CYP1B1 copy number variation is not a major contributor to primary congenital glaucoma

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Purpose: To evaluate the prevalence and the diagnostic utility of testing for *CYP1B1* copy number variation (CNV) in primary congenital glaucoma (PCG) cases unexplained by *CYP1B1* point mutations in The Australian and New Zealand Registry of Advanced Glaucoma.

Methods: In total, 50 PCG cases either heterozygous for disease-causing variants or with no *CYP1B1* sequence variants were included in the study. *CYP1B1* CNV was analyzed by Multiplex Ligation-dependent Probe Amplification (MLPA).

Results: No deletions or duplications were found in any of the cases.

Conclusion: This is the first study to report on *CYP1B1* CNV in PCG cases. Our findings show that this mechanism is not a major contributor to the phenotype and is of limited diagnostic utility.

Primary congenital glaucoma (PCG, OMIM 231300) is an important cause of glaucoma blindness in children and results from a developmental defect of the aqueous outflow system. It is characterized by increased intraocular pressure (IOP), buphthalmos, corneal clouding, and Haab's striae, and it generally manifests in the neonatal or early infantile period [1]. The level of incidence varies across different ethnic groups. A high incidence has been found among some populations (Slovakian Gypsies 1/1250 [2], Saudi Arabian 1/2500 [3]), but it is usually lower in Western countries (1/22,000–1/23,000) [2,4]. In Australia, PCG has an incidence of 1/30,000 births [5].

CYP1B1 (GLC3A, OMIM 601771) on chromosome 2p21 was the first gene discovered to cause PCG [6]. Pathogenic variants in *CYP1B1* have been identified among different populations [7-13], and they have been reported to occur in as few as 15% (in an American cohort) [12] and as high as 92% (in a Saudi Arabian cohort) [3] of PCG patients. In Australia, *CYP1B1* variants occur in 22% of PCG cases [14]. Four other PCG loci have been described (GLC3B-E), and *LTBP2* has been identified on chromosome 14q24 (GLC3C, OMIM 602091) [15]. However, a more recent study suggested that *LTBP2* variants might be responsible for primary congenital megalocornea with secondary glaucoma [16].

CYP1B1 is a member of the cytochrome P450 super-family and is composed of three exons, of which only the last two are coding. The protein is responsible for the metabolism of a wide range of diverse substrates, both endogenous and exogenous [17]. Functional studies have demonstrated that *CYP1B1* pathogenic variants reduce the enzymatic activity or stability of the enzyme [18,19]. The mechanism by which they cause glaucoma is not fully understood, but it is hypothesized that the expressions of genes important to the development of the eye may be altered by the level of key regulatory molecules or the presence of normally eliminated metabolites [17].

CYP1B1 pathogenic variants are inherited in an autosomal recessive manner. However, individuals with PCG harboring only one pathogenic variant in *CYP1B1* have been reported [7-9,13,14]. These patients might have a second variant in the promoter, a non-coding region, or a deletion of a part of or the entire gene on their other allele, explaining their phenotype. No study has evaluated the prevalence of *CYP1B1* copy number variation (CNV) among PCG cases. In this report, we investigated whether *CYP1B1* gene CNV accounts for PCG cases heterozygous for *CYP1B1* mutations or with no pathogenic sequence variant, as identified through gene sequencing, and we explored the diagnostic use of testing for *CYP1B1* CNV.

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METHODS

Recruitment of participants: Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. The study was conducted in accordance with the revised Declaration of Helsinki and following the National Health and Medical Research Council statement of ethical conduct in research involving humans.

Individuals with PCG were recruited through the Australian and New Zealand Registry of Advanced Glaucoma by referral from their eye practitioner [20]. Informed written consent and a blood sample for DNA extraction purposes were obtained. Clinical information was collected by the patient's usual clinical ophthalmologist. The diagnosis of PCG was based on combinations of corneal enlargement and buphthalmos, loss of corneal transparency, photophobia, raised IOP, or optic disc cupping.

Genetic testing: All PCG cases had bidirectional sequencing of the two *CYP1B1* coding exons and the respective intron–exon boundaries. Cases with two confirmed pathogenic sequence variants identified were excluded from the current analysis. Our cohort consisted of PCG patients with only one pathogenic sequence variant, with sequence variants of unknown clinical significance, or with no pathogenic sequence variants identified in the *CYP1B1* gene (n = 50).

CYP1B1 was analyzed for copy number abnormalities by Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA MLPA P128 Cytochrome P450 probemix (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. MLPA allows for the detection of small copy number changes to the DNA sequence of a gene [21]. The probes consisted of two oligonucleotides complementary to adjacent sequences of the target DNA. The two probe sequences were joined by DNA ligase. Perfect homology between the two probes with the target at this junction is required for this step. The probes were then denatured from the template and amplified in a multiplex PCR reaction with the use of a single primer pair. Each probe generated an amplification product of a unique length, allowing for the separation of products by capillary electrophoresis. An indirect measurement of the copy number present in the original DNA specimen was determined from the relative amplitude of each amplicon product detected using the ABI 3130*xl* Genetic Analyzer (Life Technologies, CA), as well as analyzed using the Peak Scanner Software v1.0 (Life Technologies).

The P128 Cytochrome P450 probemix contained internal controls. The kit included probes targeting copy number changes of 14 Cytochrome P450 and Glutathione S-transferase genes. In addition, 12 reference probes allowed for the detection of several different autosomal chromosomal locations and probes to the X and Y chromosome were included.

RESULTS

We studied 50 individuals with PCG, with 20 being female (40%) and 30 being male (60%). The majority were Caucasian (78%), while the rest were of African (8%), Asian (6%), Melanesian (2%), and Middle Eastern (6%) backgrounds. Most cases were diagnosed before the age of 3 years (92%). A family history of PCG was present in four patients; three had affected siblings and one had an affected parent. The mean highest IOP was 29.5±9.3 mmHg and the mean cup-to-disc ratio was 0.6±0.3.

Prior to this study, all cases had been directly sequenced for *CYP1B1* variants. Three of the included cases were heterozygous for previously reported variants (R444Q, P513_K514del, and E229K [14,22]). An MLPA analysis was successful in all 50 patients. Internal controls detected several copy number changes in some patients in regions that are known to differ in copy number within the normal population (e.g., *GSTM1* and *GSTT1* genes). Moreover, we were able to correctly identify the sex of the individuals tested with probes to both the X and Y chromosomes. No *CYP1B1* CNV of part of or the entire gene was detected in any cases.

TABLE 1. *CYP1B1* DELETIONS IN PRIMARY CONGENITAL GLAUCOMA PREVIOUSLY REPORTED (REFERENCE GENOME GRCH37).

Location (Chr2)	Size	Deletion	Genotype	Ethnicity	Family history	Ref
Intragenic	Intron 2 - exon 3	Partial	Homozygous	Turkish	Segregated in 3 affected siblings	[5,23]
38,222,086 − 38,368,231	146 kb	Whole gene	Homozygous	Cypriot	Negative	[25]
38,191,823 − 38,385,253	193 kb	Whole gene	Homozygous	Caucasian	Segregated in 2 affected siblings	[26]
38,187,289 −38,349,505	162 kb	Whole gene	Compound hetero-zygous (T404fs*30)	Spanish	Negative	[24]

DISCUSSION

CYP1B1 is the only known gene to cause PCG in our population to date. Its prevalence among PCG cases varies across different populations, but overall, it accounts for approximately one in five cases in Caucasians [9,12-14]. Cases heterozygous for known pathogenic variants have been frequently reported [7-9,11,13,14]. In these situations, the other allele could display a variant in the promoter, in a non-coding region, or a deletion of part of or the entire gene. Whole gene deletions or duplications have been identified to be causative in other glaucoma-associated genes, such as *PITX2*, *FOXC1*, or *TBK1* [23,24]. Moreover, a few isolated cases of partial or whole gene *CYP1B1* deletions have been previously reported (Table 1) [6, 25–28]. In this study, we screened 50 PCG cases, in which *CYP1B1* sequencing results did not fully explain the phenotype, for *CYP1B1* CNV. An MLPA analysis did not detect any intragenic deletions or duplications in this cohort.

The intragenic deletion reported by Stoilov et al. was identified through the sequencing of the coding exons [6,25]. Other *CYP1B1* whole gene deletions previously reported were identified because of suspicious sequencing results: two studies reported a homozygous whole-gene deletion following repeated failures to amplify the *CYP1B1* coding exons through sequencing [27,28]. A third study found an apparently homozygous pathogenic variant in a patient that was present in the heterozygous state in the mother, but not in the father. After excluding non-paternity and maternal disomy, a gene dosage assessment revealed a heterozygous deletion on the other allele in the patient [26]. Although some deletions would be suspected by gene sequencing, others would still be missed. The four cases of *CYP1B1* CNV previously reported were all detected by chance through direct sequencing. This is the first study to assess directly the prevalence of *CYP1B1* CNV and to explore whether such a test would be of diagnostic use to complement *CYP1B1* gene sequencing. Although we cannot deny that this mechanism does play a role in rare PCG, the prevalence of such mutations in Caucasian PCG cases is expected to be less than 1%. As the probes used cover exons 1 and 3 only, we cannot completely exclude the possibility of an intragenic exon 2 CNV. However, the three whole gene deletions and the intragenic deletion previously published would have been detected with the probes used. Further, as we did not detect any CNV in this gene, the diagnostic potential of such a test is not high.

In conclusion, we did not identify any *CYP1B1* CNV among 50 PCG cases recruited through the Australian and New Zealand Registry of Advanced Glaucoma. To the best of our knowledge, this is the first study to report MLPA results for the *CYP1B1* gene in PCG cases and to assess its clinical utility. *CYP1B1* CNV is not a major contributor to PCG in our cohort of cases CYP1B1-negative or heterozygous through sequencing. It would be interesting to assess the prevalence of *CYP1B1* CNV in other populations, especially in those where *CYP1B1* mutations are more prevalent. This finding does not exclude this mechanism occurring rarely, but it means that research efforts should be focused on identifying further causative genes that when mutated cause PCG, and that testing for *CYP1B1* CNV as a diagnostic procedure in PCG is likely to have a low yield.

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