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Exome sequencing identifies existing and novel variants in a South African cohort presenting with anterior segment dysgenesis

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ABSTRACT

Anterior segment dysgenesis (ASD) defines a collection of congenital eye disorders that affect structures within the anterior segment of the eye. Mutations in genes that initiate and regulate the complex pathways involved in eye development can cause a spectrum of disorders such as ASD, congenital cataracts and corneal opacity. In South Africa, causes of ASD are poorly understood with few studies looking at the possible genetic basis for these disorders. In this study, we performed exome sequencing on a cohort of South African patients with ASD, focusing on a panel of genes known to regulate eye development pathways, including the *PXDN* gene which has recently been associated with ASD. We identified novel as well as established variants: specifically, we found a disease-causing variant in *PAX6;* variants that are likely to be pathogenic in *GJA8*, *BCOR* and *EPHA2*, as well as variants of uncertain significance in *PXDN* and *LTBP2*. In conclusion, this study is the first to show disease-causing variants in South African patients presenting with ASD, including the identification of novel variants and highlights the need to expand upon such studies in understudied populations.

1. Introduction

Anterior segment dysgenesis defines a collection of various rare congenital eye disorders that affect structures within the anterior segment of the eye, including the cornea, iris, lens, trabecular meshwork and ciliary body (Kuang et al., 2023). These disorders typically involve anatomical alterations that affect adhesion between different eye structures (such as between the lens and cornea or cornea and iris) and can lead to corneal opacity; iris hypoplasia and posterior embryotoxon, which can present individually or in combination (Sowden, 2007). ASD is also accompanied by a 50 % increase in predisposition to developing glaucoma (Sowden, 2007). The most common disorders that fall under the umbrella term of ASD are: Axenfeld-Rieger-Syndrome (ARS) that shows both eye anomalies as well as systemic features (Alward, 2000); Peters anomaly, which is characterised by cornea-iris adhesion, corneal leukoma and shallow anterior chamber (Harissi-Dagher and Colby, 2008); Aniridia, which displays iris hypoplasia together with other eye abnormalities such as corneal opacity, lens dislocation and ciliary body hypoplasia as well as foveal hypoplasia, cataract, early onset glaucoma, retinal detachment and aniridia-associated keratopathy, all of which can lead to impaired or complete loss of vision (Lee et al., 2008); Sclerocornea is characterised by noninflammatory, nonprogressive asymmetric scleralisation of the cornea and can be associated with cataracts and coloboma (Kenyon, 1975); Microphthalmia, Anophthalmia and Coloboma disorders present as a syndrome with disruptions in the normal formation of the anterior segment and congenital cataracts may be seen in patients with microphthalmia (Verma and Fitzpatrick, 2007).

In an attempt to genetically characterise these disorders,

Abbreviations: ASD, Anterior Segment Dysgenesis; ARS, Axenfeld-Rieger Syndrome; BAM, Binary Alignment Map; BCOR, BCL6 corepressor; ECM, extracellular matrix; GJA8, Gap Junction Protein alpha 8; IGV, Integrative Genomics Viewer; LTBP2, Latent Transforming Growth Factor Beta Binding Protein 2; NGS, Next Generation Sequencing; PAX6, Paired box protein 6; PXDN, Peroxidasin; VCF, Variant Call Format; VEP, Variant Effect Predictor.

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investigations have been carried out in cohorts of various ethnic groups presenting with ASD that have indicated that there is wide genetic heterogeneity leading to these ocular malformations (Reis et al., 2024). Mutations have been found in various genes including those that initiate and regulate the complex pathways involved in eye development (as reviewed in 8). One such example is the well-established master transcriptional regulator paired box protein 6 (*PAX6*) that is expressed in the very early stages of eye development to drive the organisation of the anterior segment (Lima Cunha et al., 2019). The most recent addition to this panel of genes is peroxidasin (*PXDN*), which is predominantly expressed in the layers of lens and corneal epithelium where it PXDN crosslinks collagen IV in the basement membranes (Yan et al., 2014). Loss of functional PXDN through mutations is thought to cause the loss of structural integrity of the lens capsule and lens molecules such as γ-crystallin, which, as a result, seem to be extruded into the anterior and posterior chamber of the eye (Khan et al., 2011; Choi et al., 2015).

In South Africa, there is limited epidemiological data regarding the burden of eye diseases; glaucoma, cataract, corneal scarring and refractive errors have been found to be major causes of impaired vision and blindness in South Africa (Roberts et al., 2016). In recent years, there has been a drive to genetically characterise eye disease in South Africa, although there are very few studies looking at ASD (Verwey and Mahomed, 2020; Carstens et al., 2016; Carstens et al., 2022; Goolam et al., 2018). Further, the genetic diversity within the South African population, contributes to the difficulty in genetic diagnosis within this population in general (Choudhury et al., 2017). Bantu-speakers make up almost 80 % of the South African population, with the remainder composed of mostly the Coloured population (an admixed population that includes multiple ancestries), whites of European origin and an Indian population, are genetically diverse and studies have identified significant numbers of novel single nucleotide variants within this population (Choudhury et al., 2017).

In this study, we sought to identify genetic mutations in a cohort of South African patients presenting with ASD and this was achieved by performing exome sequencing on a panel of genes known to be involved in eye development pathways.

2. Methods and Materials

2.1. Recruitment of study participants

The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg, South Africa (protocol number: M131125), and written informed consent was obtained from all participants, which ranged from 4 to 53 years old. A total of 14 patients (plus the two parents of one of the patients for segregation analysis) were recruited through ophthalmology and genetics clinics hosted by the University of the Witwatersrand, Johannesburg and all patients were diagnosed or identified for the study by a qualified ophthalmologist and medical geneticist.

2.2. DNA preparation

DNA was extracted using prepIT®.L2P kit (DNA Genotek, OraSure Technologies Inc, Bethlehem, Pennsylvania, USA) for saliva samples, while Monarch® Genomic DNA purification kit (New England Biolabs® Inc, Ipswich, Massachusetts, USA) was used for blood samples. The DNA samples were tested for purity and quantified with the aid of the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Double stranded DNA was quantified and assessed using the Invitrogen Qubit 4.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) before being used for Next Generation Sequencing (NGS).

2.3. Target gene selection and Ion Torrent gene panel design

All sequencing reagents used were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Candidate genes were selected for sequencing based on their involvement in eye development pathways and were previously examined in other study cohorts (Sinn and Wittbrodt, 2013). In total, 50 candidate genes were chosen then submitted to the Ion AmpliSeq™ Designer tool, of which only 43 genes were pre-validated and could be ordered on demand, while 7 genes (*FOXC1, FOXC2, FOXE3, GDF6, SMOC1, RARB* and *MAF*) required a spike-in panel to be designed separately and as such these were not screened for genetic variants in this study.

2.4. Ion Torrent exome sequencing

The 43 pre-validated genes were uploaded onto Ion Ampliseq designer to generate a custom sequencing gene panel assay, which was uploaded onto a Ion 520™ sequencing chip. The DNA library was prepared as stipulated in the DNA library preparation protocol system user guide (Publication number: MAN0013432) of the Ion Chef™ as well as DNA templating with the aid of the Ion 520™ Kit user guide (Publication number: MAN0016854) prior to sequencing. The Ion 520™ chip was loaded into the Ion S5™ Sequencer to perform exome sequencing on the DNA samples.

2.5. Calling, prioritisation and classification of variants

The Ion Torrent Suite™ software v5.14 generated two output files per sample: a variant call format (VCF) file type and a binary alignment map (BAM) file type, which were uploaded onto the Ensembl Variant Effect Predictor (VEP) and Integrative Genomics Viewer (IGV), respectively, for quality control filtration as well as prioritisation (Thorvaldsdóttir et al., 2013; McLaren et al., 2016; Dashti and Gamieldien, 2017). Detected or called genetic variants within the VCF file, as well as read alignment data in the BAM file per patient, were contrasted to the human reference genome (GRCh37 − hg19) build 37.

The variants were submitted into various population databases including: the genome aggregation database (gnomAD); 1000 genomes project, and exome aggregation consortium (ExAC), to determine the minor allele frequency (MAF) as an indication of how common (MAF > 5 %) or rare (MAF < 1 %) these variants are in the general population of various ethnic groups. The National Centre for Biotechnology Information (NCBI) ClinVar database Variant Effect Predictor (VEP), VARSOME and American College of Medical Genetics and Genomics (ACMG) guidelines were used to assess variant effect on protein function as well as classification as to whether variants were (likely) pathogenic or (likely) benign (Richards et al., 2015). VARSOME and VEP built-in plugins enabled access to *in silico* predictive tools, including Combined Annotation Dependent Depletion (CADD); Deleterious Annotation of Genetic Variants using Neural Network (DANN); Sorting Intolerant From Tolerant (SIFT); MutationAssesor; Polymorphism Phenotyping version 2 (PolyPhen2); dbscSNV and Functional Analysis Through Hidden Markov Models (FATHMM), which enabled the prediction of pathogenicity of the variants (Kopanos et al., 2019; Rentzsch et al., 2019; Quang et al., 2015; Kumar et al., 2009; Adzhubei et al., 2013; Jian et al., 2014; Shihab et al., 2013).

3. Results

3.1. Exome sequencing quality control metrics

The quality of the NGS preparations was assessed by examining the quality control parameters of each sequencing run before and after alignment of sequencing reads. The 16 DNA samples were sequenced in batches of eight on two separate runs. The overall data indicated that both runs were of high-quality as the first run showed that 59 % of the

reads were useable, 94 % of the wells were loaded efficiently, only 7 % of the reads were of low quality and with 36 % polyclonality. The second run also showed similar quality data in that 71 % of the reads were useable while 92 % of the wells were loaded successfully, 6 % of the reads were of low quality and with 24 % polyclonality.

3.2. Variant prioritisation and classification in the study cohort

Variant prioritisation following filtration and classification, revealed a disease-causing variant in *PAX6*; three likely pathogenic variants (Table 1) in *Gap Junction Protein alpha 8* (*GJA8)*; *BCL6 corepressor* (*BCOR*) and *EPH receptor A2* (*EPHA2*), as well as four variants of uncertain significance (VUS) with two in *Latent Transforming Growth Factor Beta Binding Protein 2* (*LTBP2*) and two in *Peroxidasin* (*PXDN*) (Table 2).

A nonsense stop gained *PAX6* heterozygous variant NM_001258462.3: c.760C > T (p. Arg254Ter) in exon 10, was detected in a 31-year-old black male (ID: 15) diagnosed with aniridia and cataracts complicated by secondary glaucoma. The patient's maternal grandfather, mother, two siblings and two children are all similarly affected. The variant has three submissions on ClinVar (Accession numbers RCV000003632.3, RCV000312176 and RCV000536976.3) with two associating the variant with aniridia and classifying it as pathogenic. The vast majority of *in silico* predictions showed the variant to be pathogenic, with only one predicting a low impact effect. IGV showed that the variant call was accurate as most reads showed the presence of the alternate allele A instead of the G reference allele upon manual assessment of variant coverage (Fig. 1A).

A *GJA8* heterozygous missense variant NM_005267.5: c.22G > A (p. Gly8Arg) in exon 2 was detected in a 6-year-old black male (ID:16) diagnosed with Axenfeld-Rieger anomaly at birth (Table 1) with profound hearing loss, global neurodevelopmental delay, subtle dysmorphic features and microcephaly. This *GJA8* variant was classified as likely pathogenic and has not previously been reported in ClinVar but has a dbSNP ID of rs202041458 with MAF of 0.000185 (~ 1 in 5418) in Africans while south Asians have a frequency of 0.0000239 (~ 1 in 41902), with other ethnic groups having no entry in gnomAD. IGV indicated that the variant was called with high confidence as the majority of the reads had the alternate allele of A, whilst the reference genome sequence has a reference allele of G (Fig. 1B).

A variant in *BCOR* was identified as a frameshift NM_001123385.2: c.1257_1260delAGATinsGAC (p.Asp420ThrfsTer22), causing a truncated protein, located in exon 4 (of 15). This was detected in two patients: a 31-year-old male (ID: 15) (that also harboured the *PAX6* mutation detailed above) and a 27-year-old black female (ID: 6) with ARS as evidenced by an Axenfeld anterior segment angle with iris processes but no posterior embryotoxon and asymmetrical glaucoma (Table 1). This *BCOR* variant has no ClinVar submissions, gnomAD genomes entry or dbSNP ID. As a frameshift, the Varsome *in silico* tools could not predict the pathogenicity, however MutationTaster predicted a deleterious/pathogenic outcome, which is probable considering that this variant causes a premature stop codon early in the amino acid sequence (in exon 4 resulting in the loss of exons 5–15) which would impact protein function. The read depth of the variant was of good quality, as shown in the IGV plot (Fig. 2).

A novel *EPHA2* frameshift variant NM_004431.5: c.986_987delC-CinsT (p.Pro329LeufsTer64), causing a truncated protein, in exon 5 was detected in a 7-year-old black female (ID:13) diagnosed with Peters anomaly associated with persistent hyperplastic foetal vasculature with age-appropriate neurodevelopment, normal growth and no other congenital malformations (Table 1). The variant has a gnomAD allele frequency of 0.00006394 (~ 1 in 15639) in Africans and a global allele frequency of 0.00001636 (~ 1 in 61110). Varsome *in silico* computational tools provided no prediction data on the impact of this variant and there was also no ClinVar submission for this variant, which might be due to the variant being potentially novel. The read depth of the variant was of good quality, as shown in the IGV plot (Fig. 3).

We also identified four variants of uncertain significance (Table 2). Two appeared in *LTBP2*: the first, a heterozygous missense NM_000428.3:c.1577G > A (p.Ser526Asn) in exon 7 was detected in a 4-year-old black male (ID: 12) with Peters anomaly who presented with kerato-irido-lenticular dysgenesis and persistent hyperplastic primary vitreous. The patient's unaffected mother (ID: 11) harboured the same variant. This variant did not have a ClinVar submission, but had a MAF of 0.00259 in Africans, in contrast to 0.00000884 in Europeans (Non-Finnish) as well as 0.000347 in Latinos and has a dbSNP ID rs142979965. The second *LTBP2* variant identified was a heterozygous splice site variant NM_000428.3:c.3527-3C > A in intron 23. This was identified in a 56-year-old black female (ID: 1) with ARS; both the mother and maternal grandmother were similarly affected. She had no systemic features but had bilateral posterior embryotoxon, iris atrophy, pseudopolycoria and angle iris processes. The variant has a gnomAD entry with MAF of 0.0124 in Africans, while 0.0000617 was reported in European (Non-Finnish), 0.00055 in Latino and 0.0000327 in South Asian populations. This variant has a dbSNP ID of rs138194436 and ClinVar has three submitters for this variant (RCV000909576, RCV000305307, RCV000403118). In silico tools gave conflicting interpretation of pathogenicity from benign through to damaging.

Two variants of uncertain significance were detected in *PXDN*, both being heterozygous *PXDN* missense variants: NM_012293.3:c.2827C > T (p.Arg943Trp) variant located in exon 17 and NM_012293.3:c.1112C >

Table 1
Pathogenic variants detected in the patient cohort.

GENE	Impact	Variant	MAF gnomAD (Africans)	dbSNP ID	In Silico tools (Benign)	In Silico tools (Pathogenic)	ACMG-AMP Code	ACMG classification	Diagnosis	Patient (s) with variant
PAX6	Stop gained	NM_005267.5: c.760C > T (p.Arg254Ter)	*	rs121907917	*	DANN, FATHMM, MutationTaster	PVS1, PP5, PM2	Pathogenic	Aniridia	ID:15
GJA8	Missense	NM_001258462.3: c.22G > A (p.Gly8Arg)	0.000185	rs202041458	Mutation Assessor	DANN, SIFT, FATHMM, MutationTaster & 7 more (see Methods).	PM2, PP2, PP3	Likely Pathogenic	Axenfeld-Rieger syndrome	ID:16
BCOR	Frameshift	NM_001123385.2: c.1257_1260delinsGAC	*	*	*	MutationTaster	PVS1and PM2	Likely Pathogenic	Axenfeld-Rieger syndrome Aniridia Unaffected	ID: 6 ID: 15 ID: 11 Mother of ID: 12
EPHA2	Frameshift	NM_004431.5: c.986_987delinsT	0.00006394	*	*	MutationTaster	PVS1 and PM2	Likely Pathogenic	Peters Anomaly	ID: 13

*= Data not available; MAF = Minor allele frequency; PM2 = Absent from controls; PP2 = Missense variants common cause of disease; PP3 = Computational evidence; PVS1 = Null variant in a gene where loss of function is a known mechanism of disease; PM2 = Absent from controls.

Table 2
Variants of uncertain significance prioritised in this study.

GENE	Impact	Variant	MAF gnomAD (Africans)	dbSNP ID	In Silico tools (Benign)	In Silico tools (Pathogenic)	ACMG-AMP Code	ACMG classification	Diagnosis	Individual (s) with variant
LTBP2	Missense	NM_000428.3: c.1577G > A	0.00259	rs142979965	BayesDel_addAF, DEOGEN2, EIGEN, LIST-S2, M−CAP, MVP, PrimateAI and SIFT	DANN, FATHMM-MKL, MutationAssess and MutationTaster	PM2 and BP4	VUS	Peters anomaly Unaffected	ID: 12 ID: 11 Mother of ID: 12
LTBP2	Splice region	NM_000428.3: c.3527-3C > A	0.0124	rs138194436	DANN	MutationTaster	PM4, PP3 and PM2	VUS	Axenfeld-Rieger syndrome	ID: 1
PXDN	Missense	NM_012293.3: c.1112C > T	0.00352	rs201592851	BayesDel_addAF, DEOGEN2, MVP and PrimateAI	DANN, EIGEN, FATHMM-MKL, LIST-S2, M−CAP, MutationAssess/ Taster and SIFT	PM2 and PP3	VUS	Axenfeld-Rieger syndrome	ID: 14
PXDN	Missense	NM_012293.3: c.2827C > T	0.00491	rs200127189	BayesDel_addAF, DEOGEN2, EIGEN and MVP	DANN, FATHMM-MKL, LIST-S2, M−CAP, MutationAssessor, MutationTaster, PrimateAI and SIFT	PM2 and PP3	VUS	Peters anomaly Axenfeld-Rieger syndrome Unaffected	ID: 12 ID: 14 ID: 10 Father of ID: 12

*= Data not available; MAF = Minor allele frequency; PVS1 = Null variant in a gene where loss of function is a known mechanism of disease; BP4 = Multiple lines of computational evidence suggest no impact on gene or gene product; PM2 = Absent from controls; PP2 = Missense variants common cause of disease; PP3 = Computational evidence; PM4 = Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants; VUS = variant of uncertain significance.

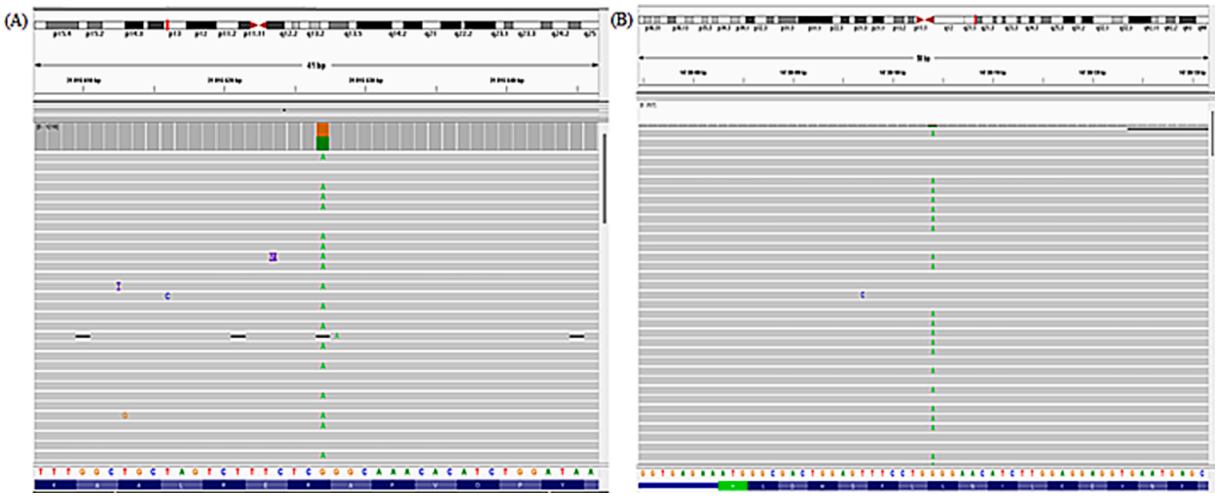

Fig. 1. IGV plot of PAX6 and *GJA8* pathogenic variants. (A) Heterozygous PAX6 c.760C > T (p. Arg254Ter) variant detected in a patient (ID:15) with aniridia. The reference genome sequence shows a reference allele as G (Mustard), which is changed to the alternate allele of A (Green). (B) Heterozygous c.22G > A (p. Gly8Arg) missense variant in exon 2 of *GJA8* in a patient (ID:16) diagnosed with ARS. The reference sequence records a G (Mustard) allele at this position, while the reads show an alternate allele of A (Green) across the multiple reads.

T (p.Pro371Leu) located in exon 10 (Fig. 4). The c.2827C > T (p. Arg943Trp) variant was identified in the 4-year-old male with Peters anomaly (ID: 12, described above), his unaffected father (ID: 10) as well as in a 34-year-old black female diagnosed with ARS (ID: 14) that presented with advanced juvenile onset open angle glaucoma with an Axenfeld angle with numerous iris processes but no posterior embryotoxon or systemic features (Table 2). The variant has the dbSNP ID rs200127189, two ClinVar submissions (RCV001546804 and RCV000533505), a gnomAD MAF of 0.00491 in Africans, in contrast to 0.00000958 in Europeans (Non-Finnish), 0.000353 in Latinos and 0.0000333 in South Asians. Eight *in silico* predictive tools predicted this variant could be pathogenic with four predicting a benign impact. AlphaMissense predicts Arg943Trp to be likely pathogenic; this amino acid change could have a significant effect on protein structure considering that the change is from a basic arginine to a nonpolar heterocyclic tryptophan. The c.1112C > T (p.Pro371Leu) variant was identified in a patient with ARS (ID: 14, as described above) and who thus harboured both *PXDN* variants identified. This variant has a dbSNP ID of rs201592851 and a gnomAD entry for MAF of 0.00352 in Africans in contrast to 0.0000558 in East Asians, 0.0000183 in European (not including Finnish), 0.000262 in Latino and 0.000131 in South Asian populations. Eight *in silico* predictive tools predicted this variant as pathogenic while only four predicted a benign impact. AlphaMissense predicts the Pro371Leu change to be likely pathogenic. In ClinVar, two submissions have been recorded (RCV001556898 and RCV000950335).

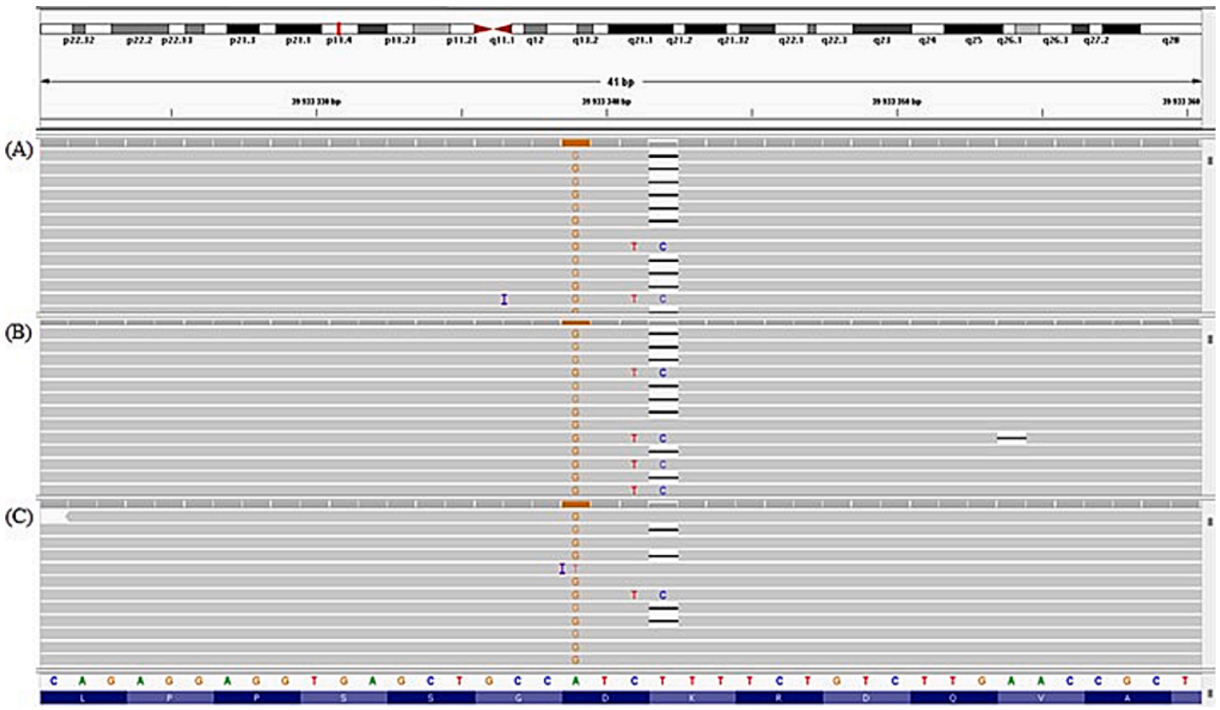

Fig. 2. ***BCOR*** **gene frameshift c.1257_1260delAGATinsGAC (p.Asp420ThrfsTer22) variant as depicted by IGV.** (A) read depth of ID:15 presenting with aniridia. (B) Patient (ID:6) presenting with ARS. (C) Mother (ID:11) of a patient (ID:12) who did not inherit the variant.

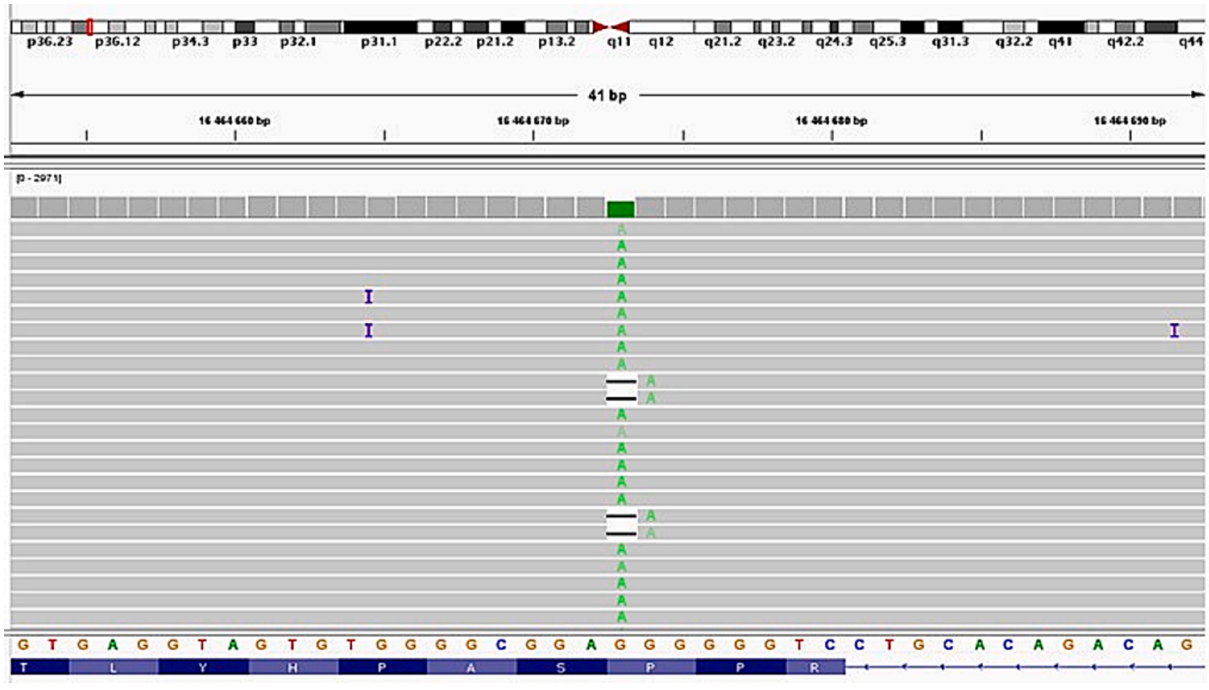

Fig. 3. ***EPHA2*** **gene frameshift c.986_987delinsT variant as depicted by IGV**. Read depth of the 7-year old female patient (ID:13) presenting with Peters Anomaly.

4. Discussion

The study presented herein focuses on a largely genetically uncharacterised group of South African patients with ASD. We performed exome sequencing on 14 patients with ASD disorders (and the two parents of one of the patients), for 43 genes available on an on-demand panel for the Ion Torrent, and we identified existing and novel disease-causing variants in *PAX6* (pathogenic); *GJA8, BCOR* and *EPHA2* (likely pathogenic), while variants of unknown significance were observed in *LTBP2* and *PXDN*.

A heterozygous stop gained *PAX6* mutation NM_001258462.3: c.760C > T (p. Arg254Ter) was detected in a single patient diagnosed with aniridia. PAX 6 is a key transcriptional regulator expressed in various structures in the eye, including the cornea, lens and retina (Sunny et al., 2022). Interestingly, this variant is located within the proline-serine-threonine transactivation domain of the transcription

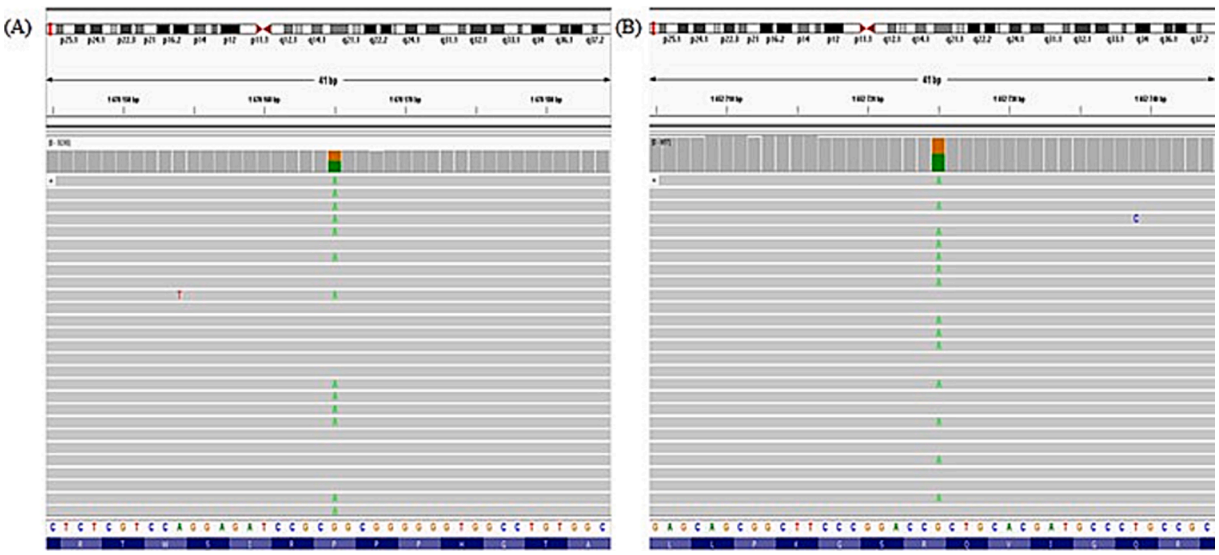

Fig. 4. *PXDN* gene missense variants c.1112C > T (p.Asp420ThrfsTer22) and c.2827C > T (p.Arg943Trp) as depicted by IGV. (A) read depth of patient (ID:14) presenting with ARS. (B) *PXDN* c.2827C > T variant read depth observed in the same patient with ARS (ID:14), a patient with Peters Anomaly (ID: 12) and the unaffected father (ID:10) of this patient.

factor, which is a highly conserved region found in all PAX family proteins. This region is tightly regulated by phosphorylation and leads to the subsequent downstream activation of PAX6 target genes, many of which are involved in eye development (Sunny et al., 2022). The family history of the patient indicates that there is an autosomal dominant inheritance pattern of aniridia in the family, in line with the inheritance pattern of aniridia; 67 % of all reported cases are familial and co-segregate in this manner with high penetrance, while the rest are sporadic [321]. Furthermore, the majority of pathogenic variants in *PAX6* that result in loss-of-function associate with aniridia, notwithstanding the potential for haploinsufficiency that can also lead to this phenotype (Vincent et al., 2003). Indeed, variants causing truncations, as the variant we identified, generate transcripts that can undergo nonsense-mediated decay (Vincent et al., 2003). Variants in *PAX6*, including this one, have previously been observed in aniridia patients from different ethnicities (Lagali et al., 2020), however this is the first study to report this variant in South Africa. Previously, a different *PAX6* variant NM_000280.3:c.622G > A (p.Gly208Arg) was identified in 27 members of an admixed South African family diagnosed with nystagmus, coloboma as well as familial congenital cataracts and co-segregation of the variant with the clinical phenotypes was observed in all 27 family members (Goolam et al., 2018). Our study has further validated the importance of screening for *PAX6* variants in South African patients diagnosed with ASD.

A missense *GJA8* heterozygous NM_005267.5: c.22G > A (p.Gly8Arg) variant was identified in a patient diagnosed with ARS. This is a novel finding since *GJA8* pathogenic missense variants have not previously been reported in this disease, but rather have previously been found to be associated with cataracts, microcornea, microphthalmia, iris hypoplasia, sclerocornea and abnormal lens development, as it is mostly expressed in the lens in lens fibre cells (Ma et al., 2016; Ma et al., 2018; Xia et al., 2012). This missense variant is located in exon 2 in the conserved intramembrane N-terminal domain of GJA8. The variant has an existing dbSNP ID of rs202041458 but has not been reported in the literature for association with any clinical phenotype. The *GJA8* gene encodes for connexin50, which belongs to a transmembrane family of proteins crucial for intercellular communication and moderating diffusion of molecules between lens fibre cells (Yu et al., 2016). Missense mutations in *GJA8* affect various regions of the protein, including the N-terminal, with three pathogenic variants observed previously and with all three mutations associated with nuclear cataracts (Ma et al., 2016; Mackay et al., 2014; Willoughby et al., 2003). To date, pathogenic variants primarily in *FOXC1* and *PITX2* genes have been established to cause ARS, although other genes have also been found to be involved (Bolton and Bohnsack, 2024). This finding therefore requires further consideration and exploration into its role in ARS in South African ASD and ARS patients. In the study presented herein, *PITX2* was successfully sequenced in all 14 patients but no pathogenic variants were identified. However, *FOXC1* was not sequenced due to the gene not being available on the gene panel. This highlights the need for an on-demand panel tailored to ASD.

A novel frameshift variant was identified in the *BCOR* gene c.1257_1260delAGATinsGAC and detected in two patients, one diagnosed with aniridia and one with ARS. The variant was classified as pathogenic due to the introduction of a pre-mature stop codon resulting in a truncated protein. *BCOR* encodes for the BCL 6 corepressor protein that functions in early development and is expressed in the lens and retina, as well as the neural tube during development (Astolfi et al., n.d; Wamstad et al., 2008). Two domains primarily regulate the function of BCOR namely the PCGF Ub-like fold discriminator (PUFD) domain, which is implicated in histone regulation, and the BCL-6 DNA binding domain that interacts with BCL-6 transcriptional repressor (41). Previously identified *BCOR* variants have been associated with oculofaciocardiodental (OFCD), lenz microphthalmia syndrome and ARS (Ng et al., 2004; Ragge et al., 2019; Reis et al., 2023). Here we identified two patients, one with ARS and one with aniridia, the latter also harbouring a pathogenic variant in *PAX6* c.760C > T (p. Arg254Ter). Due to a lack of previously identified *BCOR* variants in aniridia, it is difficult to state with certainty that this variant associates with aniridia; it is therefore important to expand this current study and consider this as a possible aniridia variant. The *BCOR* variant was also observed in the unaffected mother of the male patient ID:12 but since *BCOR* is on the X-chromosome, the male patient (ID:12) likely inherited the allele without the variant. It is also possible that the variant could be a partial sequencing artefact, which will require further validation. Since our finding together with the recent literature, have identified *BCOR* variants in ARS, further studies are warranted to understand the role of BCOR in eye development.

A novel frameshift variant was identified in *EPHA2* NM_004431.5: c.986_987delinsT (p.Pro329LeufsTer64) and observed in a patient presenting with Peters anomaly and was classified as likely pathogenic. *EPHA2* encodes for ephrin receptor tyrosine kinase-type A2 (EPHA2), a

transmembrane receptor highly expressed during embryogenesis that is required for the normal development of various organs, including the eye (mostly in the lens epithelial and fibre cells), kidney and ear (Park et al., 2013). *EPHA2* pathogenic variants cause eye development disorders such as cataracts and microphthalmia (Dave et al., 2013; Harding et al., 2021). Our finding adds support to the importance of EPHA2 in eye development and demonstrate a potential novel association of *EPHA2* gene variants with Peters anomaly. Further investigations would assist in determining whether this novel variant results in a truncated protein or may lead to nonsense mediated decay due to the premature stop codon, and as such could aid in understanding the contribution of EPHA2 to the Peters anomaly diagnosis. Previously studies have suggested that truncating variants may cause more severe phenotypes than other forms of variants (Courdier et al., 2022).

We also identified two *LTBP2* heterozygous missense variants, NM_000428.3:c.1577G > A (p.Ser526Asn) in exon 7 detected in a child patient with Peters anomaly (his unaffected mother was also a carrier of the variant), and the heterozygous splice-site variant NM_000428.3: c.3527-3C > A in intron 23 identified in a patient with ARS. LTBP2 is predominantly expressed in the ciliary zonule and is required for the normal development of the anterior chamber of the eye where it is involved in cell adhesion, tissue repair and regulates the synthesis of the aqueous humour (Shipley et al., 2000). Mutations in *LTBP2* have been previously reported in patients with primary congenital glaucoma (PCG) (Ali et al., 2009); how these variants result in clinical phenotypes such as PCG is not well understood but could involve regulating elastic fibres in developing elastic tissues and association with fibrillin 1 in the ECM (Vehviläinen et al., 2009). A previous study in South Africans found no pathogenic variants in *LTBP2* in patients with PCG (Carstens et al., 2022). *LTBP2* pathogenic variants have also been associated lens dislocation, secondary glaucoma, megalocornea, microspherophakia and Weill Marchesani syndrome (Désir et al., 2010; Kumar et al., 2010; Haji-Seyed-Javadi et al., 2012). Interestingly, the child with Peters anomaly harbouring the *LTBP2* c.1577G > A missense variant inherited from the mother also has a second heterozygous missense variant in *PXDN* c.2827C > T inherited from the patient's father. This compound heterozygosity could explain the atypical clinical phenotype in the patient.

Lastly, we found two *PXDN* heterozygous missense mutations: NM_012293.3:c.1112C > T (p.Pro371Leu) in exon 10 (rs201592851) and NM_012293.3:c.2827C > T (p.Arg943Trp) in exon 17 (rs200127189). A patient with ARS was found to have both missense variants and a child with Peters anomaly had the latter variant. PXDN is expressed in the corneal and lens epithelium where it is secreted into the extracellular matrix (Yan et al., 2014). Mutations in *PXDN* have been identified in various population groups presenting with congenital cataract, congenital glaucoma, corneal opacity, microphthalmia, developmental delays, ASD, hypotonia and sclerocornea (Khan et al., 2011; Choi et al., 2015; Micheal et al., 2016). The two *PXDN* variants identified in our study are located in exons 10 and 17; regions in which other pathogenic variants have been previously reported and fall within the Ig-like domain and haem peroxidase domain, respectively. Amino acid changes within these conserved domains are likely to affect both protein structure and enzymatic ability of PXDN, respectively. To date, only one study has looked for an association of *PXDN* gene variants with ASD in an African family (Thanikachalam et al., 2020) and none in a South African cohort, and as such our findings thereby further validate the significance of PXDN in eye development in South Africans.

In summary, we sequenced 43 genes involved in eye development pathways, in a South African cohort presenting with ASD using an on-demand panel. We identified one patient presenting with aniridia that harboured a recurrent pathogenic *PAX6* gene variant; four patients presenting with ARS that harboured variants in *PXDN, LTBP2, EPHA2* and *BCOR*, with one patient harbouring a pathogenic *GJA8* variant. All patients presenting with ARS will require additional screening for *FOXC1* mutations given that this gene was not available on this panel. For the remaining patients presenting with ASD but with no pathogenic variants detected, additional screening is warranted for genes that were unavailable on the on-demand-panel for IonTorrent sequencing. Further, Sanger sequencing of family members of the patients with pathogenic variants as well as variants of uncertain significance will also be a valuable validation. To conclude, despite the small sample size, variants of significant interest have been identified within this cohort that warrant further expansion in this understudied population.

CRediT authorship contribution statement

Tebogo Marutha: Writing – original draft, Methodology, Formal analysis, Data curation. **Sue Williams:** Writing – review & editing, Resources, Methodology, Formal analysis, Data curation. **Michael Novellie:** Methodology. **Bronwyn Dillon:** Methodology. **Nadia Carstens:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Demetra Mavri-Damelin:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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