Novel mutations of the *PAX6*, *FOXC1*, and *PITX2* genes cause abnormal development of the iris in Vietnamese individuals

Ha Hai Nguyen,[1,2] Chau Minh Pham,[3] Hoa Thi Thanh Nguyen,[1] Nhung Phuong Vu,[1] Trang Thu Duong,[2] Ton Dang Nguyen,[1,2] Bac Duy Nguyen,[4] Hiep Van Nguyen,[3] Hai Van Nong[1,2]

[1]Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Vietnam; [2]Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam; [3]Vietnam National Eye Hospital, Hanoi, Vietnam; [4]Vietnam Military Medical University, Hanoi, Vietnam

Purpose: Congenital iris abnormality is a feature of several genetic conditions, such as aniridia syndrome and anterior segment degeneration (ASD) disorders. Aniridia syndrome is caused by mutations in the *PAX6* gene or its regulatory elements in the locus 11p13 or deletions of contiguous genes, while ASDs are the result of mutations in various genes, such as *PAX6*, *FOXC1*, *PITX2*, and *CYP1B1*. This study aims to identify pathogenic mutations in Vietnamese individuals with congenital anomalies of the iris.
Methods: Genomic DNA was extracted from peripheral blood of 24 patients belonging to 15 unrelated families and their available family members. Multiplex ligation-dependent probe amplification (MLPA) was used to detect the deletions or duplications in the 11p13–14 region, including the *PAX6* gene and its neighboring genes. Direct PCR sequencing was used to screen mutations in 13 exons and flanking sequences of the *PAX6* gene. The patients without mutation in the *PAX6* locus were further analyzed with whole exome sequencing (WES). Identified mutations were tested with segregation analysis in proband family members.
Results: We identified a total of 8 novel and 4 recurrent mutations in 20 of 24 affected individuals from 12 families. Among these mutations, one large deletion of the whole *PAX6* gene and another deletion of the *PAX6* downstream region containing the *DCDC1* and *ELP4* genes were identified. Eight mutations were detected in *PAX6*, including four nonsense, three frameshift, and one splice site. In addition, two point mutations were identified in the *FOXC1* and *PITX2* genes in patients without mutation in *PAX6*. Some of the mutations segregated in an autosomal dominant pattern where family members were available.
Conclusions: This study provides new data on causative mutations in individuals with abnormal development of iris tissue in Vietnam. These results contribute to clinical management and genetic counseling for affected people and their families.

Aniridia syndrome (OMIM 106210) is a disease that features a partial or complete absence of the iris, foveal hypoplasia, and nystagmus [1,2]. This syndrome belongs to a group of anterior segment dysgenesis (ASD) disorders relating to developmental disruption of anterior segment structures of the eyeball, leading to visual impairment [2,3]. Aniridia syndrome is a rare, hereditary, panocular disorder with both eyes affected. Congenital aniridia syndrome occurs with an incidence of 1:40,000 to 1:100,000 [4]. Aniridia syndrome is characterized by a variable degree of iris hypoplasia, mild hypoplasia, or variable-sized rudimentary iris stump [5-7]. Along with iris malformation, a range of sight-threatening conditions were observed previously, encompassing reduced visual acuity, foveal hypoplasia, nystagmus, cataract, glaucoma, keratopathy, and corneal dystrophy [4,8,9]. Most patients with aniridia have poor vision, nystagmus, foveal hypoplasia, and a high incidence of secondary glaucoma later in life [7,10,11].

Two-thirds of all cases of congenital aniridia are familial with autosomal dominant inheritance and complete penetrance [4,7]. The phenotypic variation has been observed within and among families, and even between two eyes of the same patient [4,12,13]. Approximately one-third of all cases are sporadic due to de novo mutations and are inherited in an autosomal dominant fashion. Aniridia syndrome may also occur in association with Wilms tumor, aniridia syndrome, genitourinary anomalies, and mental retardation (WAGR) syndrome which is caused by deletions of the *PAX6* and *WT1* genes in the chromosome 11p13 region [1]. Nelson et al. estimated that 25% to 33% of sporadic cases may develop Wilms tumor within 3 years after birth [8].

The *paired-box gene 6* (*PAX6*; OMIM 607108) is a homeobox gene with 13 exons located on chromosome 11p13. The PAX6 protein contains a conserved paired-box and a homeobox domain, both of which serve as regulators of

Correspondence to: Ha Hai Nguyen, Institute of Genome Research, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam; Phone: +84.24.3756.2934; FAX: +84.24.3791.7039; email: nguyenhaiha@igr.ac.vn

gene expression by binding to a specific region of DNA and activating the transcription. *PAX6* was the first homeobox gene discovered to play a key role in eye development [14]. Mutations in *PAX6* were first reported for congenital aniridia by Ton et al. in 1991 [15]. Since then, at least 491 mutations in the *PAX6* gene have been reported in the Leiden Open Variation Database (*PAX6*-LOVD). As both copies of *PAX6* are essential for eye development, silencing of one of the two copies leads to a *PAX6* haploinsufficiency disease mechanism [15,16]. In most cases, the disease is caused by the loss of function of *PAX6* that results from either intragenic mutations or chromosomal rearrangements at 11p13. As regulation of the gene is driven by multiple enhancers located in an upstream or downstream region of *PAX6*, a mutation or deletion in these enhancers can also cause ocular disorders, including aniridia syndrome.

Recently, several molecular techniques, including Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA), and array comparative genomic hybridization (aCGH), have been employed to identify genetic causes of aniridia syndrome [17-24]. If these methods do not reveal any defects in the *PAX6* locus, then whole exome sequencing (WES) or whole genome sequencing (WGS) might identify the causative defect.

This study focused on investigation of pathogenic mutations in Vietnamese individuals with congenital aniridia. Sanger sequencing and MLPA were conducted to detect mutations in the *PAX6* gene and deletions or duplications in the 11p13 region. Then, WES was performed on non-*PAX6* and −11p13 mutated individuals to identify mutations in other genes associated with ASD. Briefly, this study provides confirmation of phenotypic variability as well as extends the number of causative mutations of aniridia syndrome and ASDs.

METHODS

Subjects: This study recruited total 24 patients (12 males and 12 females, 17 pediatrics and 7 adults) with congenital anomalies of iris tissue from 15 unrelated families at Vietnam National Eye Hospital. These patients were periodically monitored at Vietnam National Eye Hospital, from 2019 to 2020. Written informed consent obtained from patients or their legal guardian prior to sampling for the genetic test. The peripheral blood samples (5ml) of patients and their available family members were collected in EDTA containing tubes and stored at − 20 °C until DNA extraction. When a mutation was identified as a pathogenic or likely pathogenic candidate in a patient, his or her familial members' samples were checked for the mutation and segregation analysis. This study

was approved by the Institutional Review Board (IRB) of the Institute of Genome Research, Vietnam Academy of Science and Technology. The study protocol was conducted in accordance with the provisions of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects.

DNA extraction: Genomic DNA was isolated from peripheral blood leukocytes using the Exgene Blood SV mini Kit (Geneall Biotechnology Co. LTD, Seoul, Korea) according to the manufacturer's protocol. DNA quality was evaluated with agarose gel electrophoresis and spectrophotometry at 260 and 280 nm (BioSpectrometer Basic; Eppendorf, Hamburg, Germany).

MPLA: The SALSA MLPA probemix P219 PAX6 (MRC Holland, Amsterdam, the Netherlands) was used to detect single- to multiexon deletions or duplications in the 11p13–14 region, which includes the *PAX6* and *WT1* (Gene ID: 7490; OMIM 607102) genes. The procedure was performed according to the manufacturer's instructions using 50 ng of genomic DNA. The PCR amplicons were separated on a Genetic Analyzer 3500 (Applied Biosystem, Foster City, CA). The results were analyzed using Coffalyser.Net software with 0.7 and 1.3 as the cut-off values for heterozygous deletion or duplication, respectively (normal status is diploid).

Sanger sequencing: Mutational screening of the *PAX6* gene was conducted for the entire 13 exons and their flanking regions. PCR was amplified in a PCR Mastercycler pro S (Eppendorf, Hamburg, Germany) in total volume of 25 µl containing 25 - 50 ng of genomic DNA, 0.5 pmol of each primer and 12.5 µl of Taq 2X Master Mix (New England Biolab, Ipswich, MA). The PCR consisted of an initial denaturation for 2 min at 95 ºC, followed by 35 cycles of 30 sec denaturation at 95 ºC, annealing for 30 s at 60 ºC and extension for 60 sec at 72 ºC. The obtained amplicons were purified using MultiScreen PCR filter plate (Merck Millipore, Darmstadt, Germany). The purified products were sequenced using the ABI Big Dye Terminator v 3.1 Sequencing Standard Kit (Applied Biosystems) and run on an ABI 3500 Genetic Analyzer (Applied Biosystems). The sequence data were analyzed by comparison with the standard sequence of the *PAX6* gene (NG_008679) using BioEdit (Raleigh, NC) sequence alignment editor software. Additional information about mutations and polymorphisms of the *PAX6* gene was confirmed from the following databases including the *PAX6* variation database: PAX6-LOVD and the Human Gene Mutation Database (HGMD).

Whole exome sequencing: The DNA library construction was performed by using Sure Select V6-Post (Agilent Technologies, Santa Clara, CA) following the manufacturer's

guidelines. The Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used for enriched library quantification. The library size distribution was checked with the Bioanalyzer using a High Sensitivity DNA Chip (Agilent Technologies) with expected size range from 200 bp to 400 bp. The sequencing was performed by using an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA) with paired reads of 150 bp.

Variant calling, annotation, and prediction: The reads were mapped to the hg19/GRCh37 human reference genome with the BWA.v0.7.12 tool [25], and Picard was used to mark the duplicates. GATK and Samtools were used to detect single nucleotide variants (SNVs) and short indels. To exclude false positives, all variants with depth reads lower than 10X were removed. Short indels in the repeat regions and within a 10 bp range from the start and end of the read were also removed. Then, the remaining variants were filtered from the public databases 1000G and gnomAD. The variants were annotated with the ANNOVAR program [26].

Population database checks: The presence and frequency of causative variant candidates were further checked in the Human Mutation Database, LOVD Database, Genome Aggregation Database (gnomAD), and in-house Vietnamese database (VN WES DB). In silico analysis was performed with sorting intolerant from tolerant (SIFT) [27], PolyPhen-2 [28], and MutationTaster [29] to anticipate the functional effect of missense variants on corresponding proteins. Amino acid conservation was checked with multiz alignment (UCSC).

RESULTS

Patients: A total of 24 Vietnamese individuals with congenital iris abnormalities from 15 unrelated families were studied. They included 12 men and 12 women whose ages range from 1 to 65 years. Of the 24 cases, eight were sporadic without family history of aniridia, and 16 were familial. For non-sporadic cases, there were two to seven affected members in each family, and the phenotype varied among families and even within the same family. The clinical ocular manifestations are summarized in Appendix 1. Notably, bilateral complete absence of the iris was dominant in most cases, except the twin brothers AN11 and AN12 who presented with partial absence of the iris of two eyes, as part of Rieger syndrome. The most frequent clinical ocular features were nystagmus and foveal hypoplasia, followed by cataract, glaucoma, and keratopathy. Patient AN1 had corneal opacity in the right eye due to asymmetric glaucoma between his right and left eyes at birth. He underwent trabeculectomy in the right eye and goniotomy in the left eye at 1 month of age.

Identification of mutations in PAX6 with MLPA and Sanger sequencing: To detect causative variants in the *PAX6* locus, DNA samples of probands were screened for large insertions or deletions and small mutations. Two large deletion and eight small mutations were identified in 17 subjects. The total mutation detection rate was 70.83% of patients (Table 1). Of the two DNA deletions, one was a chromosomal deletion comprising the *DCDC1* (Gene ID: 341019; OMIM 608062) and *ELP4* (Gene ID: 26610; OMIM 606985) genes downstream at the 3'untranlated region (UTR) of *PAX6* found in sporadic patient AN2, and one was a whole *PAX6* gene deletion detected in the son AN13 and his mother AN14. Among eight point mutations, five were nucleotide substitutions (*PAX6* c.538C>T, p.(Gln180*); c.765+1G>A; c.949C >T, p.(Arg317*); c.433A>T, p.(Lys145*); c.403C>T, p.(Gln135*)), one was a nucleotide insertion (*PAX6* c.551insG, p.(Glu185Argfs*14)), and two were nucleotide deletions (*PAX6* c.375–376del, p.(Arg125Serfs*6) and *PAX6* c.112del, p.(Arg38Glyfs*16)). It is notable that only two of eight mutations (*PAX6* c.949C>T, p.(Arg317*) and *PAX6* c.112del, p.(Arg38Glyfs*16)) were reported as pathogenic variants in the Human *PAX6* Mutation Databases (LOVD), and the six remaining mutations were novel. These novel mutations, including three nonsense, two frameshift, and one splice site, could be assumed to produce the adverse changes in PAX6 protein synthesis; therefore, they were likely pathogenic variants. The sequencing chromatograms of ten detected mutations in *PAX6* are presented in Appendix 2.

Identification of mutations in ASD genes with WES: Next, whole exome sequencing was used to find causative variants in probands negative at the *PAX6* locus mutation. We focused on novel or rare variants of ASD-related genes which may be involved in the pathogenesis of iris hypoplasia. The results revealed three novel nucleotide substitutions in the coding sequences of the *FOXC1* (Gene ID: 2296; OMIM 601090), *PITX2* (Gene ID: 5308; OMIM 601542) and *CPAMD8* (Gene ID: 27151; OMIM 608841) genes, respectively (Table 2, Figure 1). The novel mutation *FOXC1* c.274C>T, p.(Glu92*) was identified in the AN1 proband as heterozygous, resulting in a premature termination codon in the forkhead domain of the protein. This is a de novo mutation because his unaffected parents were not carriers. The heterozygous missense mutation *PITX2* c.329C>G, p.(Pro110Arg) was detected in the AN9 proband. This mutation also presented as heterozygous in her affected mother, the AN10 proband. All three bioinformatics tools used in the present study pointed out the damaging effect of the *PITX2* c.329C>G, p.(Pro110Arg), which is located in the homeobox domain of the PITX2 protein. As dysfunction mutations in the *FOXC1* and *PITX2* genes are well-known to cause autosomal dominant ASD type 3 and 4, respectively,

TABLE 1. *PAX6* GENE MUTATIONS IDENTIFIED IN ANIRIDIA PATIENTS BY SANGER SEQUENCING AND MLPA METHODS.

Family .No	Patient ID	Exon/ intron	Nucleotide change	Protein change	Variant type	Reference
S2	AN2		*DCDC1, ELP4* genes deletion downstream of *PAX6*			Anna Wawrocka et al.. 2013
S3	AN3	Exon 7	c.375–376del	p.(Arg125Serfs*6)	Frameshift	novel
S5	AN5	Exon 8	c.538C>T	p.(Gln180*)	Nonsense	novel
S6	AN6	Intron 9	c.765+1G>A	p.?	Splice site	novel
S8	AN8	Exon 11	c.949C>T	p.(Arg317*)	Nonsense	LOVD
F11	AN13 AN14		Whole gene deletion			Anna Wawrocka et al.. 2013
F12	AN15 AN16	Exon 8	c.551insG	p.(Glu185Argfs*14)	Frameshift	novel
F13	AN17 AN18 AN19	Exon 7	c.433A>T	p.(Lys145*)	Nonsense	novel
F14	AN20 AN21	Exon 7	c.403C>T	p.(Gln135*)	Nonsense	novel
F15	AN22 AN23 AN24	Exon 5	c.112del	p.(Arg38Glyfs*16)	Frameshift	LOVD

Nucleotide position of *PAX6* cDNA is available from the GenBank accession numbers NM_000280.3. Amino acid numbering refers to the reference sequence for PAX6 protein, NP_000271. LOVD PAX6 version **PAX6:210304.**

a heterozygous mutation of *FOXC1* or *PITX2* could be the cause of iris hypoplasia in patients AN1, AN9, and AN10. The third mutation, *CPAMD8* c.2405G>A, p.(Cys802Tyr), was identified as heterozygous in the twin brothers (AN11 and AN12). Although the mutation was predicted by PolyPhen-2 and MutationTaster as a damaging variant, its pathogenicity is still unknown. We did not find any genetic cause in the two remaining subjects (AN4 and AN7).

Mutational localizations and genotype–phenotype correlation: The localization of a variant in a gene has an impact on its biologic function. The distribution of the mutations identified in this study is represented in Figure 2. For the *PAX6* gene, the number of mutations located in the paired domain (PD), linker region (LR), homeodomain (HD), and proline/serine/threonine-rich transactivation (PST)-rich region were two, four, one, and one, respectively. The mutation in the *FOXC1* gene was found in the forkhead domain (FD), while the mutation in the *PITX2* gene also resided in the HD. The FD and the HD are responsible for the DNA-binding function of proteins.

Appendix 3 shows clinical characteristics of the patients in this study. All had aberrant expression of iris tissue. Two-thirds of cases (75%, 12/16 patients) displayed nystagmus, which is the highest percentage of any ocular manifestation. Foveal hypoplasia was the second most prevalent feature with a prevalence of 50% (8/16 patients). In the *PAX6* mutation-harboring individuals, all had nystagmus, and three-quarters had foveal hypoplasia. Two patients developed keratopathy: One carried the whole *PAX6* gene deletion, and one carried the *FOXC1* nonsense mutation. Among patients who had glaucoma, only one child carrying the *FOXC1* mutation (AN1) was congenital. The percent frequencies of cataract, glaucoma, and keratopathy were 18.75% (3/16 patients), 35.29% (6/17 patients), and 12.50% (2/16 patients), respectively.

DISCUSSION

Aniridia or ASD disorders are associated with not only iris malformation but also many intraocular complications that could lead to visual impairment and blindness. There were in total 24 affected individuals from 15 unrelated families enrolled this study; the rate of sporadic and inherited cases was one-third and two-thirds, which is consistent with a previous review [30]. We identified in total 12 different mutations in the *PAX6*, *FOXC1,* and *PITX2* genes. Notably, eight detected mutations are novel, thus expanding our understanding of the spectrum of iris hypoplasia causative variants. As the majority of affected individuals harbor a mutation in the *PAX6* gene (approximately 70.83%), the results support the observation that aniridia syndrome in Vietnam arises from insufficiency of PAX6.

TABLE 2. SUMMARY OF GENETIC VARIANTS DETECTED BY WES.

| Samples | WES | | | | | Condition (inheritance) | Parental genotype | | *In silico* prediction | | |
	Gene (transcript ID)	cDNA change	AA change	Variant types	Zygosity	Presence in the GnomAD/ VN WES DB		Father	Mother	SIFT	Poly-phen2	Mutation taster
AN1	*FOXC1* (NM_001453)	c.274C>T	p.(Gln92*)	Nonsense	Het	No/ No	ASD 3 (AD)	CC	CC	-	-	-
AN9	*PITX2* (NM_153426)	c.329C>G	p.(Pro110Arg)	Missense	Het	No/ No	ASD 4 (AD)	CC	CG	D	D	D
AN11	*CPAMD8* (NM_015692)	c.2405G>A	p.(Cys802Tyr)	Missense	Het	No/ No	ASD 8 (AR)	NA	NA	T	D	D
AN12	*CPAMD8* (NM_015692)	c.2405G>A	p.(Cys802Tyr)	Missense	Het	No/ No	ASD 8 (AR)	NA	NA	T	D	D

AA: amino acid, No: not yet reported or found, Het: heterozygous, ASD: Anterior Segment Dysgenesis, AD: Autosomal Dominant, AR: Autosomal Recessive, NA: not available sample. D: damaging, T: Tolerant. Genetic conditions and their inheritance are available from OMIM database.
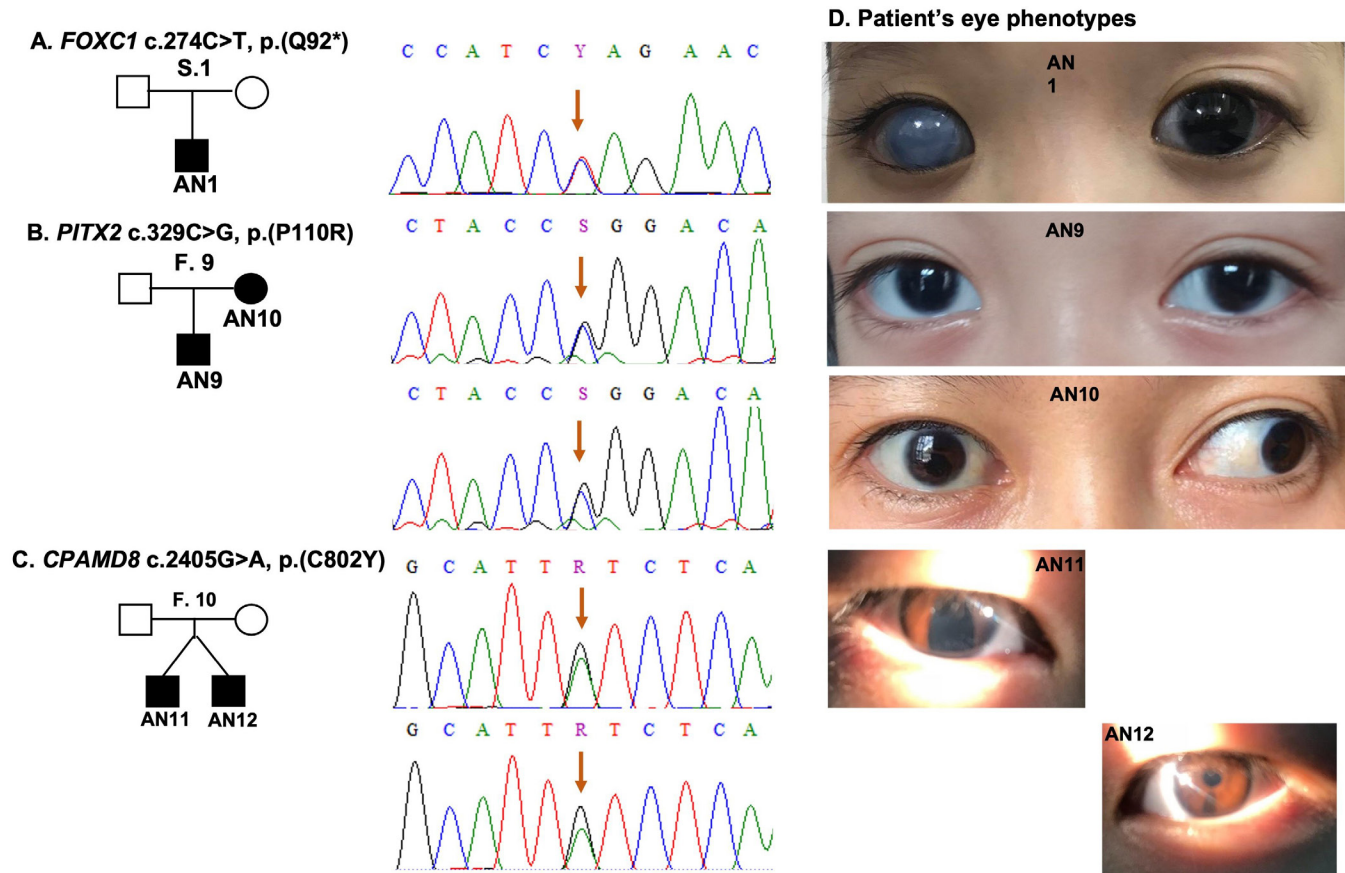
Figure 1. Novel mutations of the *FOXC1*, *PITX2*, and *CPAMD8* genes and patients' ocular phenotypes. **A–C**: Pedigrees, sequence chromatograms of the nonsense mutation (*FOXC1* c.274C>T, p.(Q92*), transcript ID NM_001453) of AN1, missense mutation (*PITX2* c.329C>G, p.(P110R), transcript ID NM_153426) of AN9 and AN10 (daughter and mother, respectively), and missense mutation (*CPAMD8* c.2405G>A, p.(C802Y), transcript ID NM_015692) of AN11 and AN12 (twin brothers). The exact positions of the mutations in the chromatograms are indicated by the arrows. **D**: Patients' ocular phenotypes.

In this study, the clinical data were available mainly in pediatric aniridia whose ages ranged from 2 to 8 years. Among the study patients, most patients with aniridia who carry a mutation in *PAX6* displayed nystagmus, foveal hypoplasia, and optic disc hypoplasia; cataract and glaucoma were presented at a low rate. Typically, cataract develops at a rate of 50% to 80% in adolescent and young adult patients with aniridia. Similarly, glaucoma develops in about 50% of people with aniridia in the first two decades of life [30]. In addition to the mutation in *PAX6*, the proband (AN1) harboring the novel mutation *FOXC1* c.274C>T, p.(Gln92*) developed glaucoma and keratopathy at an early age. In the treatment for glaucoma, he had trabeculectomy in the right eye, and goniotomy and Ahmed valve implantation in the left eye. His right eye presented with total corneal opacity, whereas his left eye was clear. The *FOXC1* gene (OMIM 601090) is a member of the forkhead family of transcription factors. The FOXC1 protein has a distinct DNA-binding forkhead domain that attaches to specific regions of DNA and thus, induces transcription. This gene participates in the regulation of embryonic and ocular development, especially in the formation of structures in the anterior segment, including the iris, the lens, and the cornea. Regarding this new finding of the *PITX2* c.329C>G, p.(Pro110Arg) mutation, both carriers (AN9 and AN10) have total iris hypoplasia, and the AN10 proband manifested glaucoma at a young age. The *PITX2* gene (OMIM 601542) is a transcription factor which plays an important role in the development of the eye. The mutation *PITX2* c.329C>G changed a very conserved amino acid proline to arginine in the homeobox domain of the protein. This mutation was predicted to have a high probability of being damaging by the SIFT, PolyPhen-2, and MutationTaster tools. It was checked and was found to be absent in the WES database of 300 Vietnamese people without any ocular disease.

In addition, we want to note the presence of the novel *CPAMD8* c.2405G>A, p.(Cys802Tyr) mutation in the twin brothers, AN11 and AN12. *CPAMD8* (OMIM 608841), a member of the complement component-3/alpha-2-macro-globulin protein family, is essential for the growth of the eye and has a likelihood of causing ASD in an autosomal recessive inheritance pattern [31]. In silico analysis showed that the *CPAMD8* c.2405G>A, p.(Cys802Tyr) mutation was deleterious, and it has not been reported in any human population database. However, multiple genomic sequence alignments demonstrated that the cysteine at position 802 of the CPAMD8 protein was conserved among various species, such as rhesus, dog, chicken, opossum, and zebrafish, suggesting that it is important to protein function. The limitation in this case was that we could not examine the parental phenotype to assess mutation segregation due to unavailability of samples. In addition, the second loss function variant possibly located in intronic regions or encompassed by a larger deletion of *CPAMD8* was also not detected. Therefore, this mutation remains a variant of uncertain significance.

Recently, MLPA and Sanger sequencing have been the most popular methods for detecting genetic mutations in the *PAX6* locus in different cohorts of aniridia [22,23,32]. In this study, we could find causative variants in 78.8% of affected individuals by using MLPA and Sanger sequencing. This detection rate was similar to that of a Korean cohort (about 70%) [12] and lower than those of Australasia and Southeast Asian (94.4%) [24] and Chinese (96.9%) [23] cohorts. Moreover, WES was a comprehensive tool allowing us to analyze the coding regions of thousands of genes simultaneously, including ocular malformation-related genes. As a result, we successfully identified two more causative mutations in the *FOXC1* and *PITX2* genes in two of six patients who did not carry a *PAX6* mutation. Four probands remained unidentified for pathogenic factors. Further analysis by using aCGH or whole genome sequencing approaches should be considered to answer the question.

In summary, this is a comprehensive analysis of the mutational spectrum in Vietnamese patients with congenital aniridia. The results revealed six novel and four recurrent causative mutations in the *PAX6* gene. Additionally, we identified two mutations in the *FOXC1* and *PITX2* genes in patients with ASD. These data provide more insight into genetic etiology of iris hypoplasia conditions and helpful information for clinical management and genetic counseling.
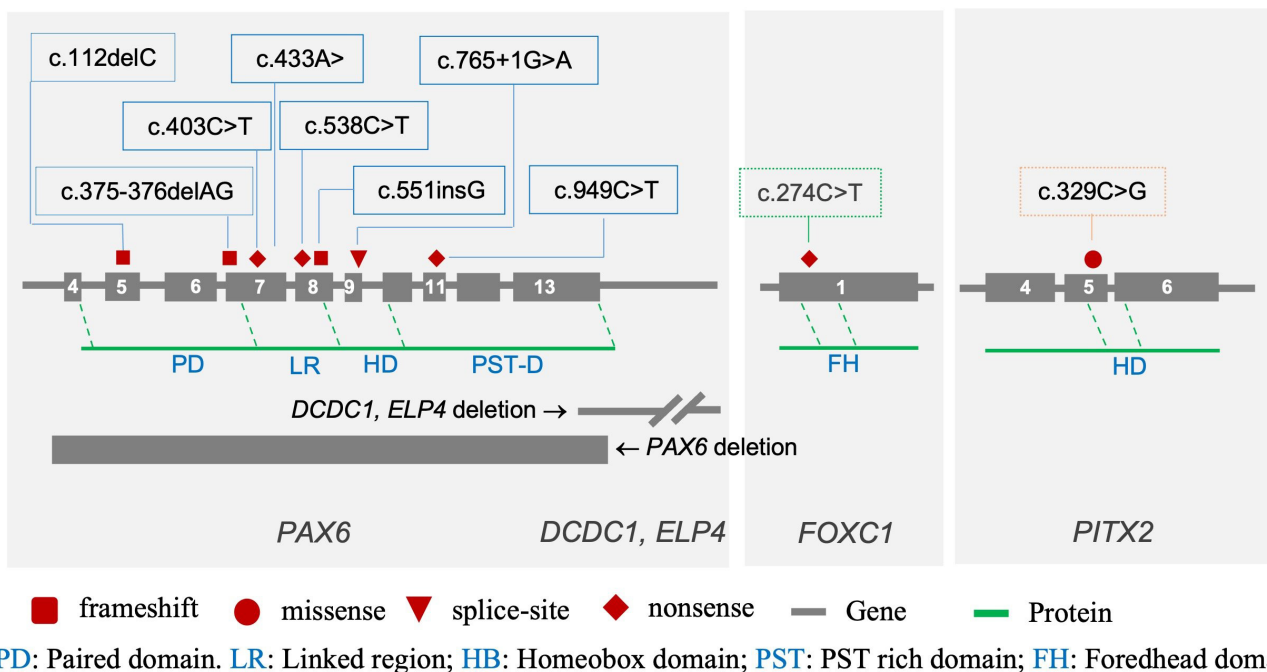

Figure 2. Schematic representation of mutations detected in the *PAX6* locus and *FOXC1* and *PITX2* genes of 20 probands. Distribution mutations were described in exons and splice sites of three genes. The frameshift (square), missense (circle), splice-site (triangle), and nonsense (diamond) mutations are indicated. A total of one partial and one whole gene deletions of the *PAX6* gene are presented as the shaded bars. The numbers in the gray boxes refer to the exons. The functional domains PD, LR, and HD and PST-D of the proteins are shown with the green line.

APPENDIX 1. CLINICAL FEATURES OF ANIRIDIA PATIENTS.

To access the data, click or select the words "Appendix 1." BCVA (RE & LE): best-corrected visual acuity (right eye & left eye), PL: perception light, F: female, M: male, B: bilateral, F: family, S: sporadic, (-) not available, ARS: Axenfeld-Rieger syndrome, (*) the first patient in the family underwent genetic testing.

APPENDIX 2. CHROMATOGRAMS OF 10 MUTATIONS DETECTED IN THE PAX6 GENE AND ITS REGULATORY REGION

To access the data, click or select the words "Appendix 2." Nucleotide numbering for PAX6 cDNA deposited in the GenBank under accession numbers NM_000280.3. Amino acid numbering refers to the reference sequence for PAX6 protein, NP_000271.

APPENDIX 3. GENOTYPE-PHENOTYPE CORRELATION OF PROBANDS

To access the data, click or select the words "Appendix 3." •: present, •: absent, -: information not available, B: affected bilateral, C: complete aniridia, P: partial aniridia.

REFERENCES

1. Tripathy K, Salini B. Aniridia. [Updated 2021 Feb 14]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021.

2. Churchill A, Booth A. Genetics of aniridia and anterior segment dysgenesis. Br J Ophthalmol 1996; 80:669-73. [PMID: 8795384].

3. Gould DB, John SWM. Anterior segment dysgenesis and the developmental glaucomas are complex traits. Hum Mol Genet 2002; 11:1185-93. [PMID: 12015278].

4. Hingorani M, Hanson I, Van Heyningen V. Aniridia. Eur J Hum Genet 2012; 20:1011-7. [PMID: 22692063].

5. Lee HJ, Colby KA. A review of the clinical and genetic aspects of aniridia. Semin Ophthalmol 2013; 28:306-12. [PMID: 24138039].

6. Edén U, Beijar C, Riise R, Tornqvist K. Aniridia among children and teenagers in Sweden and Norway. Acta Ophthalmol 2008; 86:730-4. [PMID: 18494744].

7. Brauner SC, Walton DS, Chen TC. Aniridia. Int Ophthalmol Clin 2008; 48:79-85. [PMID: 18427263].

8. Nelson LB, Spaeth GL, Nowinski TS, Margo CE, Jackson L. Aniridia. A review. Surv Ophthalmol 1984; 28:621-42. [PMID: 6330922].

9. Lee H, Khan R, O'keefe M. Aniridia: Current pathology and management. Acta Ophthalmol 2008; 86:708-15. [PMID: 18937825].

10. Hingorani M, Williamson KA, Moore AT, van Heyningen V. Detailed ophthalmologic evaluation of 43 individuals with PAX6 mutations. Invest Ophthalmol Vis Sci 2009; 50:2581-90. [PMID: 19218613].

11. Dubey SK, Mahalaxmi N, Vijayalakshmi P, Sundaresan P. Mutational analysis and genotype-phenotype correlations in southern Indian patients with sporadic and familial aniridia. Mol Vis 2015; 21:88-97. [PMID: 25678763].

12. Lim HT, Seo EJ, Kim GH, Ahn H, Lee HJ, Shin KH, Lee JK, Yoo HW. Comparison between aniridia with and without PAX6 mutations: Clinical and molecular analysis in 14 Korean patients with aniridia. Ophthalmology 2012; 119:1258-64. [PMID: 22361317].

13. Wawrocka A, Krawczynski MR. The genetics of aniridia - simple things become complicated. J Appl Genet 2018; 59:151-9. [PMID: 29460221].

14. Glaser T, Walton DS, Maas RL. Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene. Nat Genet 1992; 2:232-9. [PMID: 1345175].

15. Ton CCT, Hirvonen H, Miwa H, Weil MM, Monaghan P, Jordan T, van Heyningen V, Hastie ND, Meijers-Heijboer H, Drechsler M, Royer-Pokora B, Collins F, Swaroop A, Strong LC, Saunders GF. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. Cell 1991; 67:1059-74. [PMID: 1684738].

16. Martha A, Ferrell RE, Mintz-Hittner H, Lyons LA, Saunders GF. Paired box mutations in familial and sporadic aniridia predicts truncated aniridia proteins. Am J Hum Genet 1994; 54:801-11. [PMID: 7909985].

17. Mirrahimi M, Sabbaghi H, Ahmadieh H, Jahanmard M, Hassanpour K, Suri F. A novel PAX6 mutation causes congenital aniridia with or without retinal detachment. Ophthalmic Genet 2019; 40:146-9. [PMID: 30985247].

18. Pedersen HR, Baraas RC, Landsend ECS, Utheim OA, Utheim TP, Gilson SJ, Neitz M. PAX6 genotypic and retinal phenotypic characterization in congenital aniridia. Investig Ophthalmol Vis Sci. 2020;61(5).

19. Berntsson SG, Kristoffersson A, Daniilidou M, Dahl N, Ekström C, Semnic R, Markström A, Niemelä V, Partinen M, Hallböök F, Landtblom AM. Aniridia with PAX6 mutations and narcolepsy. J Sleep Res 2020; 29:e12982- [PMID: 31943460].

20. Wang GM, Prasov L, Al-Hasani H, Marrs CER, Tolia S, Wiinikka-Buesser L, Richards JE, Bohnsack BL. Phenotypic Variation in a Four-Generation Family with Aniridia

Carrying a Novel PAX6 Mutation. J Ophthalmol 2018; 2018:5978293-[PMID: 29850208].

21. Giray Bozkaya O, Ataman E, Kilicarslan OA, Cankaya T, Ulgenalp A. Identification of a novel frameshift heterozygous deletion in exon 8 of the PAX6 gene in a pedigree with aniridia. Mol Med Rep 2016; 14:2150-4. [PMID: 27431685].

22. Syrimis A, Nicolaou N, Alexandrou A, Papaevripidou I, Nicolaou M, Loukianou E, Christophidou-Anastasiadou V, Malas S, Sismani C, Tanteles GA. Aniridia due to a novel microdeletion affecting PAX6 regulatory enhancers: case report and review of the literature. J Genet 2018; 97:555-62. [PMID: 29932076].

23. You B, Zhang X, Xu K, Xie Y, Ye H, Li Y. Mutation spectrum of pax6 and clinical findings in 95 chinese patients with aniridia. Mol Vis 2020; 26:226-34. [PMID: 32214788].

24. Souzeau E, Rudkin AK, Dubowsky A, Casson RJ, Muecke JS, Mancel E, Whiting M, Mills RAD, Burdon KP, Craig JE. PAX6 molecular analysis and genotype-phenotype correlations in families with aniridia from Australasia and Southeast Asia. Mol Vis 2018; 24:261-73. [PMID: 29618921].

25. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009; 25:1754-60. [PMID: 19451168].

26. Wang K, Li M, Hakonarson H. ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010; 38:e164-[PMID: 20601685].

27. Hu J, Ng PC. SIFT Indel: Predictions for the Functional Effects of Amino Acid Insertions/Deletions in Proteins. PLoS One 2013; 8:77940-[PMID: 24194902].

28. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods 2010; 7:248-9. [PMID: 20354512].

29. Schwarz JM, Cooper DN, Schuelke M, Seelow D. Mutationtaster2: Mutation prediction for the deep-sequencing age. Nat Methods 2014; 11:361-2. [PMID: 24681721].

30. Calvão-Pires P, Santos-Silva R, Falcão-Reis F, Rocha-Sousa A. Congenital Aniridia: Clinic, Genetics, Therapeutics, and Prognosis. Int Sch Res Not. 2014; 2014:1-10. [PMID: 27355034].

31. Cheong SS, Hentschel L, Davidson AE, Gerrelli D, Davie R, Rizzo R, Pontikos N, Plagnol V, Moore AT, Sowden JC, Michaelides M, Snead M, Tuft SJ, Hardcastle AJ. Mutations in CPAMD8 Cause a Unique Form of Autosomal-Recessive Anterior Segment Dysgenesis. Am J Hum Genet 2016; 99:1338-52. [PMID: 27839872].

32. Goolam S, Carstens N, Ross M, Bentley D, Lopes M, Peden J, Kingsbury Z, Tsogka E, Barlow R, Carmichael TR, Ramsay M, Williams SE. Familial congenital cataract, coloboma, and nystagmus phenotype with variable expression caused by mutation in PAX6 in a south african family. Mol Vis 2018; 24:407-13. [PMID: 29930474].