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PAX6 molecular analysis and genotype–phenotype correlations in families with aniridia from Australasia and Southeast Asia

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Purpose: Aniridia is a congenital disorder caused by variants in the *PAX6* gene. In this study, we assessed the involvement of *PAX6* in patients with aniridia from Australasia and Southeast Asia.
Methods: Twenty-nine individuals with aniridia from 18 families originating from Australia, New Caledonia, Cambodia, Sri Lanka, and Bhutan were included. The *PAX6* gene was investigated for sequence variants and analyzed for deletions with multiplex ligation-dependent probe amplification.
Results: We identified 11 sequence variants and six chromosomal deletions, including one in mosaic. Four deleterious sequence variants were novel: p.(Pro81HisfsTer12), p.(Gln274Ter), p.(Ile29Thr), and p.(Met1?). Ocular complications were associated with a progressive loss of visual function as shown by a visual acuity ≤ 1.00 logMAR reported in 65% of eyes. The prevalence of keratopathy was statistically significantly higher in the Australasian cohort (78.6%) compared with the Southeast Asian cohort (9.1%, p=0.002). Variants resulting in protein truncating codons displayed limited genotype–phenotype correlations compared with other variants.
Conclusions: *PAX6* variants and deletions were identified in 94% of patients with aniridia from Australasia and Southeast Asia. This study is the first report of aniridia and variations in *PAX6* in individuals from Cambodia, Sri Lanka, Bhutan, and New Caledonia, and the largest cohort from Australia.

Aniridia (OMIM 106210) is a congenital developmental disorder of the eye characterized by bilateral complete or partial iris hypoplasia [1]. Associated ocular anomalies include foveal hypoplasia, nystagmus, corneal opacification and neovascularization, cataracts, and glaucoma, which can lead to progressive decreased visual acuity [2,3]. Aniridia is a rare disease, with an incidence of between 1:64,000 and 1:100,000 births [4-6]. Isolated aniridia is caused by sequence variants in the *PAX6* gene [7] (OMIM 607108) or chromosomal rearrangements involving *PAX6*, and is transmitted in an autosomal dominant manner with complete penetrance but variable expressivity. Aniridia is usually isolated but in some cases can be part of a contiguous gene syndrome, such as Wilms tumor, aniridia, genitourinary abnormalities, and mental retardation (WAGR; OMIM 194072) syndrome caused by hemizygous deletions of chromosome 11p13 involving *PAX6* and the adjacent Wilms tumor (*WT1*; OMIM 607102) gene [8], or Wilms tumor, aniridia, genitourinary anomalies, mental retardation, and obesity (WAGRO; OMIM 612469) syndrome involving hemizygous deletion of the region containing *PAX6*, *WT1*, and *BDNF* (OMIM 113505) associated with obesity [9].

The *PAX6* gene, which lies on chromosome 11p13, encodes a highly conserved transcription factor containing two DNA-binding domains (a paired domain and a paired-type homeodomain) and a proline-serine-threonine-rich transactivation domain [10]. *PAX6* is expressed in the ectoderm, and subsequently in the differentiating cells of the cornea, lens, ciliary body, and retina [11]. Heterozygous variants in *PAX6* in mice result in a small eye phenotype (*Sey*) whereas homozygous variants result in anophthalmia and poorly formed nasal cavities [12]. The phenotypes observed in humans and mice with PAX6 haploinsufficiency demonstrate that the gene has a pivotal role in the embryological development of the eye, especially the anterior segment structures.

The human PAX6 allelic variant database (last accessed 19 January 2018) contains 472 unique variants of *PAX6*. Several types of *PAX6* variants are associated with aniridia, including nonsense, splicing, frameshift, missense,

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extension variants, and in-frame insertions or deletions [13]. The majority of sequence variants associated with isolated aniridia are loss of function variants (nonsense, splice, frameshift variants, and in-frame insertions or deletions) that introduce premature termination codons into the *PAX6* coding region and potentially result in the degradation of the translation products by the nonsense-mediated decay process to prevent the production of truncated proteins [13]. Therefore, the phenotype results from haploinsufficiency of the *PAX6* gene. Sequence variants and deletions of the *PAX6* gene account for around 90% of all cases of familial or sporadic aniridia [14,15]. Aniridia may also result from chromosomal rearrangements involving elements with transcriptional functions downstream of *PAX6* without affecting the integrity of the structural *PAX6* gene [16,17].

This study reported on the largest cohort from Australia and is the first study of patients with aniridia from Cambodia, Sri Lanka, Bhutan, and New Caledonia. We investigated *PAX6* for sequence variants and deletions in 18 probands with aniridia and 11 of their affected relatives. We identified variants and deletions in *PAX6* in 94% of the probands, including four novel *PAX6* deleterious variants, and reported detailed associated phenotypes.

METHODS

Participant recruitment: Twenty-nine individuals from 18 families were enrolled. The study complied with the tenets of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects. Informed consent was obtained from participants or their legal guardian in the local language. Institutional board approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee, and further approval was obtained from local institutions in Cambodia, Bhutan, and Sri Lanka.

Most Australian and New Caledonian cases were part of the Australian and New Zealand Registry of Advanced Glaucoma as described previously [18]. They received a complete ocular examination, including corrected visual acuity, slit-lamp biomicroscopy of the anterior segment, and review of the fundus. In children, when performed, refraction was measured with a retinoscope following cycloplegic dilation with 1% cyclopentolate. Central corneal thickness (CCT) was measured in a minority of patients using an ultrasound pachymeter (Pachymate, DGH Technology, Exton, PA).

Fifteen patients from seven families were enrolled as a result of surveys of schools for the blind in Cambodia [19], Sri Lanka [20], and Bhutan [21] that evaluated the causes of childhood blindness within those communities. Data were collected from school staff, children, relatives, and medical records. A detailed eye examination was conducted by a team of Australian and local optometrists and ophthalmologists as previously described [19–21]. Briefly, the examination included assessment of near and distance visual acuity, the anterior segment was assessed with slit-lamp biomicroscopy, and the posterior segment was evaluated with indirect or direct fundus ophthalmoscopy. Children who had distance vision recorded as better than no perception of light underwent refractive testing and low-vision assessment. Four affected relatives of a family from Bhutan provided samples for DNA analysis but could not be examined.

Genetic analysis: DNA was extracted from whole blood anticoagulated with EDTA using the QIAcube (Qiagen, Doncaster, Australia) automated system and reagents according to the manufacturer's protocols. Alternatively, for patients either needle phobic or remote from a pathology collection center, including Cambodian, Bhutanese, and Sri Lankan participants, individuals provided a saliva specimen collected in an Oragene® DNA Self-Collection Kit (DNA Genotek Inc., Kanata, Canada), and the DNA was extracted according to the manufacturer's instructions. The entire coding sequence and intron–exon boundaries of *PAX6,* encoding the longer transcript NM_001604.5, was amplified with PCR using primer pairs detailed in Appendix 1. Each PCR was performed using 100 ng of purified genomic DNA as a template in a reaction mix containing 1X final concentration of AmpliTaq Gold 360 Master Mix. The following PCR conditions on a Veriti (Life Technologies, Mulgrave, Australia) thermal cycler were used as standard for all templates with both primer pairs: Step 1, 95 °C for 10 min; Step 2, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, repeated for 40 cycles; and Step 3, 72 °C for 7 min. PCR amplified products were prepared for DNA sequencing with the ExoSAP method using a 10 µl sample of each PCR reaction treated with 5 U of Exonuclease I (Biolabs, Ipswich, MA) and 1 U of Shrimp Alkaline Phosphatase (USB Corporation, Cleveland, OH) to remove residual primers and dNTPs. Bidirectional BigDye® Terminator Cycle Sequencing (Life Technologies) reactions of the appropriate template and *PAX6* PCR primer were resolved and base called on an Applied Biosystems 3130XL Genetic Analyzer (Life Technologies).

Detection of the sequence variants was performed using Mutation Surveyor™ v4.0.11 (SoftGenetics LLC, State College, PA). All forward and reverse sequence trace files were assembled by the program against the *PAX6* GenBank reference (assembly GRCh37.p13). Software programs Sorting Intolerant From Tolerant (SIFT) and PolyPhen-2 HumVar were used to predict the potential impact of amino acid

substitutions on the protein. Conservation among mammalian species was assessed using Homologene. Genome Aggregation Database r2.0.2 (gnomAD) was used to compare allelic frequencies to the population frequencies.

PAX6 was analyzed for copy number variation (CNV) with multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P219-B2 PAX6 probemix (MRC Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. The copy number present in the original DNA specimen was determined from the relative amplitude of each amplicon product detected using the ABI 3130*xl* Genetic Analyzer and the data analyzed using Peak Scanner v2.0 (ThermoFisher Scientific, Scoresby, Australia). Breakpoints of the identified *PAX6* deletions were further characterized using single nucleotide polymorphism (SNP) array (Illumina Infinium CytoSNP-850K BeadChip, Illumina PTY, Scoresby, Australia) in some patients. The SNP array was also performed for individual 13 following inconclusive MLPA results. Mosaic changes were detected by assessing for changes in probe intensities along with a shift in the allele frequencies of the SNP probes as previously described [22]. The percentage of mosaicism was estimated by comparing the expected allele frequency under a deletion model to various mosaic levels [22].

Data were analyzed with PASW Statistics, Rel. 18.0.1.2009 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation. The Mann–Whitney U test was used for the assessment of differences in nonparametric data. A p-value ≤ 0.05 was considered significant.

RESULTS

Phenotype: Twenty-nine patients from 18 families were evaluated in this study, comprising 14 male and 15 female patients. The mean age of the whole cohort was 26.4±14.9 years (range 9–58 years), while the mean age of the Australasian cohort was 31.2±15.9 years (range 11–58 years) and the mean age of the Southeast Asian cohort was 19.9±11.3 years (range 9–51 years). The age difference is explained by the fact that patients from Southeast Asia were children recruited from schools for the blind. The phenotype of the 25 examined individuals is described in Table 1. A family history of aniridia was reported in 10/18 probands (56%). Additional family members were available for genetic testing for five of the probands, and the pedigrees are illustrated in Figure 1A.

All individuals had total iris aplasia based on available documentation. Some of the most commonly associated complications are shown in Figure 2. After iris anomalies, the most commonly associated features were foveal hypoplasia (14/18, 78%) and nystagmus (17/19, 90%). Cataract or a history of cataract extraction was identified in 72% (18/25). Cataract extraction was performed in 50% (9/18) with a mean age at surgery of 20.4±13.9 years. Posterior subcapsular or cortical and posterior subcapsular cataracts were the predominant forms of lenticular opacification. Subluxation of the lens was identified in 36% (9/25). Glaucoma was identified in 44% (11/25), and glaucoma filtration surgery or glaucoma drainage device implantation was required by 36% (4/11) with a mean age at glaucoma surgery of 13.0±5.60 years. CCT was obtained in 12 individuals; the mean CCT was high at 645±74.0 µm (range 526–779 µm). Keratopathy was present in 48% (12/25; Figure 2), including one individual with limbal stem cell failure and complete corneal opacification who required corneal transplantation (1/12, 8%). The mean age at keratopathy diagnosis was 19.3±10.5 years (range 9–34 years). A range of refractive errors were identified within the cohort with refraction ranging from −13.00 to +7.30 (excluding individuals 15, 16, and 17 who had refractions of +8.00 to +11.00 secondary to subluxed lenses). All individuals from family 12 were myopic, including two individuals (12a and 12c) with high myopia associated with chorioretinal atrophy affecting the macula. Visual acuity ≤ 1.00 logMAR was recorded in 65% (31/48) of eyes, including 40% (8/20) of eyes from individuals aged ≤20 years compared with 82% (23/28) of eyes from individuals aged >20 years.

The prevalence of keratopathy was statistically significantly higher in the Australasian cohort (11/14, 78.6%) than in the Southeast Asian cohort (1/11, 9.1%, p=0.002). The prevalence of other aniridia-associated features, including foveal hypoplasia, nystagmus, cataracts, lens dislocation, and glaucoma, were not statistically significantly different between the two cohorts.

PAX6 genetic results: Sequence variants in the *PAX6* gene were found in 11 of the 18 probands (61.1%) and included seven previously reported and four novel variants. Eight variants were predicted to introduce a premature termination codon, one variant resulted in a C-terminal extension variant, one variant affected the initiation codon, and one was a missense variant (Table 2). Chromosomal deletions were found in an additional six probands (33.3%). No sequence variants or deletions in *PAX6* were identified in individual 14. In total, *PAX6* accounted for 94.4% (17/18) of the probands with aniridia in this study.

Among the eight variants predicted to result in premature termination codons, five were nonsense variants, two were frameshift variants, and one was a splicing variant (Table 2). Two were novel, p.(Pro81HisfsTer12) and p.(Gln74Ter), whereas the remainder have been previously reported. Variant p.(Pro81HisfsTer12) segregated in two affected family

Table 1. Phenotypic features of the patients with aniridia.

ID/ inheri-tance	Origin	Age (years) / Sex	Visual acuity (logMAR) (RE/LE)	Retinal features	Ocular motility	Kerato-pathy	Cataract	Lens sublux-ation	Glaucoma	CCT (µm) (RE/ LE)	Refraction (RE/LE)	Surgery	Associated conditions
1a/F	Australia	24/F	1.1:CF	FH	nystagmus	+	+ (PSC, C, NS)	-	OHT	na	+3.0/+3.0	Nil	Type 1 diabetes
1b/F	Australia	26/M	CF:PL	FH	nystagmus	+	+ (C)	+	+ (severe)	550/526	+1.5/+1.5	CAT (BE) VIT (BE) TRAB (BE) TUBE (BE)	Retinal detach-ment, stroke
1c/F	Australia	52/F	CF:CF	FH	nystagmus, esotropia	+	+ (Fleck)	-	-	na	+5.0/+5.0	CG (RE)	
2/F	Australia	13/M	0.8:0.9	FH	nystagmus, esotropia	+	-	+	-	628/610	+5.0/+5.0	Nil	
3a/F	Australia	47/M	NPL:CF	FH	nystagmus	+	+	-	+	na/770	na	CAT (BE) Buckle (LE)	Retinal detachment
3b/F	Australia	17/F	better than 1.00	FH	nystagmus	-	+ (dot)	-	-		+3.5/+3.5	Nil	
4/S	Australia	31/F	0.6:0.5	FH	intermittent esotropia	+ (mild)	+ (PSC, C)	-	-	628/654	−2.1/-1.9	Nil	
5/S	Australia	54/M	0.3:0.2	FH	normal	-	+ (PSC)	-	-	na	+7.1/+7.3	CAT (BE)	
6/S	Australia	20/M	0.5:0.9	FH	nystagmus	-	+ (PSC)	-	+	660/716	na	CAT (BE) TRAB (LE) TUBE (LE)	
7/S	Australia	34/M	1.0: 1.1	FH	nystagmus	+	+ (PSC, C)	-	+	na	+3.5/+4.5	CAT (RE)	Intellectual disability, obesity, IgA nephropathy
8/S	Australia	57/F	1.3:HM	na	nystagmus	+	+	-	+ (severe)	na	na	TRAB (BE) CAT (BE)	Vitreous hemorrhage from diabetic proliferative retinopathy
9/S	Australia	10/F	1.2:CF	-	nystagmus	+	+ (PSC)	-	+	601/590	+1.5/+6.4	TUBE (BE) CAT (LE)	
10/F	Australia	33/F	NPL:0.9	FH	nystagmus	+	+ (C)	+	+ (severe)	537/589	na	CAT (BE)	
11/S	New Caledonia	17/M	1.0:1.0	-	nystagmus, esotropia	+	-	-	-	697/689	−0.5/-0.8	Nil	

ID/ inheri-tance	Origin	Age (years) / Sex	Visual acuity (logMAR) (RE/LE)	Retinal features	Ocular motility	Kerato-pathy	Cataract	Lens sublux-ation	Glaucoma	CCT (µm) (RE/ LE)	Refraction (RE/LE)	Surgery	Associated conditions
12a/F	Cambodia	51/M	HM:CF	CA	nystagmus	+ (mild)	+ (dense C)	-	-	779/761	−8.0/-8.0	Nil	
12b/F	Cambodia	24/F	CF:CF	FH	nystagmus	-	+ (PSC, C)	-	-	584/607	−12.0/-12.0	Nil	
12c/F	Cambodia	21/F	1.3:1.3	CA	nystagmus	-	-	+	-	653/657	−13.0/-13.0	Nil	
12d/F	Cambodia	14/F	na	FH	nystagmus	-	-	-	-		−2.5/-2.5	Nil	
12e/F	Cambodia	12/M	CF:CF	FH	nystagmus	-	+ (PSC, C)	-	-	602/617	−3.5/-3.5	Nil	
13/F	Cambodia	15/F	0.5:0.4	na	na	-	-	+	+	na	na	Nil	
14/F	Sri Lanka	21/M	HM:NPL	na	na	-	+	+	-	na	na	CAT (LE)	Phthisical eye following surgery
15/F	Sri Lanka	17/M	0.8:0.8	na	na	-	+	-	+	na	−1.8/-2.0	Nil	
16/S	Sri Lanka	21/F	PL:NPL	na	na	-	-	+	+	na	+10.0/-1.0	Nil	Refraction RE secondary to lens subluxation
17/F	Bhutan	14/M	0.9:0.8	na	na	-	+	+	+	na	+8.0/+10.0	Nil	Refraction BE secondary to lens subluxation
18/F	Bhutan	9/F	1.0:1.1	na	na	-	-	+	-	na	+11.0/+11.0	Nil	Refraction BE secondary to lens subluxation

Abbreviations: F, familial; S, sporadic; RE, right eye, LE, left eye; BE, both eyes; CF, count fingers; PL, penetrating light; NPL, no penetrating light; HM, hand motion; FH, foveal hypoplasia; CA, chorioretinal atrophy; PSC, posterior subscapsular; C, cortical; NS, nuclear sclerotic; OHT, ocular hypertension; CCT, central corneal thickness; CAT, cataract extraction; TRAB, trabeculectomy; VIT, vitrectomy; CG, corneal graft; na, not available

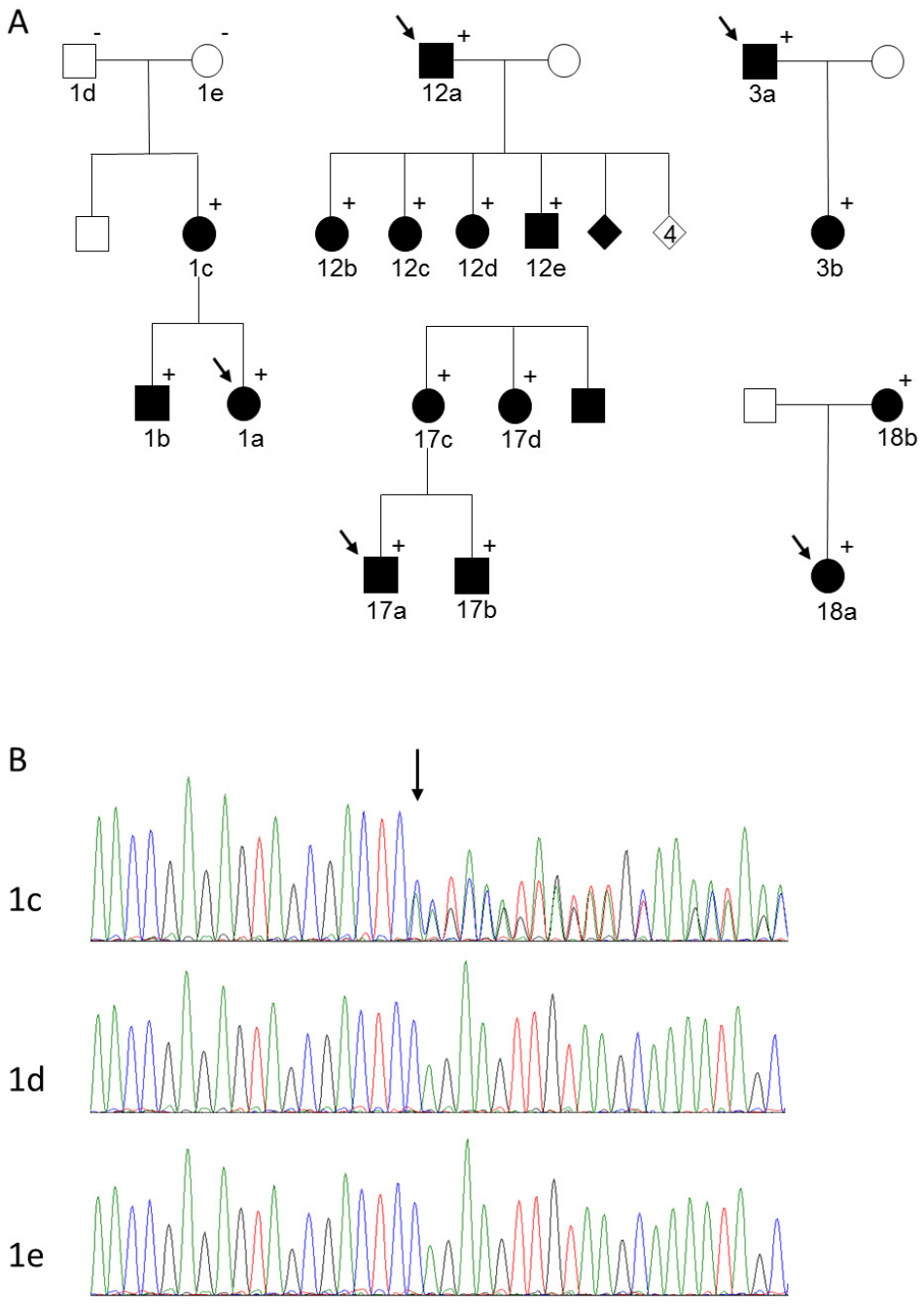

Figure 1. Pedigrees and genetic analysis. **A**: Pedigree of the families recruited. Round symbols indicate female; square, male; diamond, unspecified gender; fully filled symbols, aniridia; unfilled symbols, unaffected; arrow, proband; plus/minus, presence/absence of the familial *PAX6* variant. **B**: *PAX6*: c.238_241dupACTC, p.(Pro81HisfsTer12) sequence variant in affected individual 1c (at the top) and her unaffected grandparents 1d and 1e (at the bottom). The black arrow marks the heterozygous variant.

members of individual 1a and was absent from his maternal grandparents. The variant was confirmed to be de novo with molecular analysis (Figure 1B). Splicing variant c.1183+1G>A was predicted to result in the loss of the last exon. One variant was predicted to result in a string of lysine extension at the end of the PAX6 protein (p.(Ter423LeuextTer15)), leading to translation into the 3′ untranslated region (UTR). This variant was present in individual 12a from Cambodia and segregated in four affected children. Variant p.(Met1?) affected the initiation codon, was novel, and was considered deleterious. The

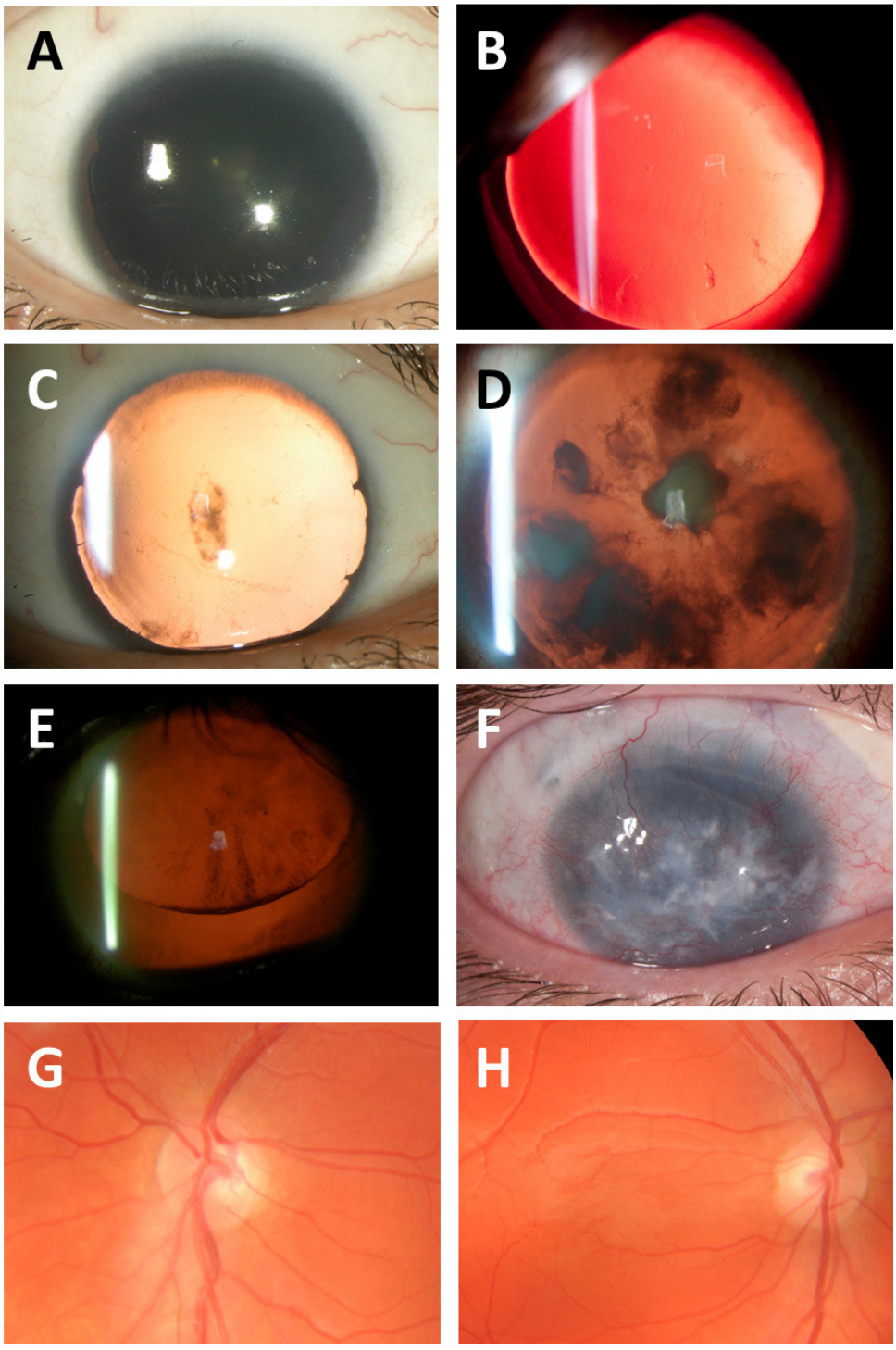

Figure 2. Clinical photographs of the eyes of individuals with aniridia. **A**: Slit-lamp photo showing total aniridia (individual 5). **B**: Slit-lamp retroillumination photo showing total aniridia (individual 2). **C**: Slit-lamp retroillumination photo showing polar cataract (individual 5). **D:** Slit-lamp retroillumination photo showing anterior polar and cortical cataract (individual 7). **E**: Slit-lamp retroillumination photo showing lens subluxation (individual 1b). **F**: Slit-lamp photo showing opacification and vascularization of the cornea due to limbal stem cell failure (individual 1b). **G**: Fundus photography showing small and tilted disc (individual 2). **H**: Fundus photography showing macular hypoplasia (individual 2).

removal of the initiation codon is expected to result in an absence of translation or translation starting at a cryptic site downstream and resulting in a truncated protein missing the N-terminal region that includes the paired DNA-binding domain. Finally, the missense variant, p.(Ile29Thr), has not been previously reported in the literature and was not present in the gnomAD populations database. The missense variant was predicted to be deleterious by SIFT and PolyPhen-2, and was highly conserved among species. As a result, this novel variant was regarded as affecting protein function.

Table 2. Sequence variants identified in the *PAX6* gene in probands.

Proband ID	Location	Domain	Nucleotide change	Amino acid change	References
1a	Exon 6	PD	c.238_241dupACTC	p.(Pro81HisfsTer12)	Novel
2	Exon 5	PD	c.114_121del8	p.(Pro39HisfsTer14)	[1]
4	Exon 10	PST	c.820C>T	p.(Gln274Ter)	Novel
6	Exon 5	PD	c.86T>C	p.(Ile29Thr)	Novel
8	Exon 8	LNK	c.538C>T	p.(Gln180Ter)	[2]
9	Exon 6	PD	c.325G>T	p.(Gly109Ter)	[3]
10	Exon 10	HD	c.781C>T	p.(Arg261Ter)	[1,3-5]
11	Exon 8	LNK	c.607C>T	p.(Arg203Ter)	[2,4-7]
12a	Exon 13	PST	c.1268A>T	p.(Ter423LeuextTer15)	[1,3,5,8]
15	Exon 1	-	c.3G>T	p.(Met1?)	Novel
17	Intron 12	PST	c.1183+1G>A	-	[5]

HD, homeodomain; LNK, linker region; PD, paired domain; PST, proline-threonine-serine-rich domain

The six chromosomal deletions are described in Table 3. MLPA of the *PAX6* gene revealed a partial terminal deletion involving exons 14 and 15 of the gene in individual 3a that segregated in the proband's affected daughter (3b). The *WT1* gene is proximal of *PAX6* and therefore, was not deleted. The karyotype of individual 7 revealed a deletion of the short arm of chromosome 11 (del11(p13-p15)). Heterozygous deletion of the entire *PAX6* gene was confirmed with MLPA. This patient was obese (weight >90th percentile) and had mild intellectual impairment and immunoglobulin A (IgA) nephropathy in addition to the typical features of aniridia, compatible with a diagnosis of WAGRO syndrome. The SNP array of individuals 16 and 18 revealed a deletion of 795 kb and 503 kb of chromosome 11, respectively, both encompassing *PAX6* but not the *WT1* gene. The SNP array of individual 13 identified a 480 kb deletion of chromosome 11 that was 100 kb distal of the *PAX6* gene. Finally, the SNP array of individual 5 identified a 96 kb interstitial deletion at chromosome 11p13 involving *PAX6* but not *WT1*. The expected probe intensities showed a decrease that was less than the expected decrease for a complete loss of one copy of the region, and the allele frequency differed from what was expected under a deletion model, both suggesting mosaicism. The estimated level of mosaicism for the deleted cell line was 30%. There was no association with the aniridia-associated features or the visual acuity between individuals with deletions in *PAX6* compared with those with sequence variants after accounting for age.

DISCUSSION

Phenotype: Variants in the *PAX6* gene have been identified in individuals with aniridia from different populations [14,23-25]. This study is the largest study that includes patients with aniridia from Australia, and the first report of patients with aniridia from New Caledonia, Cambodia, Sri Lanka, and Bhutan. Aniridia is an autosomal dominant disorder with high penetrance but variable expressivity [26]. Abnormalities of the iris are the most common feature of aniridia, and are characterized by either the absence of a visible iris or rudimentary remnant. Foveal hypoplasia is present in the majority

Table 3. 11p13 deletions identified in probands.

Proband ID	Method	Deletion (hg19)	Comments
3a	MLPA	c.(?_858)_(1207_?)del	Deletion of exons 14 & 15 of PAX6
5	MLPA+SNP array	31,742,816–31,838,768	96kb deletion involving *PAX6* in mosaic
7	Karyotype+MLPA	del11(p13-p15)	Deletion involving *PAX6*, *WT1* and *BDNF*
13	MLPA+SNP array	31,225,707–31,705,767	480kb deletion distal of *PAX6*
16	MLPA+SNP array	31,488,890–32,249,249	795kb deletion involving *PAX6*
18	MLPA+SNP array	31,530,678–32,033,826	503kb deletion involving *PAX6*

MLPA: Multiplex Ligation-dependent Probe Amplification

of patients [2,23,27], as shown in this study (78%), and results in decreased visual acuity and nystagmus [1]. Progressive loss of visual function often results from complications, such as cataracts, glaucoma, and corneal opacification, that lead to worsened vision with increasing age. In this cohort, vision ≤ 1.00 logMAR defined as severe visual impairment according to the World Health Organization [28] was present in 65% of eyes, and visual acuity progressively deteriorated with age.

Cataract is the most common complication requiring surgery in aniridia, usually observed in 50–90% of patients [1-3,23,27,29,30]. Cataracts were present in 72% of the cohort. Small lens opacities may be present from birth, but they usually do not impair the vision until the teens or early adulthood [1,3]. This is reflected in the mean age of cataract extraction at 20 years in this cohort. Lens dislocation is usually less common [3,23,30] but was present in 36% of the cohort.

The prevalence of glaucoma in aniridia is reported as 6% to 75% [1], although more recent studies suggest a prevalence around 45–65% [23,27,29,30] with an onset commonly in childhood or early adulthood [27,30]. Glaucoma was present in 44% of the cohort, and 36% needed glaucoma surgery at a mean age of 13 years. Glaucoma in aniridia is often difficult to treat and may require multiple interventions to control the intraocular pressure (IOP) to slow progression of the disease [31,32]. The mechanisms causing glaucoma in patients with aniridia are thought to result from developmental defects in the anterior chamber and impair the aqueous humor outflow through the trabecular meshwork and Schlemm's canal. However, different explanations have been provided regarding the nature of these developmental defects, including progressive angle closure and obstruction of the trabecular meshwork by the residual iris stroma extending anteriorly [33], absence of Schlemm's canal [1], and abnormalities of the canal's inner layer or the trabecular meshwork [32]. Heterozygous *Pax6*-deficient mice show impaired development of the trabecular meshwork and Schlemm's canal [34,35], and growing adhesions between the cornea and the iris periphery, resulting in complete closure of the iridocorneal angle, increased IOP, and development of glaucoma [35].

The central corneal thickness influences IOP measurements with thicker CCT leading to overestimation of IOP [36]. The mean CCT from individuals with aniridia in this study was high (645±74.0 µm) compared to the mean in healthy Australians (540±33.0 µm) [37] and in healthy Asian populations (504–556 µm) [37]. Other studies previously reported thick mean CCT measurements in children and adults with aniridia [15,27,38,39]. These findings indicate that IOP measurement can be less reliable in patients with aniridia and that examination of the optic nerve is important for detecting and managing patients' glaucoma.

Aniridia-associated keratopathy is a serious complication developing in 50–90% of patients and has been reported as the most frequent complication by previous studies [23,27,29,40]. This complication often presents in late childhood or early adulthood and less commonly among younger patients [3,29]. In the present cohort, keratopathy was present in 48% of individuals with a mean age at diagnosis of 19 years (range 9–34 years). Early features involve the peripheral epithelium, and aniridia-associated keratopathy leads to progressive opacification and vascularization of the cornea to complete corneal sclerosis and vascularization as the complication progresses centrally [29]. The keratopathy is believed to result from insufficiency of the limbal stem cell population [41]. Additionally, corneal surface problems may be complicated by abnormal tear film and Meibomian gland dysfunction [12]. In this study, keratopathy was more frequent in the Australasian cohort than in the Southeast Asian cohort. Although this may reflect true ethnic phenotypic variability, an alternative explanation could be reduced access to surgical procedures to address aniridia complications such as cataract, lens dislocation, and glaucoma, as surgical procedures are known to accelerate the development of keratopathy [42]. The latter explanation is supported by the finding that only one of the Southeast Asian individuals had a surgical procedure (cataract surgery in one eye) and could account for the lower prevalence of keratopathy in this study compared with previous reports.

Genotype–phenotype correlations: We identified 11 different variants in *PAX6*, of which four were novel and eight introduced a premature termination codon. A family history of aniridia was present in ten probands. A de novo inheritance was confirmed molecularly in one family. De novo variants in *PAX6* have previously been reported although the prevalence is not known [2,43]. Similar to other studies [13-15], we did not find a statistically significant association between the phenotype of individuals with variants resulting in premature termination codons compared with individuals with deletions in *PAX6*. The absence of phenotypic difference is consistent with the fact that truncated proteins are subject to nonsense-mediated decay [13], resulting in PAX6 haploinsufficiency in a similar manner to deletions in *PAX6*. Interestingly, nonsense suppression drugs have been shown to alter *PAX6* dosage, inhibit disease progression, and reverse some ocular phenotype in *PAX6*-transgenic mice [44]. This therapeutic approach is promising for *PAX6*-associated aniridia because the majority of cases are explained by variants resulting in premature termination codons.

Although variants and gene deletions resulting in PAX6 haploinsufficiency do not present genotype–phenotype correlations, other types of *PAX6* variants display associations. An extension variant, p.(Ter423LeuextTer15), resulting in a longer open reading frame and translation into the 3′ UTR, was identified in five affected individuals from the same family (individuals 12a–e). This variant has been reported among different populations with aniridia [14,15,25]. Partial aniridia and milder phenotypes have also been associated with *PAX6* variants in the proline-threonine-serine-rich (PST) region [26]. However, none of the patients in this study with variants in the PST region had partial aniridia. Hingorani et al. reported that glaucoma was unusual and the iris anomalies were milder in individuals with extension variants in *PAX6* compared to premature termination codon variants [2]. Interestingly, none of the five individuals carrying an extension variant in this study had glaucoma despite their age range (12–51 years) falling within the range of typical glaucoma onset. All individuals from this family had myopia, including three with high myopia (<–6.0 diopters) and two with chorioretinal atrophy consistent with degenerative myopia. Other studies have also noted an association between *PAX6*-associated aniridia and high myopia [2,45]. Interestingly, myopia has been reported in other individuals with a similar C-terminal extension variant as family 12 [2]. A genome-wide association study previously identified significant linkage to myopia on chromosome 11p13 in Caucasians, with strong evidence of linkage, but not association, for genetic markers covering the *PAX6* gene [46]. Ng et al. identified two dinucleotide repeats in the *PAX6* P1 promoter that increased transcriptional activity of *PAX6* and were associated with high myopia in a Chinese population [47]. Additional studies showed conflicting results for the association of *PAX6* polymorphisms with myopia, and a recent meta-analysis concluded that *PAX6* may confer only a small effect, if any, to myopia development [48]. Further studies are needed to clarify the potential involvement of *PAX6* in myopia.

One novel variant, p.(Met1?), was predicted to abolish the initiation codon and was considered deleterious. Although this variant has not been previously reported, other variants affecting the initiation codon of *PAX6* have been described with aniridia [24,25,49]. In the present study, individual 15 carrying variant p.(Met1?) had total aniridia, cataracts, and glaucoma. The phenotype associated with the different variants affecting the initiation codon does not seem to differ from variants resulting in premature termination codons [24,25,49].

One proband carried a missense variant predicted to be deleterious in exon 5, p.(Ile29Thr)). Missense variants associated with aniridia occur more often in exons 5–6 that encode the paired domain important for DNA binding [13]. A previous study showed that missense variants can affect protein stability or impair DNA-binding ability [50]. More recently, Xie et al. demonstrated that missense variants can affect the ability of the protein to activate its target genes [51]. Two other variants affecting the same codon, p.(Ile29Val) and p.(Ile29Ser), have been reported in patients with aniridia in the Human PAX6 Allelic Variant Database. Missense variants have been described in individuals with a range of ocular phenotypes from aniridia to milder non-aniridia phenotypes, including optic nerve anomalies, Peters' anomaly, corectopia, and foveal hypoplasia [13]. Missense variants have been associated with better visual acuity due to the lower rate of associated features [2]. In this study, individual 6 carrying the p.(Ile29Thr) novel variant had visual acuities better than 1.00 logMAR, but he presented total aniridia, glaucoma, cataracts, foveal hypoplasia, and nystagmus, which does not suggest a milder phenotype.

We identified five deletions of the *PAX6* gene using MLPA, one partial and four total deletions, as well as one deletion distal of *PAX6*. The partial *PAX6* deletion involved the last two exons of the gene, resulting in a truncated protein. Three total *PAX6* deletions did not encompass *WT1,* therefore, confirming a diagnosis of isolated aniridia. The other total deletion was part of a larger chromosomal deletion on chromosome 11p (p13-p15) as shown by the karyotype. The phenotype of the patient (individual 7) included aniridia, obesity, mild intellectual impairment, and IgA nephropathy, which is consistent with a diagnosis of WAGRO syndrome. The karyotypes of both parents were normal suggesting a de novo occurrence of the chromosomal deletion.

We identified one deletion in mosaic in 30% cells from blood. Mosaic *PAX6* deletions have been associated with milder phenotypes in some patients with low mosaicism: a 10 Mb deletion in 28% cells was reported in an individual with coloboma [52] and a 5.28 Mb deletion in 45% cells was identified in an individual with mild WAGR syndrome [53]. In comparison, bilateral aniridia and optic atrophy were present in two individuals with a 200 kb deletion in 50% and 60% cells [54]. In this study, individual 5 had bilateral total aniridia with a deletion identified in 30% cells. However, the phenotype associated with mosaic *PAX6* deletion is determined by the ocular tissue whereas the deletion was detected in cells from blood. Therefore, it is possible that the deletion is present in a greater proportion in ocular tissues, accounting for the more severe phenotype.

A deletion distal of *PAX6* was identified in one patient (individual 13). Deletions downstream of *PAX6* that leave the *PAX6* gene intact have been reported in some patients with aniridia [17,55]. These deletions abolish *PAX6* expression and cause aniridia in patients due to the loss of an evolutionary conserved regulatory region containing tissue specific enhancers that are essential for PAX6 expression [17].

The detection rate is similar to other studies that reported intragenic variants and chromosomal rearrangements in approximately 90% of patients with aniridia [14,15]. No *PAX6* variant or copy number variation could be demonstrated in one of the 18 probands (5.6%) with aniridia (individual 14 from Sri Lanka). His phenotype was not different from the other probands in the cohort. He had a visual acuity of hand motion in the right eye due to cataract and a subluxed lens, and had no perception of light in his left eye due to complication of cataract surgery resulting in a phthisical eye. His father was reported as having aniridia but was not available for examination. A single nucleotide substitution located in *PAX6* regulatory elements has been previously reported in an individual with aniridia [56]. Therefore, it is possible that deletions or variants distal of *PAX6*, or variants in other genes, may account for the aniridia in the unsolved case.

In conclusion, we reported a detection rate of 94% for variants and deletions in *PAX6* in individuals with aniridia from Australasia and Southeast Asia. To the best of our knowledge, this study is the first description of aniridia and *PAX6* variations in individuals from Cambodia, Sri Lanka, Bhutan, and New Caledonia. These results support the observation of marked allelic heterogeneity and high prevalence of loss of function variants within *PAX6*-related aniridia. Molecular diagnosis can assist in the clinical management of affected patients and may be essential to future clinical trials.

APPENDIX 1. PRIMERS USED FOR AMPLIFICATION OF THE PAX6 GENE.

To access the data, click or select the words "Appendix 1"

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