

ARTICLE

Exome sequencing in developmental eye disease leads to identification of causal variants in *GJA8*, *CRYGC*, *PAX6* and *CYP1B1*

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Developmental eye diseases, including cataract/microcornea, Peters anomaly and coloboma/microphthalmia/anophthalmia, are caused by mutations encoding many different signalling and structural proteins in the developing eye. All modes of Mendelian inheritance occur and many are sporadic cases, so provision of accurate recurrence risk information for families and affected individuals is highly challenging. Extreme genetic heterogeneity renders testing for all known disease genes clinically unavailable with traditional methods. We used whole-exome sequencing in 11 unrelated developmental eye disease patients, as it provides a strategy for assessment of multiple disease genes simultaneously. We identified five causative variants in four patients in four different disease genes, *GJA8*, *CRYGC*, *PAX6* and *CYP1B1*. This detection rate (36%) is high for a group of patients where clinical testing is frequently not undertaken due to lack of availability and cost. The results affected clinical management in all cases. These variants were detected in the cataract/microcornea and Peters anomaly patients. In two patients with coloboma/microphthalmia, variants in *ABCB6* and *GDF3* were identified with incomplete penetrance, highlighting the complex inheritance pattern associated with this phenotype. In the coloboma/microphthalmia patients, four other variants were identified in *CYP1B1*, and *CYP1B1* emerged as a candidate gene to be considered as a modifier in coloboma/microphthalmia.

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INTRODUCTION

Developmental eye diseases are those where development of the eye can be affected through a number of different processes influencing development of the various components of the eye, including the cornea, lens, iris and optic fissure. Progress in genetic diagnostic capability in patients with developmental eye diseases, including cataract/microcornea, disorders of the anterior segment and coloboma/microphthalmia/anophthalmia, has been hampered by the marked genetic heterogeneity of these conditions. Although a number of disease genes have been identified, pursuit of a molecular diagnosis is often not clear-cut as there is an overlap in the clinical features associated with mutations in the various underlying disease genes (Figure 1).^{1,2} In some cases, molecular diagnosis may be aided by syndrome identification or a chromosomal anomaly may be revealed.³ For isolated cases, which are the majority, molecular diagnosis is challenging, as the required sequential sequencing for identification of underlying mutation/s is rarely completed owing to the lack of an available panel covering all the known genes.

Congenital cataracts associated with microcornea, with or without microphthalmia, may be caused by mutations in at least nine genes,

including *GJA8*, *CRYAA*, *CRYBB1*, *CRYBA4*, *CRYBB2*, *CRYGC*, *CRYGD*, *MAF* and *FOXE3*.⁴ Other disorders of the anterior segment of the eye also show marked genetic heterogeneity and include isolated trabecular meshwork abnormality or primary congenital glaucoma, as well as iris abnormalities, including Axenfeld and Rieger anomalies, and corneal dysgenesis or Peters anomaly. In Peters anomaly, there are reports identifying mutations in a number of genes, including *PAX6*, *PITX2*, *FOXC1*, *MAF*, *FOXE3*, *PITX3* and *CYP1B1*.^{1,5,6} Overlap in clinical features is evident with some patients with mutations in a particular gene having cataract/microcornea while others may have a specific anterior segment dysgenesis phenotype, and microphthalmia may also be present.⁷ As an example, mutations in the gene *FOXE3* may be found in patients with cataract, disorders of the anterior segment and microphthalmia (Figure 1).^{8–10}

The developmental ocular anomaly of coloboma, due to delay in closure of the optic fissure, may affect the iris, choroid, retina and/or optic disc.¹¹ It is frequently associated with microphthalmia or anophthalmia.¹² In patients with microphthalmia and anophthalmia, attempts have been made to stratify patients on the basis of the

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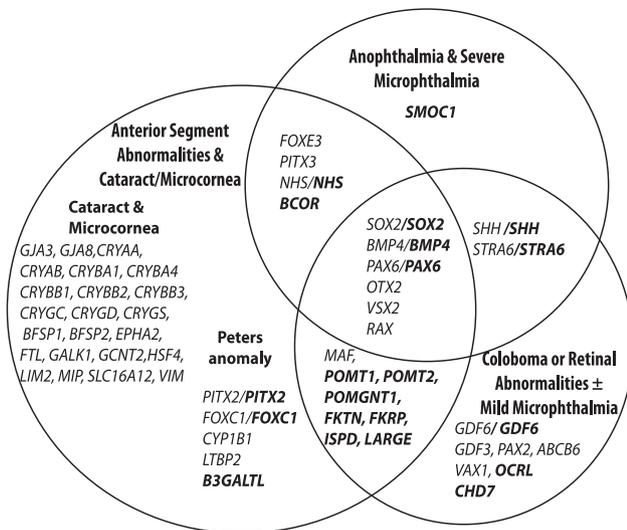


Figure 1 Genetic heterogeneity and overlap in phenotypes of developmental eye disorders. Developmental eye phenotypes are shown, and as indicated, variants in many of the genes can cause different types of developmental eye phenotypes. Normal font of the gene indicates causation of an eye disease phenotype without systemic abnormality, while bold font of the gene indicates eye disease with additional systemic/syndromic features.

presence of associated eye malformations; however, the overlap in phenotypic features makes this difficult (Figure 1).^{13,14}

In this study, we applied exome capture and sequencing in patients with developmental eye diseases, including cataract/microcornea, Peters anomaly and coloboma/microphthalmia/anophthalmia, and analysed the 56 genes currently known to be implicated in developmental eye disease (Supplementary Table S1).

MATERIALS AND METHODS

Patients

Eleven probands with developmental eye disease, four with cataract/microcornea, three with Peters anomaly and four with coloboma/microphthalmia, were chosen for the study (Table 1). Probands were selected where there were parental and/or other family member samples available so that segregation analysis could subsequently be performed (Figure 2). Probands and family members underwent full ophthalmic examination (Supplementary Table S2). All the four probands (Patients 1–4, Table 1) in the cataract and microcornea/microphthalmia group had severe bilateral eye disease that had been identified soon after birth. One proband (Patient 4, I.2 in Family 4) also had multiple iris processes adherent to the lens, highlighting that while the main ocular phenotype is recorded as cataract in this case, there can be associated anterior segment anomalies in these patients complicating decisions regarding candidate disease genes to be studied. In the three probands (Patients 5–7, Table 1) with a predominant Peters anomaly phenotype, all patients had bilateral corneal opacities and were diagnosed soon after birth. Two also had lens abnormalities, one with anterior polar lens opacities (Patient 5, II.1 in Family 5) and the other with rudimentary lens (Patient 6, II.1 in Family 6), and two also had microphthalmia (Patient 6, II.1 in Family 6, and Patient 7, II.2 in Family 7). All the four probands in the coloboma/microphthalmia group (Patients 8 to 11, Table 1) had bilateral eye disease detected at or soon after birth, and one of these also had anterior polar cataracts (Patient 8, II.1 in Family 8). In view of the overlap in phenotypic features that may occur in these groups of developmental eye conditions and the large number of candidate disease genes, we decided to take a whole-exome sequencing approach followed by analysis of known disease genes in these disorders. Genomic DNA was isolated from leukocytes of peripheral venous blood. All experiments were performed in

accordance with the ethical tenets of The Children's Hospital at Westmead, Sydney, NSW, Australia.

Exome sequencing, assembly and variant calling

Whole-exome sequence capture was performed in a proband from each of the 11 families. For Patient 1, this was performed using the TruSeq exome enrichment system (Illumina Inc., San Diego, CA, USA), and for all other probands the SeqCap EZ Human Exome Library v3.0 (Roche NimbleGen, Inc., Madison, WI, USA) was used. Sequencing was performed on the Illumina HiSeq 2000 using 100 bp paired-end read sequencing protocol (Axeq Technologies Asia, Macrogen Inc., Seoul, South Korea and at the Genome Institute of Singapore for Patient 4). Reads were aligned to the reference human genome (hg19) using the Burrows–Wheeler alignment tool¹⁵ using default parameters. Reads with a mapping quality score ≤ 10 were discarded using SAMtools¹⁶ and the Picard MarkDuplicates tool was used to identify and discard read duplicates (<http://picard.sourceforge.net>).

SNPs and indels were identified using the Genome Analysis Toolkit (GATK) version 1.6-9,¹⁷ except for Patient 4 where CASAVA 1.8 (Illumina Inc.) was used. Variants in 56 known developmental eye disease genes (Supplementary Table S1) were annotated using Annovar.¹⁸ Known polymorphisms were identified using dbSNP132, dbSNP135, 1000 genomes (30 April 2012 release) and NHLBI exome project (ESP5400 release), and minor allele frequencies were recorded from 1000 genomes and ESP5400. Minor allele frequencies were recorded manually from dbSNP when required. Coding sequence variants were annotated with prediction of pathogenicity based on the SIFT,¹⁹ PolyPhen-2²⁰ and MutationTaster²¹ algorithms using the pre-calculated data provided in dbNSFP²² (Supplementary Figure S1).

Variant prioritisation

Variants within the 56 known eye development disease genes (Figure 1, Supplementary Table S1) were selected for further analysis when all of the following conditions were met as in previous similar approaches²³ (Supplementary Figure S1). Variants that were nonsense, frameshift or canonical splice site variants, affecting the two nucleotides immediately adjacent to the splice donor or acceptor sites, were considered to be pathogenic. We used a classification system for missense variants using: (1) missense prediction software assessing pathogenicity at the amino-acid level and (2) evolutionary conservation at the nucleotide level.²⁴ We used the missense prediction programs, PolyPhen-2, SIFT and MutTaster, and the different scores from these tools were derived according to the rules described by Liu *et al.*²² Results from the three different tools were combined to give a majority vote resulting in a single classification as 'damaging' or 'tolerated'. For the classification based on evolutionary conservation, all variants with a phylo $P > 0.95$ were considered conserved (C), otherwise non-conserved (NC). Hence, missense variants were considered 'probably pathogenic' when the population frequency was $< 1\%$ in the public databases as described above, and if either the prediction programs or conservation tool predicted a damaging or highly conserved allele. Otherwise the variant was considered 'probably benign'. All 'probably pathogenic' alleles were subjected to segregation analysis. A cutoff of $< 1\%$ population frequency in the control databases was used as isolated developmental eye disorders are rare,²⁵ and in addition, each disease gene accounts only for a small proportion of cases.

Variant validation and segregation analysis

Using this approach, between 0 and 4 variants per sample were selected for validation leading to a total of 16 variants in the 11 probands (Table 1, Supplementary Table S3). Variants were validated using the conventional Sanger sequencing. Reference sequences used were: *GJA8*, NM_005267.4; *CRYGC*, NM_020989.3; *CYP1B1*, NM_000104.3; *PAX6*, NM_000280.4; *CRYBA1*, NM_005208.4; *GCNT2*, NM_145655.3; *BFSP1*, NM_001161705.1; *ABCB6*, NM_005689.2; *SLC16A12*, NM_213606.3; and *GDF3*, NM_020634.1. Primers for Sanger sequencing are available upon request. DNAs from additional family members were sequenced to enable segregation analysis, and proteins and variants were examined for previous disease reports. Variants identified in *GJA8*, *CRYBA1*, *CRYGC*, *BFSP1*, *GCNT2* and *PAX6* genes were submitted to Eye Diseases—Leiden Open Variation Databases (LOVD)

Table 1 Clinical phenotypes and causative and other prioritised variants from exome sequencing in patients with developmental eye disease

| Sample (family, pedigree ID, inheritance pattern ^a) | Ocular phenotype | Genes with causative variants, ^{b,c,d} prioritised variants with appropriate family segregation | Genes with prioritised variants, ^{c,d} but lacking appropriate family segregation | Novel |
|---|---|---|--|---|
| Congenital cataracts and microcornea/microphthalmia | | | | |
| Patient 1 (Family 1, II.1, AD) | Nuclear cataracts, microcornea | GJA8, c.593G>A, p.(Arg198Gln) | Nil | Devi and Vijayalakshmi, ²⁶ |
| Patient 2 (Family 2, II.1, AD) | Nuclear cataracts, microcornea | Nil | Nil | — |
| Patient 3 (Family 3, II.1) | Cataracts, microcornea, iris processes to lens | Nil | <i>CRYBA1</i> , c.475G>A, p.(Gly159Ser) | rs117757092 |
| Patient 4 ^e (Family 4, I.2, AD) | Nuclear cataracts, microphthalmia | CRYGC, c.497C>T, p.(Ser166Phe) | Nil | Yes |
| Peters anomaly | | | | |
| Patient 5 (Family 5, II.1) | Peters anomaly, anterior polar cataracts | CYP1B1, c.1200_1209dup, p.(Thr404Serfs*30) CYP1B1, c.171G>A, p.(Trp57*) PAX6, c.152G>T, p.(Gly51Val) | Nil | Stoilov <i>et al</i> , ²⁷ Vincent <i>et al</i> , ⁶ |
| Patient 6 (Family 6, II.1) | Peters anomaly, rudimentary lens | Nil | Nil | Yes |
| Patient 7 (Family 7, II.2) | Peters anomaly, microphthalmia | Nil | Nil | — |
| Coloboma and microphthalmia | | | | |
| Patient 8 (Family 8, II.1) | Coloboma, limbal dermoid, anterior polar cataract | Nil | Nil | — |
| Patient 9 (Family 9, II.1, AR) | Iris and fundal colobomas, microphthalmia | Nil | <i>CYP1B1</i> , c.868dup, p.(Arg290- Profs*37) <i>CYP1B1</i> , c.241T>A, p.(Tyr81- Asn) <i>BFSP1</i> , c.1620_1621del, p.(*541Lysext*7) | Yes rs9282671 recorded in 1000G |
| Patient 10 (Family 10, II.1) | Iris and fundal colobomas | Nil | <i>ABCB6</i> , c.575G>A, p.(Arg192Gln) <i>SLC16A12</i> , c.472T>C, p.(Ser158Pro) | rs150221689 rs150800688 |
| Patient 11 (Family 11, II.1) | Iris and fundal colobomas, microphthalmia | Nil | <i>GDF3</i> , c.974C>T, p.(Pro325- Leu) <i>CYP1B1</i> , c.1103G>A, p.(Arg368His) <i>CYP1B1</i> , c.685G>A, p.(Glu229Lys) <i>BFSP1</i> , c.401G>C, p.(Cys134Ser) | Yes rs79204362 rs57865060 Yes |

Abbreviations: AD, autosomal dominant, AR, autosomal recessive; Nil, no variant identified.

^aInheritance pattern given if known from pedigree.

^bGenes in bold contain clear-cut pathogenic variants with appropriate segregation in family members.

^cReference sequences used: *GJA8*, NM_005267.4; *CRYGC*, NM_020989.3; *CYP1B1*, NM_000104.3; *PAX6*, NM_000280.4; *CRYBA1*, NM_005208.4; *BFSP1*, NM_001161705.1; *ABCB6*, NM_005689.2; *SLC16A12*, NM_213606.3; and *GDF3*, NM_020634.1.

^dAll variants are heterozygous.

^eVariant in *GCNT2* (Reference sequence: NM_145655.3) identified, c.505G>A, p.(Ala169Thr) (rs56106312), that is not associated with cataract Yu *et al*.²⁸

<http://grenada.lumc.nl/LOVD2/eye/home.php> and http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6. Other variants were submitted to the ClinVar database (www.ncbi.nlm.nih.gov/clinvar/).

RESULTS

Exome and target region analysis

In the 11 developmental eye disease probands, whole-exome sequencing resulted in an average of 59 311 192 reads/sample. The mean depth of coverage of the targeted regions across the exome was 43X across all samples, with on average 94% of all targeted regions being covered by $\geq 10X$ reads (Supplementary Table S3). For the 56 developmental eye disease genes across the 11 samples, 87.8–95.6% of the coding regions were covered by $\geq 5X$ reads.

After selection for exonic and canonical splice site variants across the 56 genes in the 11 samples, 36–55 variants were identified per sample (Supplementary Table S3). After application of our systematic

variant prioritisation tool (Supplementary Figure S1), 16 predicted pathogenic variants were identified. Sanger sequencing confirmed these variants. The variants confirmed in probands were further sequenced in parental and sibling samples where available.

Five causative variants identified in cataract/microcornea and Peters anomaly patients

In two of four cataract/microcornea patients, (Table 1) two pathogenic variants with appropriate segregation were identified, and one of these was novel. In Patient 1 (Family 1, II.1), we detected a heterozygous mutation in *gap junction alpha-8* (*GJA8*), c. 593G>A, predicting a deleterious substitution of arginine by glutamine (p.(Arg198Gln)), which segregated with disease in the family (Table 2, Figure 3a, Supplementary Figure S2), and is previously reported in autosomal-dominant cataract/microcornea.²⁶ In Patient 4 (Family 4, II.1), a novel heterozygous variant was detected in *CRYGC*,

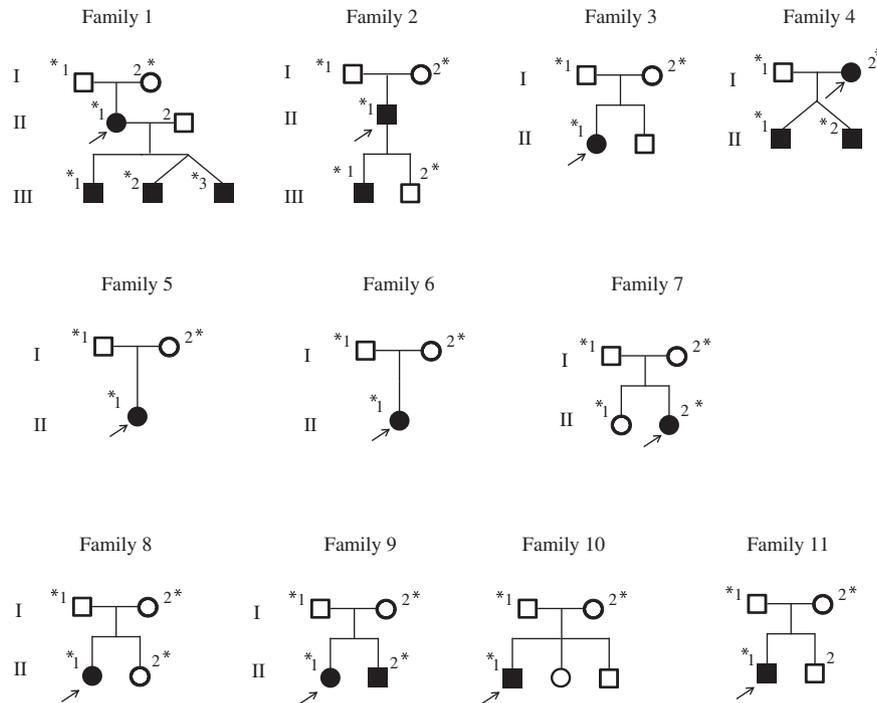


Figure 2 Pedigrees of the families studied. A proband from each of the families underwent exome sequencing. Proband is indicated by arrows. Asterisk indicates DNA sample available. Filled squares or circles indicate affected individuals.

Table 2 Causative variants—allele frequencies in public databases, conservation, prediction categories and segregation patterns

| Sample (family, pedigree ID) | Gene | Nucleotide alteration ^a (all heterozygous) | | Protein alteration | Allele frequencies in public databases | | Prediction tools (SIFT, Polyphen-2, MutTaster) | | Segregation |
|--|---------------|---|--|--------------------|--|---|--|--|--|
| | | | | | Cons ^b | | | | |
| <i>Congenital cataracts and microcornea/microphthalmia</i> | | | | | | | | | |
| Patient 1 (Family 1, II.1) | <i>GJA8</i> | c.593G>A | | p.(Arg198Gln) | Nil | C | D,D,D | | New AD in proband, also in affected children |
| Patient 4 (Family 4, I.2) | <i>CRYGC</i> | c.497C>T | | p.(Ser166Phe) | Nil | C | D,D,D | | AD, present in all affecteds |
| <i>Peters anomaly</i> | | | | | | | | | |
| Patient 5 (Family 5, II.1) | <i>CYP1B1</i> | c.1200_1209dup | | p.(Thr404Serfs*30) | Nil | | Frameshift | | Heterozygous in unaffected mother |
| | <i>CYP1B1</i> | c.171G>A | | p.(Trp57*) | ESP5400: 0.000287; 1000G: nil | C | Stop | | Heterozygous in unaffected father, therefore AR disease in proband |
| Patient 6 (Family 6, II.1) | <i>PAX6</i> | c.152G>T | | p.(Gly51Val) | Nil | C | D,D,T | | New AD in proband |

Abbreviations: D, damaging; T, tolerated.

^aReference sequences used: *GJA8*, NM_005267.4; *CRYGC*, NM_020989.3; *CYP1B1*, NM_000104.3; and *PAX6*, NM_000280.4.

^bConservation based on PhyloP score, C, conserved, NC, non-conserved.

c.497C>T, p.(Ser166Phe). This predicted deleterious variant affected a highly conserved amino acid in the second beta/gamma crystallin domain of this protein, was not present in the 1000 genome, ESP5400 or dbSNP databases and segregated with disease in the affected family members (Table 2, Figures 3b and e, Supplementary Figure S2).

Examination of exome sequence data from three individuals with Peters anomaly led to the identification of causative variants in two patients (Table 1) In Patient 5 (Family 5, II.1), two heterozygous variants in *CYP1B1* (p.(Thr404fs), p.(Trp57*)) were identified. One of the variants was a 10 nucleotide insertion, c.1200_1209dupT-CATGCCACC, which resulted in a frameshift mutation

(p.(Thr404Serfs*30)) leading to a shortened abnormal protein. This same variant has been reported in primary congenital glaucoma,²⁷ as part of a compound mutation, but not previously in Peters anomaly. The second heterozygous *CYP1B1* variant in this patient, c.171G>A, predicted a premature stop codon (p.(Trp57*)) (Table 2, Figure 3c, Supplementary Figure S2) and was reported previously in Peters anomaly.⁶

In Patient 6 (Family 6, II.1) with Peters anomaly, we detected a novel heterozygous variant in *PAX6* leading to a missense change in a highly conserved glycine in the paired box domain (c.152G>T, p.(Gly51Val)). This was predicted as a pathogenic variant due to

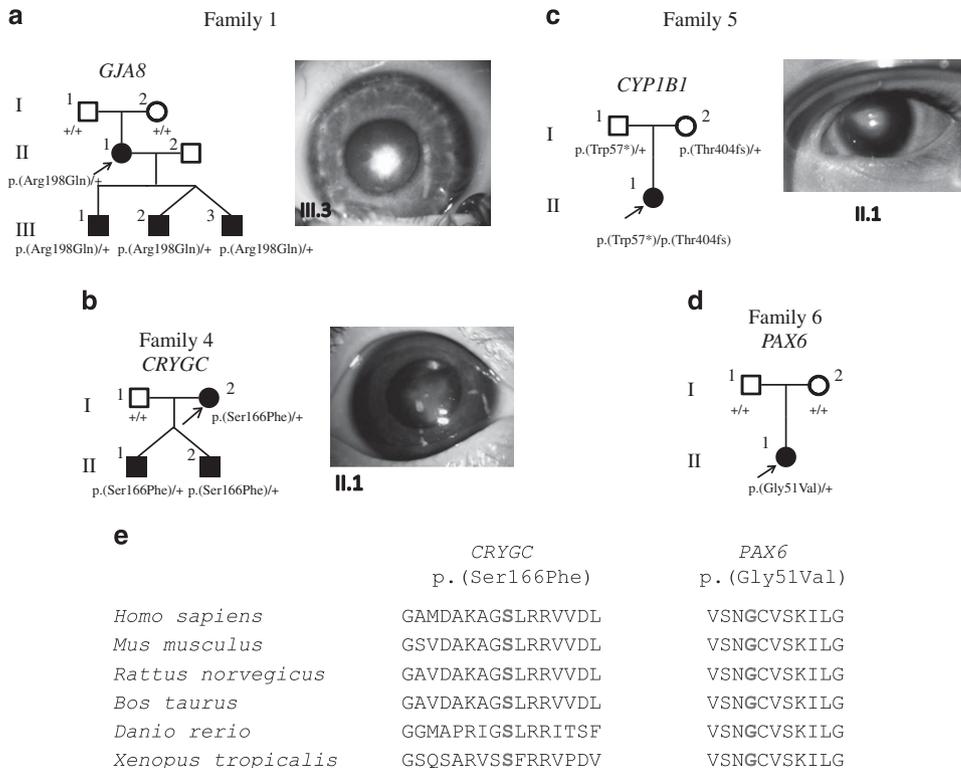


Figure 3 Pedigrees and clinical images in families with pathogenic variants and segregation indicating autosomal dominant or recessive inheritance and alignment of amino-acid sequences for two novel mutations. (a) Family 1 pedigree and nuclear cataract in patient III.1. Mutation in *GJA8* c. 593G>A, p.(Arg198Gln), *de novo* autosomal-dominant mutation in II.1 (Patient 1) transmitted to III.1, III.2 and III.3, (b) Family 4 pedigree and nuclear cataract in patient II.1. Mutation in *CRYGC*, c.497C>T, p.(Ser166Phe), autosomal-dominant mutation in I.2 (Patient 4), transmitted to II.1 and II.2, (c) Family 5 pedigree and anterior polar lens opacity in patient II.1 (Patient 5) with Peters anomaly post-penetrating keratoplasty. Compound heterozygous mutations in II.1 in *CYP1B1*, c.171G>A, (p.Trp57*), inherited from unaffected father, I.1, and c.1200_1209dup, p.(Thr404Serfs*30) inherited from the unaffected mother, I.2, (d) Family 6 pedigree. *De novo* autosomal-dominant mutation in patient, II.1, (Patient 6) in *PAX6*, c.152G>T, p.(Gly51Val). (e) Alignment of amino-acid sequences for the two novel mutations, *CRYGC*, p.(Ser166Phe) and *PAX6*, p.(Gly51Val). The reference sequences for *CRYGC* and *PAX6* correspondingly: *Homo sapiens* NP_066269.1 and NP_000271.1, *Mus musculus* NP_031801.1 and NP_001231129.1, *Rattus norvegicus* NP_264077.1 and NP_037133.1, *Bos taurus* NP_001013613.1 and NP_001035735.1, *Danio rerio* NP_001018630.1 and NP_571716.1, and *X.tropicalis* XP_002937158.1 and NP_001006763.1.

a high conservation score and damaging scores using missense prediction tools, and this variant has not been identified in any control panels. Sanger sequencing showed that this was a *de novo* heterozygous variant in the patient, with absence of the mutation in the unaffected parents (Table 2, Figures 3d and e, Supplementary Figure S2).

Two other variants were prioritised in the cataract/microcornea patients. One of these, in Patient 4 (Family 4, I.2), was not considered further as it was a variant in an isoform of *GCNT2*, c.505G>A, p.(Ala169Thr), (rs56106312, Reference sequence: NM_145655.3), that is not expressed in lens and hence not associated with cataract.²⁸ In Patient 3 (Family 3, II.1) with cataract/microcornea, a heterozygous variant in *CRYBA1*, c.475G>A, predicted a probable pathogenic variant, p.(Gly159Ser), (rs117757092, frequency in controls ESP5400, 0.00316 and 1000 genomes, 0.0009), but this was also found in the patient's unaffected mother (Table 3). Variable penetrance and expression occur in developmental eye diseases and are reported in cataract/microcornea patients, including those with mutations in crystallin genes.²⁹ Whether this variant may contribute to this patient's phenotype will be determined as more variants in these genes are identified through broader sequencing strategies in patient cohorts made available by Next-Generation Sequencing (NGS).

Variants with incomplete penetrance in coloboma/microphthalmia patients

A number of variants were prioritised in three out of four of our coloboma/microphthalmia patients (Table 1), and we first considered genes previously reported as known disease genes particularly in this disorder (Figure 1). Patient 11 (Family 11, II.1), with microphthalmia and coloboma, was heterozygous for a novel missense variant in *GDF3*, c. 974C>T, p.(Pro325Leu). This variant affects a conserved residue in the TGF-beta domain of this protein and is predicted to be a damaging variant using Polyphen-2, SIFT and Mutation Taster (Table 3). This patient was of Indian ethnicity, and the variant was not present in ESP5400 or 1000 genome controls, including 70 individuals of South Asian origin. This variant was also present in the patient's unaffected father (Table 3, Supplementary Figure S3). Other patients with microphthalmia and coloboma have been found to have heterozygous missense variants in the TGF-beta domain of *GDF3*, and variable penetrance has been previously noted.³⁰

In Patient 10 (Family 10, II.1), we detected a rare heterozygous variant in *ABCB6*, c.575G>A, p.(Arg192Gln) (Table 3, Supplementary Figure S3). *ABCB6* encodes a protein responsible for porphyrin transport and is located in the outer mitochondrial membrane. A missense variant in this gene has been found to segregate in an autosomal-dominant manner in a family with

Table 3 Other variants lacking appropriate segregation—allele frequencies in public databases, conservation and prediction categories

| Sample (family, pedigree ID) | Gene | Nucleotide alteration ^a (all heterozygous) | Protein alteration | Allele frequencies in public databases | | Prediction tools (SIFT, Polyphen-2, MutTaster) | | Segregation |
|--|-----------------|---|--------------------|--|-------------------|--|--|--|
| | | | | | Cons ^b | | | |
| <i>Congenital cataracts and microcornea/microphthalmia</i> | | | | | | | | |
| Patient 3 (Family 3, II.1) | <i>CRYBA1</i> | c.475G>A | p.(Gly159Ser) | ESP5400: 0.00316; 1000G: 0.0009 | C | T,T,D | | Heterozygous in unaffected mother |
| <i>Coloboma and microphthalmia</i> | | | | | | | | |
| Patient 9 (Family 9, II.1) | <i>CYP1B1</i> | c.868dup | p.(Arg290Profs*37) | Nil | | Frameshift | | Not present in more mildly affected brother, heterozygous in unaffected mother |
| | <i>CYP1B1</i> | c.241T>A | p.(Tyr81Asn) | ESP5400: 0.00327; 1000G: 0.0032 | C | D,D,D | | Heterozygous in affected brother and unaffected father |
| | <i>BFSP1</i> | c.1620_1621del | p.(*541Lysext*7) | ESP5400: N/A; 1000G: 0.0014 | | Frameshift | | Heterozygous in affected brother and unaffected mother |
| Patient 10 (Family 10, II.1) | <i>ABCB6</i> | c.575G>A | p.(Arg192Gln) | ESP5400: 0.002975; 1000G: 0.0014 | NC | D,D,D | | Heterozygous in unaffected father |
| | <i>SLC16A12</i> | c.472T>C | p.(Ser158Pro) | ESP5400: 0.000651; 1000G: Nil | C | T,T,D | | Heterozygous in unaffected mother |
| Patient 11 (Family 11, II.1) | <i>GDF3</i> | c.974C>T | p.(Pro325Leu) | Nil | C | D,D,D | | Heterozygous in unaffected father |
| | <i>CYP1B1</i> | c.1103G>A | p.(Arg368His) | ESP5400: 0.001496; 1000G: 0.0009 | C | D,D,D | | Heterozygous in unaffected mother |
| | <i>CYP1B1</i> | c.685G>A | p.(Glu229Lys) | ESP5400: 0.003817; 1000G: 0.0027 | C | D,D,T | | Heterozygous in unaffected father |
| | <i>BFSP1</i> | c.401G>C | p.(Cys134Ser) | Nil | C | T,D,T | | Heterozygous in both parents |

Abbreviations: D, damaging; T, tolerated.

^aReference sequences used: *CYP1B1*, NM_000104.3; *PAX6*, NM_000280.4; *CRYBA1*, NM_005208.4; *BFSP1*, NM_001161705.1; *ABCB6*, NM_005689.2; *SLC16A12*, NM_213606.3; and *GDF3*, NM_020634.1.^bConservation based on PhyloP score, C, conserved, NC, non-conserved.

coloboma, and another heterozygous missense variant was found in three isolated cases. Results from zebrafish rescue experiments indicated that these variants lead to loss of protein function.³¹ However, in two more recent studies, several individuals homozygous for deleterious loss of function mutations, including frameshift and nonsense mutations, were found to have a newly identified rare Lan blood group antigen. There were no reports of coloboma or eye defects in individuals homozygous or heterozygous for these variants³². In our family (Family 10), the unaffected father also carried *ABCB6*, c.575G>A (p.(Arg192Gln)) in the heterozygous state, indicating that this is a variant with incomplete penetrance and that other factor/s are contributing to the disease phenotype in Patient 10.

Variants in developmental eye disease genes across the cohort

As there can be overlap in clinical features in patients with developmental eye disorders, we considered prioritised variants from known developmental eye disease genes across the 11 probands with developmental eye disease. This revealed one novel and three rare variants in *CYP1B1* in two out of four coloboma/microphthalmia patients (Table 1). In Patient 9 (Family 9, II.1), there was a novel heterozygous duplication in *CYP1B1*, c.868dup, predicting a shortened protein with the normal amino-acid

sequence finishing at amino acid 290, p.(Arg290Profs*37), compared with the normal 543 amino-acid protein. This variant was not present in any of the control databases, was present in the patient's unaffected mother but was not present in her more mildly affected brother (Table 3, Supplementary Figure S3). Patient 9 also had a rare missense mutation in *CYP1B1*, c.241T>A, p.(Tyr81Asn), which was present in her affected brother, her unaffected father but not her unaffected mother (Table 3, Supplementary Figure S3). This missense mutation is in a conserved residue in the P450 domain of this protein (<http://smart.embl.de>), and previous enzymatic studies have indicated that it is a hypomorphic allele.³³ Patient 11 (Family 11, II.1) who had a heterozygous change in *GDF3*, was also a compound heterozygote for two missense mutations in *CYP1B1*, c.1103G>A, p.(Arg368His), and c.685G>A, p.(Glu229Lys) (Table 3, Supplementary Figure S3). Both of these variants have been found to have higher frequencies in individuals with primary congenital glaucoma than in control populations, including in controls with this patient's ethnicity. Enzymatic assays indicate c.1103G>A, p.(Arg368His) has markedly reduced activity while c.685G>A, p.(Glu229Lys) is a hypomorphic allele.^{33,34} There was no evidence of glaucoma or Peters anomaly in Patient 9 (Family 9, II.1) or Patient 11 (Family 11, II.1). *CYP1B1* can contribute to retinoic acid synthesis in embryonic development,³⁵ and retinoic

acid receptor signalling regulates choroid fissure closure.³⁶ The presence of variation in two genes, *GDF3* and *CYP1B1*, which may modulate choroid fissure closure, may be relevant as contributing factors to the disease in Patient 11 (Family 11, II.1).

Patients 9 (Family 9, II.1) and 11 (Family 11, II.1), both with coloboma/microphthalmia, also had rare heterozygous variants in *BFSP1*, which encodes a beaded filament lens protein. Patient 9, in addition to her variants in *CYP1B1* described above, had a 2-bp deletion in *BFSP1* that changed the stop codon to a lysine, resulting in the addition of seven random amino acids to the end of the protein, c.1620_1621del, p.(^{*}541Lysex^{*}7). For Patient 9, this variant was also present in her affected sib, as well as her unaffected mother (Table 3, Supplementary Figure S3). Patient 11, in addition to his variants in *GDF3* and *CYP1B1* described above, also had a variant in *BFSP1*, which was a missense change in a conserved amino acid in the coiled coil domain of the protein, c.401G>C, p.(Cys134Ser). In Patient 11's family, the *BFSP1* variant was present in heterozygous form in both his parents (Table 3, Supplementary Figure S3).

Patient 10 (Family 10, II.1), also with coloboma/microphthalmia, in addition to his variant in *ABCB6* described above, also had a rare heterozygous variant in *SLC16A12*, c.472T>C, p.(Ser158Pro) (Table 3, Supplementary Figure S3), a cataract disease gene previously identified in an autosomal-dominant cataract family.³⁷ This variant was in a conserved predicted transmembrane domain of the protein and was also present in Patient 10's unaffected mother. Patients with mutations in other lens-related genes are reported with coloboma, as lens factors may influence anterior segment development.¹ Hence, while these *BFSP1* and *SLC16A12* variants are not causative variants by themselves, as they are present in unaffected parents, it is possible that they may be modifying factors in coloboma/microphthalmia, and observation for variations in these and similar genes is warranted in future exome and genome-wide studies of other coloboma/microphthalmia patients.

DISCUSSION

In this study, we developed an investigation tool for developmental eye disease, consisting of exome sequencing of patients, followed by systematic analysis of the known disease genes in the disorders under study and interpretation of all detected genetic variants across the cohort. The clinical utility of this approach is highlighted by the diagnostic yield in our sporadic cases, revealing both autosomal recessive and *de novo* dominant mutations. The presence of *de novo* dominant mutations confers a significant risk for transmitting the disease to the patient's offspring. The presence of compound heterozygous mutations confers a significant recurrence risk for the patient's parents. These findings illustrate the molecular diagnostic power of exome capture followed by targeted analysis in these heterogeneous eye disorders and also the huge impact of this method for affected families.

The strength of our approach lies in the parallel analysis of all known eye developmental disease genes in our patients with unknown molecular cause of disease. Systematic variant prioritisation reduced the initially high number of identified variants (~45 per patient) by 97% to a number that is manageable to validate by Sanger sequencing (approximately 1.4 variants per patient). Using a systematic approach for assessment of pathogenicity of the validated variants, our NGS approach resulted in a clear molecular diagnosis in 4 out of 11 patients, which is an encouraging detection rate in a group of diseases where molecular diagnosis is often not undertaken because of low detection rates in each of the genes which, up until now, have required individual sequential sequencing.

Several individuals in our cohort were the first affected person in their family, where there were concerns about recurrence risk expressed by family members and the individuals themselves. Cataract associated with microcornea is usually an autosomal-dominant condition, although autosomal-recessive cases are reported.³⁸ With sequential Sanger sequencing of several genes in research cohorts, the mutation detection rate is in the vicinity of 10–50%.^{4,39} Such testing is not possible to be undertaken in the clinical setting, because no panels of these genes are available for diagnostic screening. Using our approach, we have detected a novel heterozygous pathogenic mutation in *CRYGC* in Patient 4 and a known pathogenic variant in *GJA8* in Patient 1. In both the families, infantile cataract management has been complicated by secondary glaucoma and requirement for repeated surgical procedures and impaired vision in all cases. Provision of molecular genetic diagnosis significantly improves the genetic information and options available for these families.

Peters anomaly is a severe disorder affecting vision that is extremely genetically heterogeneous with autosomal-dominant and -recessive forms described. In two out of the three isolated cases in our cohort, we have identified disease-causing mutations in two different genes. In Patient 5, two previously reported mutations in *CYP1B1* were present. One of the mutations had been reported in Peters anomaly while the other was in a patient with primary congenital glaucoma. It has been previously noted that mutations in *CYP1B1* can lead to Peters anomaly or primary congenital glaucoma, and this suggests that they may have the same etiology due to the abnormal migration of neural crest cells.⁴⁰ Each of the unaffected parents was found to be heterozygous for one of the variants. Before this information, the parents were given a recurrence risk varying from <1% (new autosomal-dominant condition) to 25% (autosomal-recessive condition). Molecular diagnosis in the proband clarifies the recurrence risk to 25% for the parents, and the transmission risk for the affected individual is low.

In Patient 6 with Peters anomaly, a novel variant in *PAX6* has been identified. This is in the paired domain and is predicted to be a damaging variant with high conservation across species. Neither of the unaffected parents has this change. For this couple, the recurrence risk has now been clarified to <1% on the basis that this is a new autosomal-dominant change in the patient. However, for the affected individual, the transmission risk is 50%. Peters anomaly is frequently seen as an isolated condition, and provision of recurrence and transmission risk information lacks clarity in the absence of a molecular diagnosis.

Molecular detection rates in any of the possible known genes in patients with coloboma/microphthalmia have been generally very low in those patients who do not have associated anophthalmia in the contralateral eye. This has been confirmed in this study where a combination of pathogenicity assessment and segregation analysis has not found a primarily pathogenic gene in any of the coloboma/microphthalmia patients. Patient 11 has a heterozygous probably pathogenic mutation in *GDF3*, which is also present in his unaffected father, and while variations in this gene have been noted in association with microphthalmia and coloboma, variability in penetrance has also been described. This patient is also a compound heterozygote for two probably pathogenic missense variants in *CYP1B1*. Deleterious homozygous or compound heterozygous mutations in *CYP1B1* are found in patients with primary congenital glaucoma and Peters anomaly, and *CYP1B1* may also regulate retinoic acid signalling for proper optic fissure closure. It is possible that variation in both *GDF3* and *CYP1B1* may be contributing to Patient

11's phenotype of coloboma and microphthalmia. Interestingly, one other microphthalmia/coloboma patient, Patient 9, had a heterozygous frameshift variant in *CYP1B1*. This was also present in her unaffected mother and was not present in her affected brother who had a less severe phenotype with only a subtle retinal coloboma revealed by fundal examination. This suggests that Patient 9 and her more mildly affected brother may have pathogenic variation in an as yet undiscovered coloboma/microphthalmia disease gene and that the disease severity may be modulated by the presence of the frameshift variant in *CYP1B1* in Patient 9. This marks *CYP1B1* as a candidate to be considered as a modifier gene in other coloboma/microphthalmia cases.

In the whole-exome sequencing approach we undertook in the 11 probands in our study, we achieved a mean depth of coverage of the targeted regions across the exome of 43X across all samples. In addition, there was incomplete coverage, as for the 56 developmental eye disease genes across the 11 samples, 87.8–95.6% of the coding regions were covered by $\geq 5X$ reads. The lack of coverage was identified particularly in GC-rich and repetitive regions. Ideally, to maximise the likelihood of mutation variant detection, a higher depth of coverage and capture in all of the possible disease-causing regions would be preferred. Nevertheless, when we compared detection rates from this study, with previous research studies involving conventional sequencing techniques to screen mutations in genes known to be associated with developmental eye diseases, we found comparable detection rates. Sanger sequencing studies have shown a detection rate of approximately 10–50% in patients with cataract/microcornea,^{4,39} and our approach has resulted in the identification of a clear-cut disease causing variant in two out of four of our cataract/microcornea patients. For the Peters anomaly group, there are mostly isolated case reports of mutations in different genes for these patients, although in one study there were four genes investigated, and clear-cut mutations were identified in 20% (3/15) of patients.⁴¹ In patients with microphthalmia/coloboma, detection rates for clear-cut pathogenic variants are generally very low, in the vicinity of 1–2% or less as found in our study.^{13,42,43}

A targeted NGS strategy of the known disease genes could possibly lead to a higher detection rate, as a higher depth of coverage can be achieved with this. However, where such a strategy was undertaken in the severe anophthalmia/microphthalmia group, where there is a known higher detection rate, no disease gene was identified in almost one-third of the patients.⁴⁴ This suggests that there are still a significant proportion of unknown disease genes in patients with developmental eye disease. The approach we followed provides an option for a mutation detection route for at least a proportion of patients with these conditions, as service laboratories are now offering whole-exome sequencing followed by an analysis of a target list of genes. Any likely pathogenic variants can be confirmed by Sanger sequencing, and segregation can be confirmed in family members. As strategies for capture of GC-rich and repetitive regions improve, and increased depth of coverage can be undertaken for less cost in the future, it is likely that detection rates will improve. For patients where no disease gene variant has been identified and with suitable family structure and availability of samples for analysis, research studies may be able to identify novel disease genes.

This paper presents an exome-sequencing strategy for disease gene identification in genetically heterogeneous developmental eye diseases, including cataract/microcornea, Peters anomaly and coloboma/microphthalmia. Overall, we identified causative mutations in known disease genes, *GJA8*, *CRYGC*, *CYP1B1* and *PAX6*, in 36% (4/11) of families, an encouraging detection rate for diseases where molecular

genetic diagnosis is frequently not undertaken owing to the high heterogeneity and lack of available panels for clinical diagnosis. These results provided clinically useful information in all cases. Clear-cut detection rates were better for the cataract/microcornea (2/4 families) and Peters anomaly (2/3 families) groups, compared with the coloboma/microphthalmia (0/4 families) group. Variants associated with variable penetrance were identified in *CRYBA1* in the cataract/microcornea group and *ABC6* and *GDF3* in the coloboma/microphthalmia group. Our broad strategy in disease gene examination also identified *CYP1B1* as a new potential modifier in coloboma/microphthalmia. Our results support the high heterogeneity among patients with developmental eye diseases and show that the exome-sequencing strategy can be an expeditious approach for the identification of disease-causing and modifier variants in these conditions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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