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***CYP1B1*, *MYOC*, and *LTBP2* Mutations in Primary Congenital Glaucoma Patients in the United States**

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Abstract

PURPOSE—To screen primary congenital glaucoma patients in the United States for sequence variants within the *CYP1B1*, *LTBP2*, and *MYOC* genes using Sanger and whole exome sequencing.

DESIGN—Retrospective case-control study.

METHODS—Fifty-seven primary congenital glaucoma patients (47 families), 71 unaffected family members of the primary congenital glaucoma probands, and 101 healthy unrelated individuals were recruited from a single institution. Sanger sequencing of the primary congenital glaucoma gene, *CYP1B1*, was performed on 47 proband deoxyribonucleic acid samples. Simultaneously, whole exome sequencing was conducted on 3 families, each including more than 1 affected individual. Concurrently, 33 of 47 primary congenital glaucoma probands with extended family deoxyribonucleic acid samples were screened for *LTBP2* and *MYOC* gene mutations. Exome-sequenced variations were validated by additional Sanger sequencing to confirm segregation of filtered disease-causing single nucleotide variations.

RESULTS—Seven primary congenital glaucoma families (14.9%) manifested disease phenotypes attributable to *CYP1B1* mutations. One primary congenital glaucoma family possessed homozygous mutant alleles, whereas 6 families carried compound heterozygous mutations. Five novel combinations of compound heterozygous mutations were identified, of which 2 combinations were found with whole exome sequencing. No disease-causing mutations within the *LTBP2* and *MYOC* genes were discovered.

CONCLUSIONS—This study analyzed *CYP1B1*, *LTBP2*, and *MYOC* mutations in a cohort of primary congenital glaucoma patients from the United States, applying whole exome sequencing as a complementary tool to Sanger sequencing. Whole exome sequencing, coupled with Sanger sequencing, may identify novel genes in primary congenital glaucoma patients who have no mutations in known primary congenital glaucoma genes.

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Primary congenital glaucoma (OMIM 231300) is a rare but devastating eye disease. It is characterized by congenital elevation of intraocular pressure (IOP) resulting from significantly reduced aqueous outflow through a malfunctioning trabecular meshwork. The raised IOP results in progressive optic nerve damage, potentially leading to blindness.^{1,2} Other clinical features include buphthalmos, corneal edema and opacification with rupture of the Descemet membrane, thinning of the anterior sclera, iris atrophy, and an anomalously deep anterior chamber.² The disease presents classically in infants and toddlers with the symptoms of epiphora, blepharospasm, and photophobia. Typically, the diagnosis is made within the first year of life.²

The incidence of primary congenital glaucoma varies based on ethnicity and ranges from as high as 1 in 1250 persons in the Gypsy population of Slovakia to as low as 1 in 18,500 to 1 in 30,000 persons in Western populations.^{2,3} The disease accounts for 5% of childhood blindness and approximately 18% of children in blind institutions worldwide.⁴ It can occur in both sporadic and familial patterns.¹ Inheritance usually is autosomal recessive in familial cases, with increased association with consanguinity.¹ Three genetic loci—GLC3A (OMIM 231300), GLC3B (OMIM 600975), and GLC3C (OMIM 613085)—have been identified by linkage analyses in large multigenerational pedigrees.² A new locus, *GLC3D* (OMIM 613086), on 14q24, recently was characterized.^{5,6} To date, 3 genes have been implicated, and include cytochrome P450, subfamily I, polypeptide 1 (*CYP1B1*; NM_000104.3), latent transforming growth factor β binding protein 2 (*LTBP2*; NM_000428.2), and myocilin (*MYOC*; NM_000261.1).²

The *CYP1B1* gene was the first gene in which mutations were found to cause primary congenital glaucoma.⁷ It is located on chromosome 2p22–p21 within the GLC3A locus. Its protein oxidizes compounds important to eye structure and function, including steroids, retinoids, arachidonate, and melatonin.² Studies have demonstrated its expression in fetal and adult ciliary body and neuroepithelium, but not in the trabecular meshwork.⁷ It is speculated that the enzyme metabolizes an unknown molecule critical to eye development.⁸ The proportion of patients with pathogenic *CYP1B1* sequence variants varies with ethnicity, ranging from 100% in Slovakian Romas to 20% in Japanese individuals.^{9,10}

LTBP2 is located on chromosome 14q24 within the GLC3D locus.⁶ In nonocular tissues, *LTBP2* is involved in tissue repair and cell adhesion.² Ocular expression studies have determined its presence in both the trabecular meshwork and ciliary processes.¹¹ Several studies have shown that *LTBP2* mutations are associated with secondary glaucoma.^{12,13} The role of *LTBP2* in primary congenital glaucoma remains unclear, because some studies did not discover any mutations within the gene, but null mutations have been found in consanguineous Slovakian Roma, Pakistani, and Iranian families.^{14,15}

MYOC also is associated with juvenile and primary open-angle glaucoma and is located on chromosome 1q24.3–q25.2.^{16,17} Pathogenic *MYOC* variant proteins can alter the trabecular meshwork and ciliary body architecture, obstructing the outflow and increasing IOP.^{18,19} Disease-causing *MYOC* sequence variants, in the presence or absence of *CYP1B1* mutations, have been reported in families with primary congenital glaucoma.^{16,17}

In this study, we sought to determine *CYP1B1*, *LTBP2*, and *MYOC* sequence variations in a large multiethnic cohort of families and cases with primary congenital glaucoma in the United States. In most instances, Sanger sequencing was performed initially. In select families, whole exome sequencing was used as the initial screening tool for genetic mutations. Compared with other sequencing methods, whole exome sequencing technology has matured in recent years to emerge as an accurate and efficient means for detecting both known and novel genetic mutations in a range of ocular diseases, including corneal

dystrophy and inherited retinal disorders.^{20,21} To our knowledge, this is the first study to perform whole exome sequencing analysis in a primary congenital glaucoma cohort. Sanger sequencing was performed to confirm identified mutations.

METHODS

• SUBJECTS

The participants were recruited at the Duke University Eye Center, from the clinical practices of 2 of the authors (S.F.F. [a majority] and T.L.Y.). The retrospective case-control study was approved by the Duke University Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. The Ophthalmic Genetics Institutional Review Board (protocol no. 00008040) approved the recruitment of individuals and family members with hereditary developmental ophthalmologic disorders, the collection of blood or cheek cell samples for deoxyribonucleic acid (DNA) extraction, the screening for genetic mutations, and the making of genotype and phenotype correlations. Written informed consent for study participation was obtained from the subject or the subject's parents, as appropriate. The study also complied with the Health Insurance Portability and Accountability Act. Primary congenital glaucoma was defined by the following characteristics: (1) age of onset of 3 years or younger; (2) increased corneal diameter of more than 10 mm accompanied by either corneal edema, Haab striae, or both; and (3) increased IOP of more than 21 mm Hg, optic nerve cupping of more than 0.4, asymmetry of more than 0.2, or a combination thereof. Any patient with other ocular abnormalities or systemic conditions, other than iris stromal hypoplasia, was excluded from the study. Excluded ocular anomalies included posterior embryotoxon, corectopia, cataract, Axenfeld Reiger syndrome, Peters anomaly, and aniridia. A questionnaire regarding family and medical history was completed by the subject or the subject's parents, or both. Blood or saliva samples were collected from the subject and family members; genomic DNA was extracted using AutoPure LS DNA Extractor and PUREGEN reagents (Gentra Systems Inc, Minneapolis, Minnesota, USA).

A total of 47 unrelated primary congenital glaucoma probands, 10 additional affected siblings, and 71 unaffected family members of the probands were enrolled in this study. A second dataset of 101 healthy unrelated individuals with normal ophthalmic examinations and no family history of hereditary ocular diseases were recruited randomly as control subjects. The ethnic distribution of this control group was matched to our primary congenital glaucoma cohort.

Before DNA sample submission of 3 primary congenital glaucoma families for whole exome sequencing, Sanger sequencing was conducted on family 32043 to search for candidate gene mutations. However, only known combinations of compound heterozygous mutations were assessed. This family demonstrated a novel *CYP11B1* compound heterozygous mutation revealed by whole exome sequencing, and Sanger sequencing was repeated to confirm the finding. In total, DNA samples of 7 individuals from 3 families underwent whole exome sequencing.

• WHOLE EXOME SEQUENCING

Because of resource availability and the timing of experiments, 2 sequencing service providers were used: the Hudson Alpha Institute, Huntsville, Alabama, and the Beijing Genomics Institute, Hong Kong, China, with different library captures and coverage depths for exome sequencing.

SeqCap EZ Human Exome Library version 2.0 exome sequencing—DNA samples (7 µg) of 2 affected individuals from family 32057 and of 1 affected individual

from family 32071 were submitted to the Hudson Alpha Institute for exome sequencing (Figures 1 and 2). The DNA was sheared randomly between 150 to 200 base pairs (bp), and adapters were ligated to both ends of the fractions. Biotinylated ribonucleic acid capture probes from the SeqCap EZ Human Exome Library version 2.0 (Roche NimbleGen, Madison, Wisconsin, USA) were hybridized to the prepared DNA fractions. The hybrid-selected enriched output library was amplified by polymerase chain reaction before targeting the sequencing depth of $\times 50$. The SeqCap EZ Human Exome Library version 2.0 was used as target covering of approximately 36.5 Mb of the human genomic regions of consensus coding sequence exons.

Agilent SureSelect 38-Mb exome sequencing—Seven- μ g DNA samples of 3 affected siblings and 1 unaffected sibling from family 32043 were submitted to the Beijing Genomics Institute for independent processing (Figure 3). Exome libraries were generated using the Agilent SureSelect 38-Mb kit (Agilent Technologies, Santa Clara, California, USA), which captured 1.22% of human genomic regions covering consensus coding sequence exons. Samples were processed similarly as above and were sequenced to obtain an average exome coverage depth of $\times 30$.

• BIOINFORMATICS: READ MAPPING AND QUALITY FILTERING

The paired reads were aligned first to the National Center for Biotechnology Information GRCh37 lite reference genome (ftp://ftp.ncbi.nih.gov/genbank/genomes/Eukaryotes/vertebrates_mammals/Homo_sapiens/GRCh37/special_requests), with the Burrows-Wheeler Alignment tool and Sequence Alignment/Map tools (<http://samtools.sourceforge.net/>).^{22,23} All aligned reads in the merged binary alignment and map file were processed with the Genome Analyzer Toolkit (version 4333; Broad Institute, Cambridge, Massachusetts, USA).²⁴ Consensus calling was performed with the Genome Analyzer Toolkit framework to obtain single nucleotide variants (SNVs). SNVs were filtered by quality and depth, minimum total read depth (coverage), and occurrence of other SNVs or microinsertions or microdeletions (microindels) close to the variant position. SNVs passing these filter thresholds were annotated with information from the University of California, Santa Cruz genome annotation database (<http://genome.ucsc.edu/index.html>), 1000 Genomes Project (<http://www.1000genomes.org/>), consensus coding sequence (<http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi>), Ensembl (<http://www.ensembl.org>), RefSeq (<http://www.ncbi.nlm.nih.gov/RefSeq/>), MirBase (<http://www.mirbase.org/>), and EntrezGene (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>).^{25–29}

To remove common SNVs, variants present in dbSNP132 or the 1000 Genomes database were removed, unless the minor allele frequency was less than 3% or unknown. Subsequently, only coding nonsynonymous variants or splice site mutations were analyzed. Two programs, SIFT (<http://sift.jcvi.org/>) and PolyPhen2 (version 2.1.0 r367; <http://genetics.bwh.harvard.edu/pph2/>), were used to predict the functional impact of the sequence variants on the gene-encoded protein.^{30,31}

• VARIANT SCREENING USING NEXT-GENERATION SEQUENCING

Variant Call Format files generated were used for analysis, reviewing all variants within *CYP11B1*, *MYOC*, and *LTBP2* genes. Concurrently, binary alignment and map files were integrated into the Integrative Genomics Viewer (<http://www.broadinstitute.org/igv/>) to confirm coverage by visualization. Previously reported pathogenic mutations or novel variants identified were Sanger sequenced for confirmation.

• POLYMERASE CHAIN REACTION AND DEOXYRIBONUCLEIC ACID SEQUENCING

Concurrent with or after whole exome sequencing, Sanger sequencing was carried out on all affected individuals to screen for *CYP1B1* sequence variants. Sequencing of the *LTBP2* and *MYOC* genes was performed selectively on 33 affected individuals for whom DNA samples of additional family members were available to confirm cosegregation with disease status.

The primers were designed using the Primer3 program (<http://frodo.wi.mit.edu/>; Supplemental Table 1, available at AJO.com). Standard polymerase chain reaction conditions were run to amplify all exons, intron and exon boundaries, and untranslated regions. Amplicons were visualized after electrophoresis on a 2% agarose gel and were purified with Quickstep 2 SOPE Resin (Edge BioSystems, Gaithersburg, Maryland, USA). Using ABI BigDye chemistry (Applied Biosystems Inc, Foster City, California, USA), Sanger sequencing was carried out on the polymerase chain reaction amplicons and was processed through an automated ABI 3730 Sequencer (Applied Biosystems, Inc). The sequences were scrutinized for variations alongside reference sequences from the University of California, Santa Cruz genome browser website (<http://genome.ucsc.edu/index.html>) using Sequencher 5.0 software (Gene Codes, Ann Arbor, Michigan, USA). Sanger sequencing subsequently was performed on unaffected family members of cases with identified sequence variants and on 101 unaffected control samples.

RESULTS

A total of 47 families with 1 or more individual(s) affected by primary congenital glaucoma were studied: 26 (55.3%) subjects were male, whereas 21 (44.7%) subjects were female. With respect to ethnicity, 27 (57.5%) were white, 16 (34.0%) were black, 2 (4.3%) were Hispanic, 1 (2.1%) was Asian, and 1 (2.1%) had a multiracial background. Forty (85.1%) of the 47 families had only 1 affected individual.

DNA samples of 3 affected individuals from 2 families were exome sequenced (2 from family 32057, 1 from family 32071) using the SeqCap EZ Human Exome Library version 2.0 at $\times 50$ coverage. An average of 85 million reads overlapped with targeted positions, with a mean coverage of $\times 77$. On average, 96% of the targeted exons had at least $\times 1$ coverage, and an average of 88% of the exons were captured at $\times 10$ coverage depth (Supplemental Table 2, available at AJO.com). Gene-specific coverage of *CYP1B1*, *LTBP2*, and *MYOC* demonstrated mean coverage depths of $\times 50$, $\times 33$, and $\times 51$, respectively. In family 32071, a nonsense mutation (p.Trp57*) and a 10-bp insertion (rs72466463) were identified as a compound heterozygous state in the *CYP1B1* gene (Figure 1 and Table 1). In family 32057, a missense mutation (p.Glu387Lys) and a 13-bp deletion (rs72549380) in a compound heterozygous state were identified in both affected individuals in the *CYP1B1* gene (Figure 2 and Table 1).

DNA samples of 3 affected individuals and 1 unaffected individual from family 32043 were exome sequenced using the Agilent SureSelect 38-Mb library capture system with a target depth of $\times 30$ coverage (Supplemental Table 2, available at AJO.com). On average, more than 95% of the targeted exons were covered with at least $\times 1$ coverage, and an average of 74% of the exons were captured at $\times 10$ in DNA samples of 4 individuals (Supplemental Table 2, available at AJO.com). The average gene-specific coverage depths for *CYP1B1*, *LTBP2*, and *MYOC* were $\times 16$, $\times 27$, and $\times 45$, respectively. Within family 32043, Variant Call Format alone identified a nonsense mutation p.Trp57* (rs72549387) and a missense mutation p.Ala106Glu in heterozygous states in affected individuals 4, 5, and 6 (Figure 2 and Table 1). Both mutations were located within the *CYP1B1* gene. Visualization using the Integrative Genomics Viewer determined unaffected individual 7 to have too low of a coverage depth to identify variants at the same positions.

• SUMMARY OF *CYP1B1* SEQUENCE VARIATIONS

Fifteen coding sequence variants were identified within the *CYP1B1* gene (Table 2). Six variants were located in exon 2, whereas 9 variants were in exon 3. Eleven variants were nonsynonymous, whereas 2 were synonymous, comprising 6 transitions and 7 transversions. The transitions were 2 nonsense variants: c.171G→A (p.Trp57*, rs72549387), c.1063C→T (p.Arg355*, rs72549381), and 4 missense variants: c.1159G→A (p.Glu387Lys, rs55989760), c.1168C→T (p.Arg390Cys, rs148542782), c.1347T→C (p.Ala443Gly, rs1056837), and c.1358A→G (p.Asn453Ser, rs1800440). The 7 transversions were c.142C→G (p.Arg48Gly, rs10012), c.317C→A (p.Ala106Asp, no rs identification), c.355G→T (p.Ala119Ser, rs1056827), c.564C→A (p.Gly188Gly, rs9341247), c.729G→C (p.Val243Val, rs9341249), c.1294C→G (p.Leu432Val, rs1056836), and c.1328C→G (p.Ala443Gly, rs4986888). There was a 10-bp insertion c.1209_1210insTCATGCCACC (rs72466463) and a 13-bp deletion c.1064_1076delGAGTGCAGGCAGA (rs72549380), and both resulted in sequence frameshifts. Seven of the 47 primary congenital glaucoma families carried known disease-causing *CYP1B1* mutations determined to be absent in the 101 control subjects (Figure 3). Of the 7 families, an affected black individual had a homozygous change, whereas affected individuals from 1 black and 5 white families had compound heterozygous changes. To our knowledge, 5 of the 6 combinations of compound heterozygous variants are novel (Table 3). Clinically, members of all 7 families had severe disease phenotypes that required trabeculectomy or placement of aqueous drainage devices within 6 months of diagnosis because of poorly controlled IOP.

Homozygote mutation family 32012 (black)—The homozygous change was that of the SNV c.1063 C→T. The unaffected mother was heterozygous, whereas the affected child was homozygous (Figure 2). The SNV replaced arginine with a premature stop codon.

Compound heterozygote mutations family 32016, 32021, 32071 (white) and 32072 (black)—All 4 families were heterozygous for the 10-bp insertion, c.1209_1210insTCATGCCACC, which caused a change in the reading frame (Figures 1 and 2). In white family 32016, the unaffected father was heterozygous for the 10-bp insertion, whereas the mother showed heterozygosity for the SNV c.1168C→T. This SNV led to the substitution of the positively charged arginine by cysteine, which is nonpolar. The affected child inherited both heterozygous changes. In white family 32021, one unaffected parent was heterozygous for the 10-bp insertion, whereas the other unaffected parent was heterozygous for SNV c.1159G→A. This SNV resulted in the replacement of a negatively charged glutamic acid by a positively charged lysine. Their affected child acquired both heterozygous changes. In white family 32071, the father did not possess the SNV c.171G→A or the 10-bp insertion, whereas the mother was heterozygous for the p.Trp57* amino acid change. The affected twins were compound heterozygous for the 10-bp insertion and the SNV. As a result, a premature stop codon replaced tryptophan and produced a truncated protein of 56 amino acids. In black family 32072, the proband was heterozygous for both the SNV c.1063C→T (p.Arg355*) and 10-bp insertion. The proband's parental DNA samples were not available for sequencing.

Family 32057 (white)—The unaffected mother harbored a heterozygous change for SNV c.1159G→A (p.Glu387Lys) in the *CYP1B1* gene, whereas the 3 affected siblings were compound heterozygous for the 13-bp deletion and the p.Glu387Lys amino acid change (Figure 2).

Family 32043 (white)—The unaffected mother was heterozygous for SNV c.171G→A (p.Trp57*), whereas the father most likely was heterozygous for amino acid change p.Ala106Asp, inferring from the knowledge that the paternal grandmother was heterozygous

for p.Ala106Asp. As a result, all 3 affected siblings acquired both heterozygous changes. Interestingly, 1 unaffected sister shared the same genotype as the 3 affected individuals. P.Ala106Asp caused alteration of a nonpolar to a negatively charged amino acid (Figure 2).

• SUMMARY OF *LTBP2* AND *MYOC* SEQUENCE VARIATIONS

LTBP2 DNA sequencing of individuals with primary congenital glaucoma identified 14 sequence variants comprising 7 transitions and 7 transversions. These variants were inherited in a heterozygous manner and included 3 coding nonsynonymous and 11 coding synonymous SNVs. Two nonsynonymous amino acid substitutions were novel (Supplemental Table 3, available at AJO.com). However, one amino acid change (p.Ser518Ile) was present in healthy controls and the other amino acid change (p.Arg548*) did not cosegregate. *MYOC* sequencing revealed 8 sequence variants that comprised 7 transitions and 1 transversion. These variants were inherited in a heterozygous manner and produced 4 coding nonsynonymous and 4 coding synonymous alterations (Supplemental Table 4, available at AJO.com). One amino acid substitution (p.Val1361Met) was novel but did not display cosegregation.

DISCUSSION

• *CYP1B1*, *LTBP2*, AND *MYOC* SEQUENCE VARIATIONS

CYP1B1, *LTBP2*, and *MYOC* have been identified as primary congenital glaucoma-causing genes. To date, we report the largest study of sequence variants for these genes in a cohort of nonconsanguineous primary congenital glaucoma cases and families from the United States. Seven of the 47 families with primary congenital glaucoma (14.9%) had disease attributable to *CYP1B1* alterations. This percentage is much lower than the 90% to 100% found in Slovakian Romas, the 44% reported in Indian patients, and the 50% reported in Brazilian patients with primary congenital glaucoma.^{2,9} This incident rate is comparable with the 15% to 20% rate reported in Japanese and Chinese primary congenital glaucoma populations.^{10,32} In our cohort, the pathogenic *CYP1B1* sequence variants comprised 3 missense amino acid changes, 2 nonsense amino acid changes, a 10-bp insertion, and a 13-bp deletion (Figure 3). All 7 pathogenic variants were determined by both SIFT and Polyphen2 programs to be damaging to the resultant protein integrity and functional activity.

The missense amino acid substitution p.Ala106Asp is located within helix B, whereas the other 2 missense amino acid changes, p.Glu387Lys and p.Arg390Cys, form the construct of the consensus sequence GluXXArg within helix K, where X can be any amino acid.^{33–36} Both helices B and K form part of the 2 highly conserved interaction clusters that are situated mainly around the heme group and the proximal site of the P450 enzyme.^{36,37} Hence, missense mutations in both helices can severely disrupt *CYP1B1* enzymatic activity.^{36,37} For the 2 nonsense amino acid changes, p.Trp57* and p.Arg355*, they lead to the truncation of the protein at amino acids 56 and 354, respectively, disrupting proper protein folding to create the heme binding domain essential for enzymatic activity of *CYP1B1*.^{36,37} It is also possible that the transcripts harboring premature termination codons can be degraded selectively by the nonsense-mediated messenger ribonucleic acid decay pathway. However, the 10-bp insertion (c.1209_1210insTCATGCCACC) and the 13-bp deletion (c.1064_1076delGAGTGCAGGCAGA) result in a frameshift followed by premature stop codons, causing amino acid changes p.Arg355fs*69 and p.Thr404fs*30, respectively, in its new reading frame.^{7,36} Such frameshifts change the composition of amino acids making up the *CYP1B1* enzyme and drastically change the primary, secondary, and tertiary structure of *CYP1B1*, rendering the protein functionally inactive.

Compound heterozygous variations were observed in 6 primary congenital glaucoma families from our cohort, of which 5 combinations were novel.³² Interestingly, the combination of amino acid change p.Trp57* and the 10-bp insertion (c. 1209_1210insTCATGCCACC) observed in family 32071 has been reported in a Rieger anomaly patient.³⁸ *CYP1B1* mutations in patients with glaucoma secondary to anterior chamber malformations also have been noted in other studies.^{39–41} Also, all 7 families with pathogenic *CYP1B1* mutations exhibited complete penetrance, except for family 32071 and 32043. In family 32071, both twins carried heterozygous changes for the 10-bp insertion, c. 1209_1210insTCATGCCACC, which was not present in both parents, implying the possibility of a de novo mutation. In family 32043, an unaffected sister shared the same compound heterozygous *CYP1B1* amino acid changes (p.Trp57* and p.Ala106Asp) with the 3 affected individuals. The age of these patients at the time of study ranged from 17 to 30 years. Phenotypic variability has been documented for *CYP1B1* mutations in individuals with primary congenital glaucoma.³³ Although primary congenital glaucoma symptoms usually manifest at birth or infancy, some family members of the probands were diagnosed with glaucoma presenting later in early adulthood or were thought to represent cases of incomplete penetrance.⁴² Nonetheless, it is important that the unaffected sibling in this family undergo regular ophthalmic examinations to allow prompt diagnosis and early treatment if needed. With respect to genotype-phenotype correlations, Martin and associates speculated that subjects with compound heterozygous *CYP1B1* alterations may exhibit less disease severity than those with homozygous alterations.⁴³ However, in our cohort, both the homozygous and compound heterozygous cases had indiscriminate severe disease clinical courses. Most of them were diagnosed at birth or within 2 weeks after birth and required trabeculectomy procedures or aqueous drainage device placements within 6 months of diagnosis because of poor IOP control. A previous study demonstrated the possibility of a digenic mechanism involving mutant alleles of both *MYOC* and *CYP1B1* genes.⁴² In our cohort, no *MYOC/CYP1B1* digenic mutations were identified that were disease causing.

• EXOME SEQUENCING

Exome sequencing has demonstrated the powerful capabilities of next-generation sequencing for primary congenital glaucoma screening. All disease-causing sequence variations in the DNA of 7 individuals were identified as a result of combining both SNV filtering and visualization. The mean coverage for *CYP1B1* from the Hudson Alpha Institute was $\times 50$, with an average of 38355 total reads across the gene (Supplemental Table 2, available at AJO.com). *LTBP2* and *MYOC* also had similar coverage depths, with an average of $\times 33$ and $\times 51$ depth and average total reads of 258329 and 92209, respectively. Strategies to capture exomes at $\times 30$ depth coverage across family 32043 generally were successful, but low or undetected variants were evident because of insufficient coverage in certain areas within the genes (Table 1). Average total reads across *CYP1B1*, *LTBP2*, and *MYOC* in these samples were 24000, 233104, and 63790, respectively (Supplemental Table 2, available at AJO.com). Of note, variant detection analyzed with either library capture systems is not reflective of the quality of the library capture kits themselves, but rather of the strategy and resources applied to sequence samples at certain coverage depths. As a result, a fair comparison between the 2 library capture kits used cannot be made within our study. Although our strategy involved the use of different capture systems with different exome coverage expectations because of resource availability, we demonstrated that both exome capture technologies detected pathogenic variations with high confidence. The amount of data and coverage produced by next-generation sequencing, the tedious preparatory steps, lack of standard analytical pipeline strategies, and automation are some of the technical challenges of performing exome sequencing and subsequent analysis.⁴⁴

In conclusion, this study screened for *CYP1B1*, *LTBP2*, and *MYOC* mutations in a large primary congenital glaucoma cohort. One homozygous and 6 compound heterozygous *CYP1B1* mutations were determined in this cohort, of which 5 compound heterozygous combinations were novel. With regard to genotype-phenotype correlation, both the homozygous and compound heterozygous cases had indiscriminate severe disease clinical courses. In addition, this study reaffirms our current understanding that *CYP1B1* is the main causative gene for primary congenital glaucoma cases worldwide. However, mutations in *CYP1B1* are less common in this United States cohort (14.9%) than in Arabic or Romany populations. This highlights the fact that other genes are accountable for primary congenital glaucoma in the United States population, reinforcing the idea that it is an ocular disease of genetic heterogeneity. More studies need to be undertaken to identify novel genes causing primary congenital glaucoma. Whole exome sequencing can be used as a complementary tool to traditional Sanger sequencing in identifying novel genes in patients who have no mutations in known disease-causing genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biography

Sing-Hui Lim is currently a medical student at Duke-National University of Singapore Graduate Medical School, Singapore, Republic of Singapore, and will be graduating in 2013. Under the tutelage of Dr Terri L Young, a pediatric ophthalmologist at the Duke Eye Center, Ms Lim spent her third year conducting research work at Duke University. Her research interests are focused on ophthalmology genetics in a range of ocular diseases, including primary congenital glaucoma, myopia and optic nerve hypoplasia.

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Family 32012

Family 32016

Family 32021

Family 32071

FIGURE 1.

Pedigrees with *CYP11B1* sequence variants in primary congenital glaucoma families 32012, 32016, 32021, and 32071 in the United States. Both alleles of the genotypes are listed below each individual. Deoxyribonucleic acid (DNA) sample from individual 1 was sent for whole exome sequencing. Circles indicate females and squares indicate males. Shaded shapes refer to affected individuals. Arrowheads indicate the proband. WT = wild type; + = DNA was collected and analyzed from that individual.

FIGURE 2.

Pedigree showing the *CYP11B1* sequence variants in primary congenital glaucoma families 32072, 32057, and 32043 in the United States. Both alleles of the genotypes are listed below each individual. Deoxyribonucleic acid (DNA) samples from individuals 2 through 7 were sent for whole exome sequencing. Circles indicate females and squares indicate males. Shaded shapes refer to affected individuals. Arrowheads indicate the proband. WT =wild type; +=DNA was collected and analyzed from that individual.

FIGURE 3.

Locations of identified mutations in the *CYP11B1* gene from a study on primary congenital glaucoma individuals in the United States. The locations of the 5 amino acid substitutions, a 10-bp insertion, and a 13-bp deletion identified in this study on primary congenital glaucoma individuals in the United States are annotated in the diagram as p.W57*, p.A106D, c.1064_1076delGAGTGCAGGCAGA, p.R355X, p.E387K, p.R390C, and c.1209_1210insTCATGCCACC. Conserved regions are shown in black with the protein structure annotation listed below.

TABLE 1

CYP11B1 Variants from Exome Sequencing on Primary Congenital Glaucoma Families in the United States

Primary Congenital Glaucoma Family	Individual Number	Mutation	dbSNP Identification Data	Base Pair Location ^a	Next-Generation Sequencing Coverage ^b
32071	1	p.Trp57*	rs72549387	38302361	×4
		10-bp insertion	rs7246463	38298287_38298288insGGTGGCATGA	×19
32057	2	p.Glu387Lys	rs55989760	38298338	×11
		13-bp deletion	rs72549380	38298421_38298433deTTCTGCCCTGCACTC	×6
32043	3	p.Glu387Lys	rs55989760	38298338	×16
		13-bp deletion	rs72549380	38298421_38298433deTTCTGCCCTGCACTC	×7
32043	4	p.Trp57*	rs72549387	38302361	×13
		p.Alal106Asp	Novel	38302215	×11
32043	5	p.Trp57*	rs72549387	38302361	×7
		p.Alal106Asp	Novel	38302215	×8
32043	6	p.Trp57*	rs72549387	38302361	×9
		p.Alal106Asp	Novel	38302215	×15
32043	7 (Unaffected)	p.Trp57*	rs72549387	38302361	N/A
		p.Alal106Asp	Novel	38302215	N/A

N/A = not applicable.

^aGRCh37.p5.

^bNumber of reads present at the particular location using Variant Call Format files or visualization using Integral Genomics Viewer.

TABLE 2

CYP1B1 Coding Sequence Variants Identified in Primary Congenital Glaucoma Individuals in the United States

Exon	rs Number	Function	Protein Residue (Amino Acid) Change
2	rs10012C→G	CNS	p.R48G
2 ^a	rs72549387G→A	CNS	p.W57X
2 ^a	(no rs number) C→A	CNS	p.A106D
2	rs1056827G→T	CNS	p.A119S
2	rs9341247C→A	CS	p.G188G
2	rs9341249G→C	CS	p.V243V
3 ^a	rs72549381C→T	CNS	p.R355X
3 ^a	rs55989760G→A	CNS	p.E387K
3 ^a	rs148542782C→T	CNS	p.R390C
3	rs1056836C→G	CNS	p.L432V
3 ^a	rs72466463	10-bp insertion	Frameshift
3 ^a	rs72549380	13-bp deletion	Frameshift
3	rs4986888C→G	CNS	p.A443G
3	rs1056837T→C	CS	p.D449D
3	rs1800440A→G	CNS	p.N453S

bp = base pair; CNS = coding nonsynonymous; CS = coding synonymous.

^aSequence variants determined to be associated with primary congenital glaucoma in this study.

TABLE 3Pathogenic *CYP11B1* Mutations in 7 Primary Congenital Glaucoma Families in the United States

Primary Congenital Glaucoma Family	Homozygous/Heterozygous	<i>CYP11B1</i> Amino Acid Changes	Combination Previously Reported
32012	Homozygous	p.Arg355*/p.Arg355*	Yes
32016	Compound heterozygous	p.Arg390Cys/c.1209_1210insTCATGCCACC	No
32021	Compound heterozygous	p.Glu387Lys/c.1209_1210insTCATGCCACC	No
32071	Compound heterozygous	p.Trp57*/c.1209_1210insTCATGCCACC	Yes
32072	Compound heterozygous	p.Arg355*/c.1209_1210insTCATGCCACC	No
32057	Compound heterozygous	p.Glu387Lys/c.1064_1076delGAGTGCAGGCAGA	No
32043	Compound heterozygous	p.Trp57*/p.Alal06Asp	No