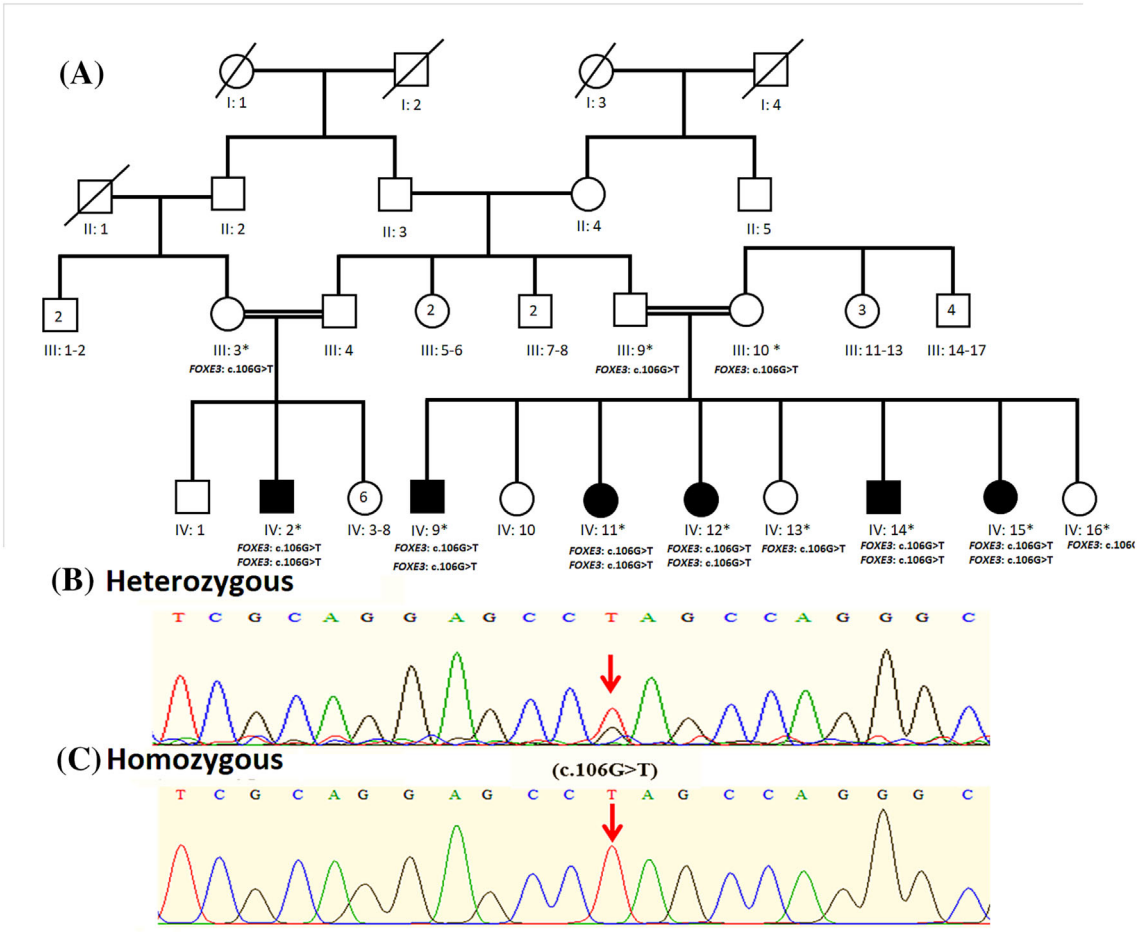

FIGURE 1 (A) Pedigree of the family. The rectangles showed males and the circles females. The filled rectangles and circles represent affected members however; unfilled rectangles and circles represent unaffected members. An asterisk (*) represents the ndividuals who participated in the research study. (B–C) Sanger sequencing chromatogram of *FOXE3* gene showing the identified variant in heterozygous and homozygous states, respectively.

examination, he was an irritable emaciated child. He is hyper pigmented with all normal joints and no contractual formations. Systemic examination is unremarkable. Magnetic resonance imaging (MRI) findings of the patient showed altered signal intensity areas in the left anterior temporal lobe following cerebrospinal fluid signal along with focal T2 weighted imaging/fluid attenuated inversion recovery hyperintense signals in the adjacent parenchyma in keeping with gliosis. The interhemispheric fissure is centered on the midline. The central ventricles are of normal size and symmetrical with normal circulation of cerebrospinal fluid. There are no signs of increased intracranial pressure. The cortex and white matter showed normal development and normal signal intensity, especially in the periventricular white matter. No abnormality was seen in the basal ganglia, internal capsule, corpus callosum or thalamus. The brain stem and cerebellum showed no abnormal changes in signal characteristics. The sella and pituitary are normal, and parasellar structures are unremarkable. The cerebellopontine angle area appears normal on each side. The internal acoustic meatus has normal width. Visualized paranasal sinuses and mastoid air cells showed normal development and pneumatization. Bilateral orbits are small in size. The overall impression drawn from the MRI findings was of altered signal intensity areas in the left anterior temporal lobe, indicative of gliosis. Additionally, features of volume loss were observed, which could be sequelae of a previous traumatic ischemic insult.

Patient IV-15 was a 7-year-old female at the time of recruitment. She was delivered through normal delivery with immediate cry. There were no maternal risk factors and no natal, antenatal and post-natal complications. She was born with bilateral anophthalmia. She is globally delayed child. She has only achieved sitting without support so far. Her disease is progressively increasing in the form of poor food intake and behavioral problems. She is unable to perform her routine tasks. She has been admitted to the hospital multiple times due to nutritional anemia and has had several visits to the outpatient department for chest infections. On examination, she is microcephalic child with hearing loss and aphasia, irritable with emaciated look and hyper pigmented. Her limbs are normal with no contractures. There is no facial dysmorphisms, obvious asymmetry or scar marks. Her systemic examination is unremarkable. All reflexes are illimitable.

These patients have another affected cousin (IV-2), who is 7 years old. He is developmentally delayed with behavioral issues and hearing

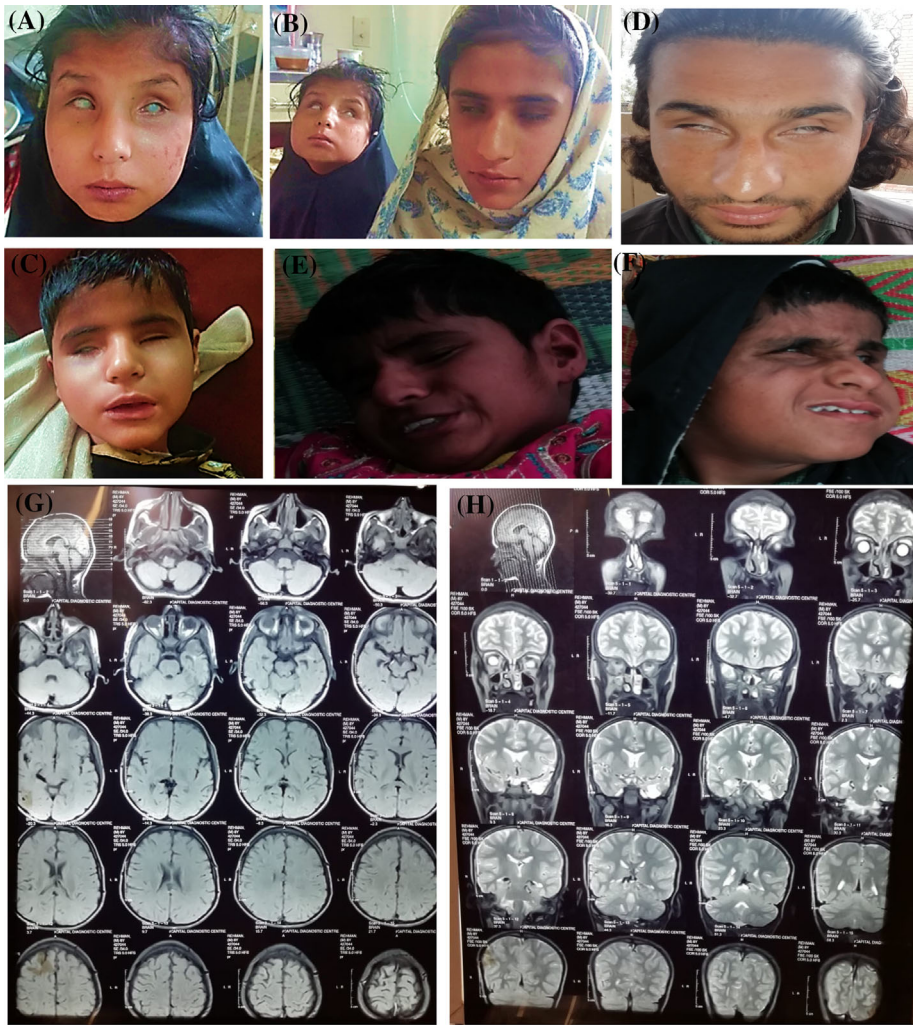

FIGURE 2 (A–F) Clinical features of three affected individuals of the family. (A–D) Showing bilateral anophthalmia and microphthalmia. The affected individuals have variable degree of cerebral palsy. (G–H) MRI of patient IV-14 showed altered signal intensity areas in the left anterior temporal lobe following cerebrospinal fluid signal along with focal T2 weighted imaging/fluid attenuated inversion recovery hyperintense signals in the adjacent parenchyma in keeping with gliosis.

loss. He is not able to stand without support even at the age of 7 years. He has vision impairment since birth. He cannot support himself in his daily life activities. He has behavioral problems and cannot even speak a few words.

Parents of the affected individuals are phenotypically healthy. Both the loops have consanguinity; therefore, the pedigree was concluded to segregate the disease in autosomal recessive form. Variability in the severity of phenotypes was observed in the patients of present family.

3.2 | Whole exome and sanger sequencing

The exome data analysis of the family revealed 19 rare variants in candidate genes shared by both affected individuals (IV-11 and IV-14). A stop gain variant (c.106G>T: p.Glu36*) in the *FOXE3* gene segregated with the phenotype in family. The p.Glu36* variant is neither found in gnomAD, nor the 1000 genomes browser (https://www.internationalgenome.org). Moreover, this variant is not reported in the ClinVar database and Human Gene Mutation Database. The CADD_phred score of the variant was 35. None of the 130 in-house exomes of matched control individuals was found to be homozygous for the identified variant in *FOXE3*. ACMG guidelines predicted the variant (p.Glu36*) in *FOXE3* to be disease-causing. In silico analysis using the GeneMANIA network showed that *FOXE3* has strong genetic and physical interactions with *ZEB2* and *MAB21L1* (Figure 3D).

The affected individual IV-14 has addition neurological phenotypes associated with anophthalmia. Therefore, we analyzed the exome data of both individuals for the identification of variant(s) underlying the additional phenotypes. Reanalysis of the exomes of both patients for homozygous variants that are only present in IV-14 revealed a pathogenic variant c.953G>A: p.Arg318Gln in *AP4M1*. ACMG guidelines predicted the identified variant pathogenic for the disease phenotypes in the present patients (IV-14).

3.2.1 | In silico protein structural modeling

Upon molecular visualization of wild and mutant FOXE3 protein, we found that the novel stop gain variant terminates the translation at position 36 resulting in the truncation of protein (p.Glu36*), as shown

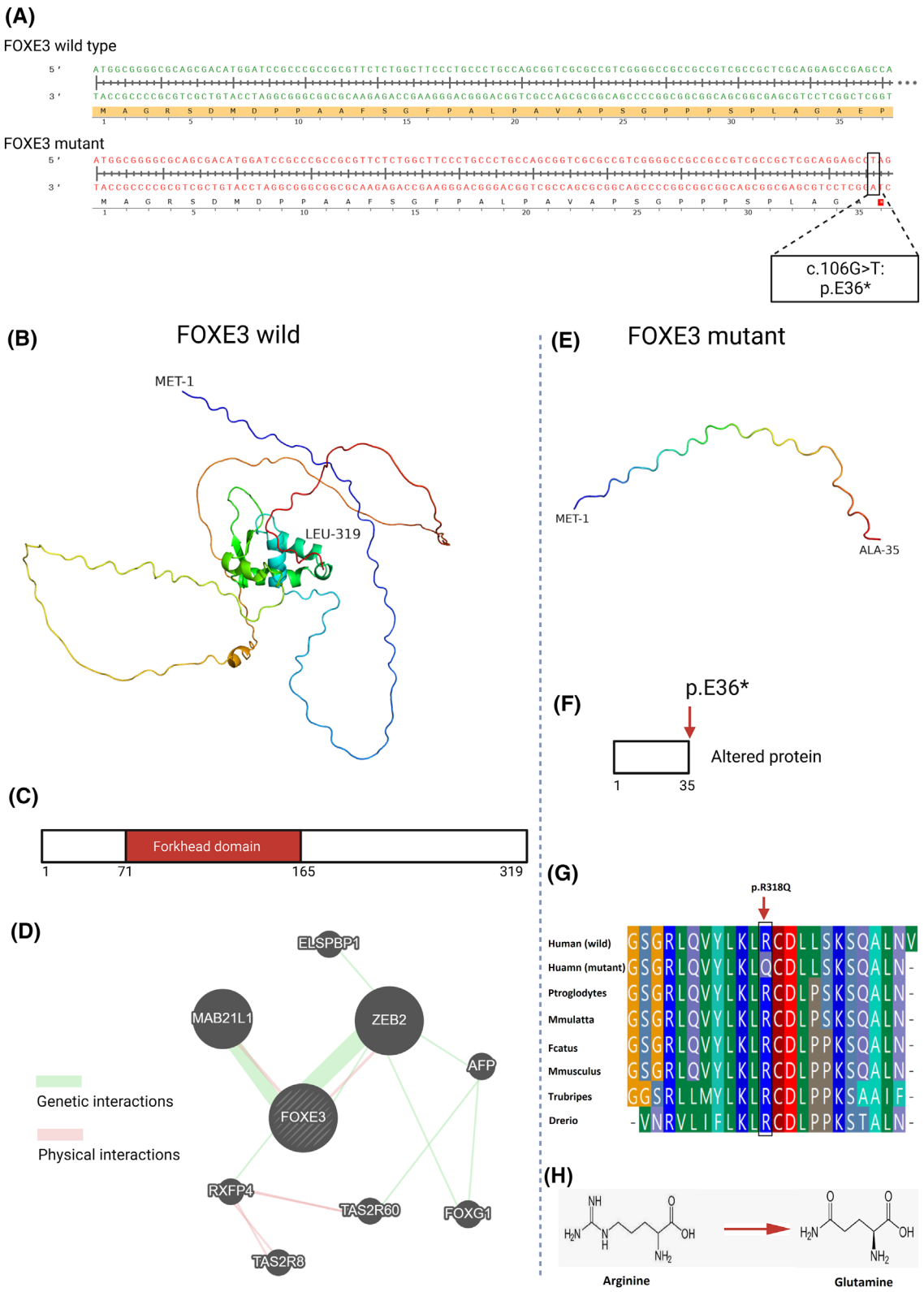

FIGURE 3 In silico molecular characterization of FOXE3 wild-type and mutant proteins. (A) cDNA and amino acid sequences of FOXE3 wild-type and mutant proteins indicating the point of mutation c.106G>T and p.Glu36*. (B, E) Predicted 3D structure of wild-type FOXE3 protein with 319 amino acids and mutant FOXE3 protein with only 35 amino acids. (C, F) Showing the forkhead domain of the wild-type FOXE3 and the mutant with p.Glu36* mutation consequently lacking the forkhead domain. (D) Genetic and physical interaction network of FOXE3 with other proteins and genes. (G) Arginine (R) within the box in AP4M1 protein indicates a conserved residue across different species. (H) Structures of amino acids arginine and glutamine.

in Figure 3. The protein resulting from a premature termination codon brings about structural changes in the protein, leading to a shorter version of the FOXE3 protein that might not be able to perform its proper function and may affect downstream signaling. To determine relatedness among the genes, GeneMANIA network analysis showed that *FOXE3* has strong genetic and physical interactions with *ZEB2* and *MAB21L1*. The arginine at amino acid position 318 (p.Arg318Gln) is completely conserved among various species (Figure 3G).

4 | DISCUSSION

Anophthalmia and microphthalmia are the most severe developmental eye disorders that occur as a result of mutations in a small percentage of genes and still 50%–60% of anophthalmia and microphthalmia cases need to be determined.[12,16] In the present study, we analyzed a consanguineous family of Pakistani origin having clinical features of anophthalmia/microphthalmia associated with cerebral palsy, hearing loss, behavioral issues and global developmental delay. Analysis of the exome data followed by a segregation analysis using Sanger sequencing revealed a novel homozygous stop gain variant [NM_012186; p.Glu36*] in the *FOXE3* gene. The variant segregated with the disorder within the family. Previously, sequence variants in the gene have been reported in isolated anophthalmia.

The affected individual IV-14 showed additional phenotypes of developmental delay, intellectual disability, sensorineural hearing loss, aphasia, behavioral issues and cerebral palsy. Therefore, reanalysis of the exomes for additional homozygous pathogenic variants in IV-14 led to the identification of c.953G>A: p.Arg318Gln in *AP4M1*. The identified variant c.953G>A in *AP4M1* was present in heterozygous form in IV-11.

AP4M1-encoded protein is a member of the heterotetrameric AP-4 complex. The complex is involved in intracellular transport of proteins having a unique role in neurons. The identified variant c.G953A in *AP4M1* leads to the replacement of arginine at amino acid position 318 to glutamine p.R318Q of AP4M1 protein. Unlike positive charge on arginine, the glutamine is uncharged. The conversion of a positively charged amino acid to an uncharged amino acid may affect intra- and inter-molecular interactions of the AP4M1 protein. Structure wise, arginine has long side chain, whereas glutamine has a shorter side chain (Figure 3H). Because of the different chemical properties of the two amino acids, the present variation may affect the protein's structure, stability and interactions with other molecules. This may lead to disruption of AP-4 complex. The formation of the disrupted complex because of a mutated AP4M1 could lead to defects in vesicle trafficking, affecting protein sorting and cellular processes. A homozygous nonsense variant at the same amino acid (c.952C>T (p.Arg318Ter)) in *AP4M1* in a Turkish family caused spastic tetraplegia, severe intellectual disability, stereotypic laughter, limited or absent speech, microcephaly and seizures.[20] Based on these findings, we hypothesize that the family under study has segregated two different disorders (anophthalmia/microphthalmia and spastic paraplegia associated with intellectual disability) caused by two different genes (*FOXE3* and *AP4M1*). All of the individuals have segregated homozygous variant in *FOXE3* underlying anophthalmia and microphthalmia, whereas individual IV-14 has segregated both the variants in *FOXE3* and *AP4M1*, therefore showing phenotypes of anophthalmia and microphthalmia associated with additional neurological phenotypes. Because of the higher consanguinity in the Pakistani population, several previous studies have reported dual diagnosis of phenotypes in the population.[21,22]

FOXE3 gene belongs to a large family of forkhead transcription factors and is located on chromosome 1p33. It consists of a single coding exon, which encodes FOXE3 protein composed of 319 amino acids.[23] A wide range of eye diseases such as aphakia, sclerocornea, anterior segment anomalies and microphthalmia have been associated with autosomal dominant and autosomal recessive mutations in *FOXE3* gene.[24,25] No pathogenic mutation in *FOXE3* has been found to cause anophthalmia in association with other sensory processing disorder till date.

In the present study, six patients showed anophthalmia. In addition to ocular anomalies, three of the patients (IV-2, IV-14 and IV-15) showed additional phenotypes of developmental delay, sensorineural hearing loss, aphasia, behavioral issues and cerebral palsy. One of the patients (IV-15) was found with microcephalic head and fits. *FOXE3* is highly expressed in anterior segment of the eye, cerebral cortex and basal ganglia and is involved in the formation of lens placode.[26]

GeneMANIA network analysis showed that *FOXE3* has strong genetic and physical interactions with *ZEB2* and *MAB21L1*. Both these genes (*ZEB2* and *MAB21L1*) have been reported to associate with neurological disorders including Mowat syndrome (microcephaly, structural brain abnormalities and intellectual disability) and cerebellar, ocular, craniofacial and genital syndrome (COFG) respectively.[27,28]

In mouse, *FOXE3* gene mutation associated with eye deformities is first reported in the dysgenetic *lens* mutant mouse.[29,30] The dysgenetic lens mutant mice showed single eye abnormality, with small lenses, which is incompletely separated from the cornea.

In humans, the first *FOXE3* mutation was a heterozygous single base duplication (c.942dupG; p.Leu315AlafsX117) found in a family segregating congenital cataract and posterior embryotoxon.[31] Subsequently, another heterozygous mutation (c.269G4T; p.Arg90Leu) in the forkhead binding domain of the gene was reported in single individual affected with Peter's anomaly.[32] The first homozygous mutation in *FOXE3* gene was reported in a consanguineous family with microphthalmia, sclerocornea, buphthalmos and congenital primary aphakia.[33] Previously, seven different homozygous variants have been reported in *FOXE3* underlying different ocular phenotypes in Pakistani families. In one study, a family of Pakistani origin segregating aphakia in autosomal recessive form revealed a premature stop gain variant (p.Cys240X) in *FOXE3*.[34] Similarly, three additional families segregating bilateral anophthalmia revealed homozygous variants (p.Met7Ilefs*216 and p.Ile97Val) in *FOXE3*.[35] Moreover, two homozygous variants (p.Asn117Lys and p.Glu103Lys) in *FOXE3* contributing to Peters anomaly through transcriptional regulation of an autophagy-associated protein termed DNAJB1 were identified in Pakistani families.[36]

A homozygous missense variant (p.(Glu103Lys)) was reported in *FOXE3* in patients underlying autosomal recessive congenital cataracts.[37] Recently, a homozygous missense variant (p.(Ile97Val)) in *FOXE3* underlying congenital anterior segment dysgenesis, keratoconus, congenital bilateral corneal haze and apparent microphthalmia in five affected individuals belonging to a large consanguineous Pakistani family was reported by Rashid et al.[38] Here, we present another Pakistani family with six affected individuals showing syndromic features (anophthalmia associated with other sensory processing disorders including sensorineural hearing loss, aphasia, developmental delay, behavioral issue and microcephaly) caused by a novel nonsense p.Glu36* variant in *FOXE3* and a missense variant in *AP4M1*. The inter- and intra-familial variability in the phenotypes of present and previous cases might be a result of different familial backgrounds, age of the patients, nature and position of the variants, and/or dual molecular diagnosis.

To date, 35 pathogenic mutations have been reported in *FOXE3* gene including 27 missense, five small deletions and three small insertions (http://www.hgmd.cf.ac.uk/ac/all.php).[25,33,39–50] Most of the missense mutations in *FOXE3* gene were reported in the forkhead domain, which acts as a DNA binding domain of *FOXE3*. These mutations alter the function of DNA binding domain, resulting in reduced transcriptional activation and affecting the normal development of the eye.[48] Other mutations reported in *FOXE3* gene such as stop gain or frame shift are distributed throughout the gene sequence.[49] The variant p.Glu36* identified in the present study locates upstream to nuclear localization signal and the forkhead domain. It is predicted that the identified variant affects the protein structure (Figure 3) and may lead to loss of function through the production of truncated protein.

5 | CONCLUSIONS

The mutation spectrum of *FOXE3* gene is clinically variable. Here, we present the clinical and genetic analysis of a consanguineous family of Pakistani origin displaying features of anophthalmia associated with hearing loss, aphasia and cerebral palsy caused by novel variants in *FOXE3* and *AP4M1*. These findings not only expand the genetic and clinical spectrum of FOXE3 and AP4M1-related phenotypes, but also are helpful in prenatal screening, carrier testing and genetic counseling. Moreover, the study highlights the importance of analysis of next generation sequencing data for dual diagnosis in families where there is higher rate of consanguinity and variable phenotypes in patients. To prevent the segregation of rare inheritable diseases to the next generations, proper genetic counseling for the affected family is critical. Furthermore, parenteral genetic screening/diagnosis in conjunction with non-invasive prenatal testing and newborn screening are some of the suggested procedures to cope with rare inheritable conditions for which there is presently no treatment.[51–53] Additional cases related to this gene might aid in the identification of genotype–phenotype connections, potentially paving the way for clinical trials in the near future.[54]

AUTHOR CONTRIBUTIONS

WA and SB designed and supervised the study experiments. AU, NH, MAS, AAS and FUK collected data and conducted the clinical assessments. WA and FUK performed initial laboratory work. SB and AU performed whole exome sequencing and data analysis. WA and AU wrote the manuscript. All authors revised and approved the final version of the manuscript submitted for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request.

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