

Screening of *CYP1B1* and *LTBP2* genes in Saudi families with primary congenital glaucoma: Genotype-phenotype correlation

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Purpose: Primary congenital glaucoma (PCG) is a severe form of glaucoma that presents early in life. PCG is a clinical and genetic entity that is distinct from juvenile forms of glaucoma. Inheritance is usually autosomal recessive and therefore the disease might be more common in societies where consanguinity is high. We studied the prevalence of cytochrome P450, family 1, subfamily B, polypeptide 1 (*CYP1B1*) and latent-transforming growth factor beta-binding protein 2 (*LTBP2*) mutations in a group of Saudi PCG patients and attempted to correlate the mutation status with the disease severity.

Methods: Genomic DNA was collected from 54 unrelated Saudi PCG families (74 patients) who were diagnosed as having PCG by standard ophthalmological examinations and screened for mutations in *CYP1B1* and *LTBP2* by sequencing. We also examined the effect of mutations on the phenotype of patients with PCG (phenotype-genotype correlation).

Results: Mutations in *CYP1B1* were identified in 41 (75.9%) of affected patients. No mutation in *CYP1B1* was found in 13 (24.1%) affected persons. We detected a total of 13 mutations: 9 missense mutations (G61E, A119S, R390H, P437L, D441G, A443G, G466S, G466D, and R469W), 2 deletions (g.4238_4247del and g.7901_7913del), and 2 nonsense mutations (R355X and R444X). Two mutations, G466S and D441G, were novel. The G61E mutation was by far the most common mutation detected. PCG cases with *CYP1B1* mutation(s) presented with a high degree of haze and greater cup/ disc ratio than those with no mutation(s). Also, PCG cases with a mutation had higher post operative indices in terms of post operative haze and the need for anti-glaucoma medications. Additionally, the surgical success rate was higher 13/14 (92.9%) among cases without mutation than those with mutation 42/60 (70%). No mutation(s) were found in *LTBP2* in any of the tested patients.

Conclusions: *CYP1B1* mutations are the predominant cause of PCG in the Saudi Arabian population with G61E as the dominant disease-associated allele. PCG cases with a mutation had higher last postoperative visit indices in terms of postoperative haze and the need for anti-glaucoma medications. This will be a valuable parameter in predicting disease severity earlier on and might help in predicting the surgical outcome.

Primary congenital glaucoma (PCG; OMIM 231300) is a severe form of glaucoma that presents early in life [1]. PCG is a clinical and genetic entity that is distinct from juvenile forms of glaucoma [2,3]. Despite its rarity, PCG and other forms of childhood glaucoma were once the leading cause of admission to schools for the blind in the United States in the first part of the 20th Century [4]. PCG results from developmental abnormalities (trabeculodysgenesis) that affect the aqueous humor outflow pathway. These changes cause elevated intraocular pressure (IOP) and secondary glaucomatous optic nerve damage [5]. PCG clinical features include elevated IOP, corneal edema, enlargement of the globe (buphthalmos), corneal enlargement, rupture of Descemet's membrane (Haab's Striae), and optic nerve damage [6]. Additional clinical features include, photophobia, ephiphora, and blepharospasm [7].

The prevalence of PCG ranges widely. The highest prevalence is found in the Gypsy population of Slovakia where 1 in 1,250 is affected. The prevalence of PCG is 1 in 2,500 among Saudi Arabians [8]. In Western populations the prevalence is approximately 1 in 20,000 [9,10]. The high rate of consanguinity among Slovakian Gypsies and Saudi Arabians accounts for the increased prevalence of PCG in these populations. In Southern India, the prevalence of PCG is estimated at 1 in 3,300 live births accounting for 4.2% of overall childhood blindness [11]. In a retrospective study covering the period from 2006 to 2010, the Glaucoma unit at King Abdulaziz University Hospital (KAUH, Riyadh, Saudi Arabia) where approximately 600 new glaucoma patients are seen annually, pediatric glaucoma accounts for about 3% of which 2.7% are PCG.

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| TABLE 1. PCR PRIMERS FOR AMPLIFICATION OF CYP1B1. | | | | | |
|---|----------------------|------------------------|--------------------|--|--|
| Primer pair | Forward (5'-3') | Reverse (5'-3') | Length (bp) | | |
| Exon 2 | TCTCCAGAGAGTCAGCTCCG | CTACTCCGCCTTTTTCAGA | 1230 | | |
| Exon 3 | GTCACTGAGCTAGATAGCCT | GGACAGTTGATTTATGCTCACC | 844 | | |

Two genes have been reported to cause PCG, *CYP1B1* (OMIM *601771; cytochrome P450, subfamily I, polypeptide I) and *LTBP2* (OMIM *602091; latent transforming growth factor beta binding protein 2) [7,12]. Both genes cause a recessive form of this disease. *CYP1B1* is the most common identifiable cause of PCG. There are over 70 reported disease-associated mutations in *CYP1B1*. In Saudi Arabia, *CYP1B1* mutations account for 96% of PCG cases [6,8]. *LTBP2* is a much rarer cause of PCG, being reported only in Pakistan and European gypsies [13].

This study was conducted to examine the role of *CYP1B1* and *LTBP2* in the Saudi Arabian population and to examine the effect of mutations on the phenotype of patients with PCG and the influence of the mutation on the surgical outcome.

METHODS

Patients: This research adhered to the tenets of the Declaration of Helsinki. All participants or their legal guardians signed an informed consent after being informed about the nature of the research. The study was approved by the ethical committee (proposal number # 08-657) of College of Medicine at King Saud University, Rivadh, Saudi Arabia. All study subjects were self identified as Saudi Arabian ethnicity. Family names were all present in the database of Arab families of Saudi Arabian origin. Additionally, these names indicated that all five major Saudi Arabian provinces were represented in the study population. Expatriates were excluded from this study. Subjects with clinically diagnosed PCG and healthy controls were recruited into the study at the glaucoma clinic of King Abdulaziz University Hospital (KAUH) in Riyadh, Saudi Arabia. All patients were unrelated to each other and never participated in a similar study or genetically tested. Controls were unrelated to the patients and to each other. All patients underwent complete eye examination under general anesthesia. Examination included assessment of intraocular pressure (IOP) with Perkins tonometer and/or Tonopen, anterior segment examination using a portable slit lamp, measurement of corneal diameter, and gonioscopy. Dilated fundus examination for optic disc evaluation was performed with an indirect and direct ophthalmoscope. Inclusion criteria were: increased corneal diameter (>12.0 mm), raised IOP (>21 mHg under sedation) and optic disc changes when clarity of the media permitted. Combined trabeculectomy and trabeculotomy versus nonpenetrating deep sclerectomy with adjuvant mitomycin C were the initial surgical procedures. Patients associated with other ocular or systemic anomalies were excluded. Non glaucomatous controls (n=50) were recruited from KAUH. Entry criteria for those subjects were age >18 years, normal IOP, open angles on gonioscopy, and normal optic nerves on examination.

CYP1B1 and LTBP2 mutation screening: DNA was extracted using the illustra blood genomicPrep Mini Spin Kit from GE Healthcare (Buckinhamshire, UK), and stored at -20 °C in aliquots until required. The coding exons of CYP1B1 were amplified using the primers listed in Table 1. Each 25 µl PCR reaction contained 2.5 µl of 10× reaction buffer with MgCl₂ (Amersham Pharmacia Biotech, Piscataway, NJ); 10 pmol of each primer; 100 pmol/ul each of deoxyATP, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate in Tris HCl buffer (Perkin-Elmer Corporation, Foster City, CA); 1 unit Taq DNA polymerase (Amersham Pharmacia Biotech); and 100 ng genomic DNA template. The mixture was denatured at 95 °C for 5 min and the PCR reaction was performed for 35 cycles, in a GeneAmp 9700 PCR system (Applied Biosystems, Foster city, CA), under the following conditions: denaturation at 95 °C for 1 min, annealing at 56 °C for 30 s and extension at 72 °C for 1 min. The final extension cycle of 72 °C was for 10 min.

Successfully amplified fragments were sequenced in both directions using the forward and reverse primers and the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). Fragments were then run on the 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer protocol. All the sequenced fragments were then analyzed using SeqScape software v2.6 (Applied Biosystems). PCG patients which were *CYP1B1* mutation-negative were screened for mutation in the *LTBP2* gene using primers and PCR conditions described previously [14] (Table 2).

Criteria for surgical success: Criteria for surgical success were IOP ≤ 21 mmHg without glaucoma medications. Qualified success was IOP of ≤ 21 mmHg with glaucoma medication. Surgical failure was all cases that required further surgical intervention to control IOP.

Prediction of pathogenicity: Pathogenic characteristics of detected non-synonymous sequence changes (those that change an amino acid in the resultant protein) in *CYP1B1* or *LTBP2* were assessed through evaluation of interspecies conservation and an assessment of the possible effect of the sequence change on protein function using PolyPhen [15].

Data collection and statistical analysis: Data were collected from medical records then entered and stored in a database

| exon | Forward (5'-3') | Reverse (5'-3') | Annealing |
|-------|-------------------------|------------------------|-----------|
| 1a | CCCAGAGCAGGAGAAAGG | GGAACAGACTGTACACCTTGG | 56 |
| 1b | GCCCCTAGACTCAGAGAAG | AATCTTCCAATCCCGATTTT | 58 |
| 2 | AATGGCAGAGTCAGGATTCA | CTTCAGGACGCAGACTAGGA | 55 |
| 3 | CTGAGGCCAGGAGAGTGG | CCAGCCCCAACACCTACT | 58 |
| 4 | AAGCCTGGTGATTCCACATA | CACAAAGCAGGTGCTCAAC | 60 |
| 5 | GCGTCCAGTAGGTACTCAGC | AGCTAGGCTGCCAAGTGAG | 60 |
| 6 | GGGGCTGGTTATTATCCACT | GGCTGAGAAGTTGAGGGAAT | 57 |
| 7 | GGGATCATTCTGGGGTTCTA | CTGTGTGCCTGGTATTGACA | 55 |
| 8 | ACTCCCTTCTCCCCTTCTTT | ACAGACTGCACCAGCAGAG | 60 |
| 9 | GCTGAGAGGAGTCTGGTGAG | TGGCTTCCTCTGTCACTCTC | 60 |
| 10 | GGAGAGGAATCCCACTGAAT | ATCTCTGTTCCAGCAGGATG | 60 |
| 11 | ATTCCACTACGCCTCTTCCT | GCAGGGAAGGCTACTTCAG | 58 |
| 12 | ACGTGCTTATCCCAACCTG | TCTTGACCCCATATGGAAGA | 58 |
| 13 | AAGAGTCCACGCTTTCTGTG | ATGGCTGCTCCATAAACAAG | 60 |
| 14 | GTAAAGTGCCTGGCAGAATG | GGTGTATAGAGAGCTCCCAGAA | 59 |
| 15 | TTAGACTGGATGTGCTCCAAC | AGAGGGACCCTGTGTTCTTT | 58 |
| 16 | CCCCTAGGGTCTTATGCAAG | GAGACTGGTCTTCCCCTGAA | 55 |
| 17 | CCCACTGGGCTGACTTTAT | AGGCTGGAGTTCTGGTCTCT | 56 |
| 18 | GGGCCTGAGCTAGATCATTT | AAGGGCTCAGGAATTCTCAT | 55 |
| 19 | GGCAGCTCTCATTCTTTCCT | TGAATATGGCCAAAGAGGAG | 60 |
| 20-21 | CATGCAGAGTGCTCTGAGTTAC | GGTCCATTTATGGGGTCTTC | 60 |
| 22 | TTCTAGGGAGGGGGTTTTAG | AAGCTTGTGAGCGACTCTTG | 59 |
| 23-24 | CCCAAGAGTCGCTCACAA | ACTCCTCGCTCCCATCTTC | 60 |
| 25 | CGAGCCTTTTCCTACATAAGC | CAGCACGAAGATGATGATTG | 58 |
| 26-27 | GGAATAGATCAAGAACCCCAGA | CTTCTTTGAAGCCTCCCTTG | 59 |
| 28 | TCTGTCCATTGGTTCCTCCT | TGTAGCTCCTGGTTTTGCTG | 60 |
| 29-30 | GGCCACTTCTTAGGGTTGTG | ACAGAAAAGGTGGAGGCAAC | 58 |
| 31 | GTAGGAACCGGAGGCAAG | CCTGGGGACAATCTCTGAC | 58 |
| 32-33 | GTGGGCTGTCAGAGATTGTC | CTACTTTGTCCCCAAACAGC | 59 |
| 34 | ATCTCCCAGAGGGTACCAGT | CCTGGGCGTATGTACTTGTC | 60 |
| 35 | TCCACAAGAATTTTATGATCCTC | TTGTCTTTTGTCTGGGAACC | 60 |
| 36 | TGTCCTTGAGTTGCTTGGTT | TCAGGATGATGGTGGATTGT | 59 |

TABLE 2. PCR PRIMERS FOR AMPLIFICATION OF LTBP2.

using Microsoft Access 2007[®] (Microsoft Corporation, Redmond, WA). Both descriptive and inferential statistical analyses were conducted to describe different indices and determine potential associations between phenotype and genotype profiles. Statistical analysis was done using SPSS v. 19 (IBM, Armonk, NY) and StatsDirect version 2.7.2 (StatsDirect, Ltd, Cheshire, UK). Odds ratios and the corresponding 95% confidence intervals were calculated to determine potential associations. Mann–Whitney U test was done to compare means of pre and post intervention indices across groups. A p value of <0.05 was used for evidence of statistical significance.

RESULTS

Clinical features of the patients: Fifty four unrelated families with at least one affected member with PCG were enrolled into this study. The pattern of inheritance was consistent with an autosomal recessive mechanism of transmission in 50 families (92.6%). In 4 families (7.4%) there appeared to be a pseudo-dominance mode of inheritance and none of the families examined had an autosomal dominance inheritance. Out of the 54 families recruited, 13 (24.1%) were multiplex

and in 41 families (75.9%) only one family member was affected. Consanguinity was a factor in 37 of 54 families (68.5%). In the 54 families examined, there were a total of 74 affected subjects with PCG, 38 males and 36 females. Among the PCG probands, the disease was bilateral in 20 (37%) and unilateral in 34 (63%). At the preoperative assessment, the mean (SD) age at surgery was 8.4 months (std 18.5), the preoperative IOP was 31.1 (std 9.3), with a corneal diameter of 12.8 (std 1.2), cup/disc ratio of 0.66 (0.8), and an average degree of haze of 2.0 (std 1.2) graded according to the Fantes scale [16].

CYP1B1 mutation analysis: Mutations in *CYP1B1* were identified in 41 (75.9%) families. No mutation in *CYP1B1* was found in 13 (24.1%) families. Among patients with mutations (n=41), 35 (85.3%) had homozygous mutations, 6 (14.7%) had compound heterozygous mutations (Table 3). We detected a total of 13 mutations: 9 missense mutations (G61E, A119S, R390H, P437L, D441G, A443G, G466S, G466D, and R469W), 2 deletions (g.4238_4247del and g.7901_7913del), and 2 nonsense mutations (R355X and R444X). Two mutations, G466S and D441G had not been previously

| CVP1R1 mutations | | | | | | |
|------------------|----------|--------------------------------------|-------------------|---------------|--------------|------------------------|
| Drohand I D | Sar | Mutation (1) | Mutation (2) | Mutation (2) | Conc | Mode of inheritance |
| 100 | Sex F | $\sigma 8147 C > T$ | Wittation (2) | Wittation (5) | Ves | Recessive |
| 109 | M | g.0147 C > 1 $g.3087 C > \Lambda$ | _ | | No | Pseudo D |
| 237 | M | $g_{3087} G > \Lambda$ | _ | | Ves | Recessive |
| 257 | M | $g_{.3}^{.3}$ $G > A$ | - | - | Vas | Pagassiva |
| 239 | E | $g_{.398}/G > A$ | - | - | Vas | Recessive |
| 202 | F | g.3000 G > A | - | - | No | Pagassiva |
| 207 | M | g.398/ U > A | - | - | No | Recessive |
| 270 | E | - 9724 C>A | - | - | No | Recessive |
| 274 | Г | g.0234 G/A | - | - | I es | Recessive |
| 296 | M | g.398/G>A | - | - | Yes | Recessive |
| 303 | M | g.398/G>A | - | - | i es | Recessive |
| 307 | M | - 2007 (> 4 | - | - | NO Nu | Recessive |
| 314 | M | g.398/G>A | g.4238_424/ del | - | INO Maria | Recessive |
| 329 | M | - 2007 (> 4 | - | - | Yes | Recessive |
| 338 | F | g.398/G>A | g.8233 G>A | - | NO | Recessive |
| 494 | M | g.8242 C >1 | - | - | Yes | Recessive |
| 505 | F | g.3987 G>A | - | - | No | Recessive |
| 517 | F | g.3987 G>A | - | - | Yes | Recessive |
| 556 | F | g.3987 G>A | - | - | Yes | Recessive |
| 624 | М | - | | - | No | Recessive |
| 631 | F | g.3987 G>A | g.7900 C>T | - | Yes | Recessive |
| 638 | М | - | - | - | No | Recessive |
| 675 | М | g.8167 C >T | - | - | Yes | Recessive |
| 696 | F | g.3987 G>A | - | - | Yes | Recessive |
| 702 | F | - | - | - | No | Recessive |
| 752 | Μ | - | - | - | No | Recessive |
| 819 | F | - | - | - | Yes | Recessive |
| 839 | F | g.3987 G>A | - | - | Yes | Recessive |
| 844 | М | - | - | - | No | Pseudo D |
| 849 | М | g.3987 G>A | - | - | Yes | Recessive |
| 873 | F | g.3987 G>A | - | - | Yes | Recessive |
| 896 | М | g.3987 G>A | - | - | Yes | Recessive |
| 922 | М | g.3987 G>A | - | - | Yes | Pseudo D |
| 950 | F | g.3987 G>A | - | - | Yes | Recessive |
| 973 | F | - | - | - | Yes | Recessive |
| 979 | М | g.3987 G>A | - | - | No | Recessive |
| 1041 | F | g.3987 G>A | - | - | Yes | Recessive |
| 1114 | M | g 3987 G>A | - | - | Yes | Recessive |
| 1124 | F | g 3987 G>A | - | - | Yes | Recessive |
| 1125 | M | - | - | - | Yes | Recessive |
| 1130 | F | - | - | - | No | Recessive |
| 1136 | F | σ 3987 G>A | - | - | Yes | Recessive |
| 1208 | M | g 3987 G>A | - | - | Yes | Recessive |
| 1200 | M | g 3087 G>A | _ | _ | Ves | Recessive |
| 1240 | F | g.3987 C>A | σ 8165 C>C | a 8150 A>G | No | Recessive |
| 1207 | F | g 3087 G>A | g.8105 C>G | g.0157 A>0 | Ves | Recessive |
| 1472 | M | 2001 7012 dal | - | - | Vas | Recessive |
| 1472 | M | 7901_7915 del | - a 4160 C>T | - | I es | Recessive |
| 1481 | IVI N | g.370/ G-A | g.4100 G>1 | - | INO Var | Recessive Recessive |
| 1541 | M | g.398/ G>A | - | - | Y es | Recessive |
| 1/24 | M | - 2007 (25.4 | - | - | Y es | Recessive |
| 214/ | F F | g.398/G>A | - | - | Y es | Recessive |
| 2154 | F | g. 8242 C>1 | - | - | Yes | Recessive |
| 2266 | M | g.3987 G>A | g.4160 G>T | - | No | Recessive |
| 2303 | M | g.3987 G>A | - | - | Yes | Recessive |
| 2333 | F | g.3987 G>A | - | - | Yes | Pseudo D |
| | - | 0 | | | | |

Mutations in **bold** were inherited in a Heterozygous status, all other mutations were inherited in a homozygous status. Pseudo D=Pseudo-dominance; **Cons**=Consanguineous.

reported (Table 3). Ten mutations were either frameshift mutations leading to premature truncation of the protein or were predicted to be pathogenic based on in silico analysis and on interspecies conservation (Table 4). Three mutations (A119S, A443G, and G466S) were predicted to be "benign." The p.G61E accounted for 59% of mutations in probands

(homozygous in 29/54 probands and heterozygous in 6/54 probands). The allele frequency for this mutation was 0.03% in controls and was heterozygous in all instances.

LTBP2 mutation analysis: No mutations in *LTBP2* were found in the 13 PCG subjects who were negative for *CYP1B1* mutations.

| | | TABLE 4. AN | ALYSIS OF THE NON-SYNONYM | AOUS SEQUENCE CHANGES DETE | SCTED IN THE PCG PATIE | NTS. | | |
|-------------------|-----------------|-----------------------|---------------------------|----------------------------|------------------------|---------------------|-----------------|-----------------|
| Nucleotide change | Protein | Mutation type | Allele frequency in | Allele frequency in | Interspecies | PolyPhen | Novel | Reference |
| | change | | PCG | controls | conservation | | | |
| g.3987G>A | G61E | Missense | 0.57 | 0.03 | High | Probably | No | [8, 32, 40, 41] |
| | | | | | | damaging | | |
| g.4160G>T | A119S | Missense | 0.02 | 0 | Low | Benign | No | [8,42] |
| g.4238_4247del | Del+FS | Del+FS | 0.01 | 0 | High | | No | [9] |
| g.7900C>T | R355X | Nonsense | 0.01 | 0 | High | | No | [18] |
| g.7901_7913del | Del+FS | Del+FS | 0.02 | 0 | High | | No | [27] |
| g.8006G>A | R390H | Missense | 0.02 | 0 | High | Probably | No | [18, 29] |
| | | | | | | damaging | | |
| g.8147C>T | P437L | Missense | 0.02 | 0 | High | Probably | No | [18, 25, 40] |
| | | | | | | damaging | | |
| g.8159A>G | D441G | Missense | 0.02 | 0 | Medium | Possibly | Yes | This report |
| | | | | | | damaging | | |
| g.8165C>G | A443G | Missense | 0.01 | 0 | Low | Benign | No | [25, 41, 43] |
| g.8167C>T | R444X | Nonsense | 0.02 | 0 | High | | No | [18] |
| g.8233G>A | G466S | Missense | 0.01 | 0 | Low | Benign | Yes | This report |
| g.8234G>A | G466D | Missense | 0.02 | 0 | High | Probably | No | [24] |
| | | | | | | damaging | | |
| g.8242C>T | R469W | Missense | 0.04 | 0 | High | Probably | No | [8, 18, 40] |
| | | | | | | damaging | | |
| PolyPhen nathoo | enicity nredict | isii bessesse sew uci | no the PolyPhen2 dataha | se ''Prohahly Damaoino' | " constitutes a high c | مەلتەمەردە مۇ ھۆلەر | ting nrotein fi | inction |

rouvened partocal partocal was assessed using the rouvened statease. Froving Damaging consumes a mail contractice of anecung protein function or structure, while "Benign" changes most likely lack phenotypic effect. "Unknown" means that PolyPhen could make no prediction due to lack of data and (-) indicate that PolyPhen is not designed to predict the effect on protein function for deletions and nonsense mutations.

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TABLE 5. CLINICAL INDICES AT PRESENTATION AND POST OPERATIVE FOR PATIENTS WITH CYP1B1 MUTATION(S) AND PATIENTS WITHOUT MUTATION(S).

| Index | Cases with mutation (mean±SD) | Cases without mutation (mean±SD) | p value |
|---|----------------------------------|-------------------------------------|---------|
| Preoperative IOP | 30.59 (9.5) | 33.36 (8.4) | 0.322 |
| Preoperative corneal diameter | 12.74 (1.1) | 13.15 (1.3) | 0.260 |
| Preoperative degree of haze | 2.08 (1.1) | 1.57 (1.3) | 0.113 |
| Preoperative cup/disc ratio | 0.70 (0.9) | 0.53 (0.3) | 0.490 |
| Preoperative number of medication | 2.09 (0.6) | 2.21(0.4) | 0.548 |
| Age at surgery (M) | 7.61 (15) | 11.21 (28.2) | 0.521 |
| Last postoperative visit | | | |
| IOP | 16.65 (5.3) | 16.43 (3.9) | 0.886 |
| Last postoperative visit corneal diameter | 12.45 (1.1) | 12.85 (1.1) | 0.264 |
| Last postoperative visit degree of haze | 0.49 (0.9) | 0.00(0) | 0.025 |
| Last postoperative visit number of medication | 0.59 (0.9) | 0.00 (0) | 0.015 |

Table 6. Clinical indices at presentation and post operative for patients with specific g.3987 G>A (Homo) mutation and patients without any mutation(s).

| Index | Cases with g.3987 G>A (mean±SD) | Cases without any mutation (mean±SD) | p value |
|---|------------------------------------|--------------------------------------|---------|
| Preoperative IOP | 30.73 (9.3) | 33.36 (8.4) | 0.485 |
| Preoperative corneal diameter | 12.42 (0.9) | 13.15 (1.3) | 0.103 |
| Preoperative degree of haze | 2.20 (1.1) | 1.57 (1.3) | 0.027 |
| Preoperative cup/disc ratio | 0.76(1) | 0.53 (0.3) | 0.518 |
| Preoperative number of medication | 2.22 (0.6) | 2.21 (0.4) | 0.450 |
| Age at surgery (M) | 5.91 (14.7) | 11.21 (28.2) | 0.267 |
| Last postoperative visit IOP | 16.69 (5.2) | 16.43 (3.9) | 0.795 |
| Last postoperative visit corneal diameter | 12.35 (1.2) | 12.85 (1.1) | 0.077 |
| Last postoperative visit degree of haze | 0.59 (1.1) | 0.00 (0) | 0.017 |
| Last Postoperative visit number of medication | 0.44 (0.8) | 0.00 (0) | 0.049 |

Phenotype-genotype correlation: We studied the possible correlation between the mutation status (present or absent) with various clinical indices at presentation and at last postoperative visit. We found that PCG cases with CYP1B1 mutations presented with a high degree of haze than those with no mutation(s), shown in Table 5. Additionally, PCG cases with a mutation had higher last postoperative visit indices in terms of final visit haze and the need for anti-glaucoma medications. The differences in both last postoperative visit degree of haze and number of needed anti glaucoma medications were statistically significant (p=0.025, and 0.015, respectively). Comparing cases with the specific g.3987 G>A mutation to those without any mutation revealed that they were presented to hospital with a significantly higher degree of haze (p=0.027), and a higher cup/disc ratio, however, insignificantly (p=0.518). In regards to the last postoperative visit indices, cases with g.3987 G>A mutation show higher degree of haze and number of needed medication than cases without any mutation(s), where both differences were statistically significant (p=0.017 and 0.049, respectively; Table 6). Additionally, we found that among cases without mutation, the surgical success rate was 13/14 (92.9%) which was much higher than the success rate among those with mutation 42/60 (70%). This may reflect the impact of severity due to mutation where the Odds Ratio of failure among cases with mutation to those without was 5.6, however, insignificantly (p=0.110).

DISCUSSION

We screened a large cohort of well defined Saudi PCG patients and perform detailed genotype-phenotype correlations. The mode of inheritance in the majority of the families was autosomal recessive, as may be expected in a society where consanguinity can reach up to 65% in some parts of the country [17]. Mutations in CYP1B1 were present in over 75% of patients. As high as this rate is, it is substantially lower than that reported by Bejjani et al. [8] where the observed rate was 96% in this population. We detected 13 mutations, 11 have been previously reported in various populations [7,18] and two were novel. Three mutations (A119S, A443G and G466S) are predicted to be benign based on in-silico analysis and low interspecies conservation. Therefore, the pathogenicity of these three mutations on the development of PCG is unclear. In the literature, Certain CYP1B1 mutations have been analyzed in-silico for their possible impact on the protein structure and function. Comparative modeling of the human CYP1B1 using the X-ray structure of CYP2c9 as a template along with molecular dynamics simulations has provided

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evidence for several structural differences that could impact the functional domains of this protein [19]. In vitro studies to determine the effect of CYP1B1 mutations on the stability and function of the protein was performed by Jansson and coworkers [20]. These investigators studied the effect of two missense mutations (G61E and R469W) on the stability and enzymatic activity of CYP1B1. It was observed that the G61E mutant had lost 60% of its stability, while the R469W mutant retained about 80% of the stability compared to the wild type. The effects of the mutants on the function of protein were further determined by an enzymatic assay that further confirmed their decreased metabolic activity (50%-70%) for all the substrates when compared to the wild protein [20]. Similar studies for the variants described as "benign" by in silico analysis would improve our understanding of these variants.

We detected *CYP1B1* mutations in 75.9% of our PCG probands, whereas 24.1% had no mutation(s). This rate (75.9%) is higher than the rate observed in less homogeneous populations where consanguinity is rare or less common like Japan (20%) [21], China (17.2%) [22], Indonesia (33.3%) [23], India (44%) [24], Brazil (50%) [25],Turkey (42.8%) [26], Morocco (46.5%) [27], Australia (21.6%) [28], France (48%) [29], and Spain (34.2%) [30]. However, the rate observed here was comparable to the rates observed in Iran (70%) [31], Kuwait (70.6%) [32], and the Slovakian gypsies (100%) where consanguinity is more common [33]. It is interesting to observe that *CYP1B1* appears to play a larger role in PCG in more homogenous and inbred populations.

The G61E mutation was the most common mutation detected (63% among PCG patients). The high frequency of the G61E is likely a founder effect. This mutation was detected at a 0.03% allele frequency in 50 normal controls. Since the G61E mutation in CYP1B1 was found in 63% of the tested PCG patients and constitutes over 80% of the total mutations detected in this population, screening for this mutation first would be appropriate.

We screened PCG patients which were CYP1B1 negative for LTBP2 and we found no mutation(s) after screening the full gene. Ali et al. [14] first reported that the *LTBP2* mutations c.412delG, c.895C>T, c.1243–1256del, and c.331C>T caused PCG in four consanguineous families from Pakistan and in persons of Gypsy ethnicity. Narooie-Nejad et al. [34] subsequently reported two LTBP2 loss of function mutations in Iranian families with PCG, homozygosity for the deletion c.5376delC in exon 36 and homozygosity for the deletion c.1415delC in exon 7. Although double heterozygosity (i.e., heterozygosity for a mutation at each of two separate genetic loci) for a CYP1B1 mutation and an LTBP2 mutation were reported by Azmanov et al. [35], the observed combination is of no clinical significance and digenic inheritance is unlikely. Apart from these reports, no further studies showing *LTBP2* mutations in PCG patients had been reported thus far. The negative results obtained here and the lack of further evidence that *LTBP2* mutations are involved in the pathogenesis of PCG raises questions about the role of this gene in PCG.

Hollander and coworkers [36,37] correlated *CYP1B1* mutations with the degree of angle dysgenesis observed histologically, as well as disease severity in terms of age at diagnosis and difficulty in controlling IOP in six congenital glaucoma patients. Their findings suggest that *CYP1B1* mutations may produce allele associated histological findings that may be correlated with disease severity. We were unable to identify any phenotypic traits that correlated with *CYP1B1* mutations or between the most common G61E mutations and other *CYP1B1* mutations. This may be due to the fact that effects are small or that this study lacked the power to determine differences.

In our study, we found that PCG cases with *CYP1B1* mutations had a last postoperative visit degree of haze and a greater cup/disc ratio than those with no mutation. In particular, the last postoperative visit degree of haze and number of medication were zero for cases without mutation(s). Moreover, PCG cases with the G61E mutation show even higher rate of the same indices. Additionally, we found that among cases without mutation, the surgical success rate was much higher than the rate among those with mutation. To the best of our knowledge, this is a novel and unique observation which might be valuable in term of predicting the severity of the disease during an early stage, choosing the best treatment regimen and surgical procedure and in providing counseling for families with affected children.

In general, genotype-phenotype correlation is highly variable. Walton and coworkers [38] have shown that the phenotype can vary significantly in the same individual (one eye being more severely affected than the other). No consistent correlation has been observed between the severity of the glaucoma phenotype and the molecular *CYP1B1* genotype among individuals with identical mutations within the same family [8], and among families with identical mutations [6,8]. No information is available on correlation between the success of surgical therapy and the type of *CYP1B1* mutation detected. However, it has been reported that patients with *CYP1B1* mutations need more surgical procedures to control intraocular pressure than individuals with congenital glaucoma without *CYP1B1* mutations [39].

In summary, this report confirms that recessive mutations in *CYP1B1* are the predominant cause of PCG in the Saudi population with p.G61E as the dominant disease-associated allele. While over 75% of PCG patients carried *CYPB1* mutations this was substantially less than previous reports. This finding suggests that this population may be powerful to detect novel genes that cause PCG. Additionally, PCG cases with a mutation had higher last postoperative visit indices in

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terms of postoperative haze and the need for anti-glaucoma medications. Genetic testing for patients suspected of PCG would be highly beneficial in term of predicting the disease severity. Testing for families with previous history of PCG would be beneficial and can provide a sense of relief from uncertainty and help people make informed decisions about managing their health care.

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REFERENCES

- Sarfarazi M, Stoilov I, Schenkman JB. Genetics and biochemistry of primary congenital glaucoma. Ophthalmol Clin North Am 2003; 16:543-54. [PMID: 14740995]vi.
- Lichter PR. Genetic clues to glaucoma's secrets. The L Edward Jackson Memorial Lecture. Part 2. Am J Ophthalmol 1994; 117:706-27. [PMID: 8198155]
- Anderson KL, Lewis RA, Bejjani BA, Baird L, Otterud B, Tomey KF, Astle WF, Dueker DK, Leppert M, Lupski JR. A gene for primary congenital glaucoma is not linked to the locus on chromosome 1q for autosomal dominant juvenileonset open angle glaucoma. J Glaucoma 1996; 5:416-21. [PMID: 8946299]
- Biglan AW. Glaucoma in children: are we making progress? J AAPOS 2006; 10:7-21. [PMID: 16527674]
- deLuise VP, Anderson DR. Primary infantile glaucoma (congenital glaucoma). Surv Ophthalmol 1983; 28:1-19. [PMID: 6353647]
- Bejjani BA, Stockton DW, Lewis RA, Tomey KF, Dueker DK, Jabak M, Astle WF, Lupski JR. Multiple CYP1B1 mutations and incomplete penetrance in an inbred population segregating primary congenital glaucoma suggest frequent de novo events and a dominant modifier locus. Hum Mol Genet 2000; 9:367-74. [PMID: 10655546]
- Kaur K, Mandal AK, Chakrabarti S. Primary Congenital Glaucoma and the Involvement of CYP1B1. Middle East Afr J Ophthalmol 2011; 18:7-16. [PMID: 21572728]
- Bejjani BA, Lewis RA, Tomey KF, Anderson KL, Dueker DK, Jabak M, Astle WF, Otterud B, Leppert M, Lupski JR. Mutations in CYP1B1, the gene for cytochrome P4501B1, are the predominant cause of primary congenital glaucoma in Saudi Arabia. Am J Hum Genet 1998; 62:325-33. [PMID: 9463332]
- Gencik A. Epidemiology and genetics of primary congenital glaucoma in Slovakia. Description of a form of primary congenital glaucoma in gypsies with autosomal-recessive inheritance and complete penetrance. Dev Ophthalmol 1989; 16:76-115. [PMID: 2676634]
- Genĉîk A, Gencikova A, Ferak V. Population genetical aspects of primary congenital glaucoma. I. Incidence, prevalence, gene frequency, and age of onset. Hum Genet 1982; 61:193-7. [PMID: 7173860]

- Dandona L, Williams JD, Williams BC, Rao GN. Populationbased assessment of childhood blindness in southern India. Arch Ophthalmol 1998; 116:545-6. [PMID: 9565065]
- Choudhary D, Jansson I, Schenkman JB. CYP1B1, a developmental gene with a potential role in glaucoma therapy. Xenobiotica 2009; 39:606-15. [PMID: 19622003]
- Ali M, McKibbin M, Booth A, Parry DA, Jain P, Riazuddin SA, Hejtmancik JF, Khan SN, Firasat S, Shires M, Gilmour DF, Towns K, Murphy AL, Azmanov D, Tournev I, Cherninkova S, Jafri H, Raashid Y, Toomes C, Craig J, Mackey DA, Kalaydjieva L, Riazuddin S, Inglehearn CF. Null mutations in LTBP2 cause primary congenital glaucoma. Am J Hum Genet 2009; 84:664-71. [PMID: 19361779]
- Ali M, McKibbin M, Booth A, Parry DA, Jain P, Riazuddin SA, Hejtmancik JF, Khan SN, Firasat S, Shires M, Gilmour DF, Towns K, Murphy AL, Azmanov D, Tournev I, Cherninkova S, Jafri H, Raashid Y, Toomes C, Craig J, Mackey DA, Kalaydjieva L, Riazuddin S, Inglehearn CF. Null mutations in LTBP2 cause primary congenital glaucoma. Am J Hum Genet 2009; 84:664-71. [PMID: 19361779]
- Sunyaev S, Ramensky V, Koch I, Lathe W 3rd, Kondrashov AS, Bork P. Prediction of deleterious human alleles. Hum Mol Genet 2001; 10:591-7. [PMID: 11230178]
- Fantes FE, Hanna KD, Waring GO 3rd, Pouliquen Y, Thompson KP, Savoldelli M. Wound healing after excimer laser keratomileusis (photorefractive keratectomy) in monkeys. Arch Ophthalmol 1990; 108:665-75. [PMID: 2334323]
- Al-Odaib AN, Abu-Amero KK, Ozand PT, Al-Hellani AM. A new era for preventive genetic programs in the Arabian Peninsula. Saudi Med J 2003; 24:1168-75. [PMID: 14647548]
- Vasiliou V, Gonzalez FJ. Role of CYP1B1 in glaucoma. Annu Rev Pharmacol Toxicol 2008; 48:333-58. [PMID: 17914928]
- Achary MS, Reddy AB, Chakrabarti S, Panicker SG, Mandal AK, Ahmed N, Balasubramanian D, Hasnain SE, Nagarajaram HA. Disease-causing mutations in proteins: structural analysis of the CYP1B1 mutations causing primary congenital glaucoma in humans. Biophys J 2006; 91:4329-39. [PMID: 16963504]
- Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. Effect of two mutations of human CYP1B1, G61E and R469W, on stability and endogenous steroid substrate metabolism. Pharmacogenetics 2001; 11:793-801. [PMID: 11740343]
- Kakiuchi-Matsumoto T, Isashiki Y, Ohba N, Kimura K, Sonoda S, Unoki K. Cytochrome P450 1B1 gene mutations in Japanese patients with primary congenital glaucoma(1). Am J Ophthalmol 2001; 131:345-50. [PMID: 11239867]
- Chen Y, Jiang D, Yu L, Katz B, Zhang K, Wan B, Sun X. CYP1B1 and MYOC mutations in 116 Chinese patients with primary congenital glaucoma. Arch Ophthalmol 2008; 126:1443-7. [PMID: 18852424]
- Sitorus R, Ardjo SM, Lorenz B, Preising M. CYP1B1 gene analysis in primary congenital glaucoma in Indonesian and European patients. J Med Genet 2003; 40:e9. [PMID: 12525557]
- Reddy AB, Kaur K, Mandal AK, Panicker SG, Thomas R, Hasnain SE, Balasubramanian D, Chakrabarti S. Mutation spectrum of the CYP1B1 gene in Indian primary congenital

Molecular Vision 2011; 17:2911-2919 < http://www.molvis.org/molvis/v17/a315>

glaucoma patients. Mol Vis 2004; 10:696-702. [PMID: 15475877]

- Stoilov IR, Costa VP, Vasconcellos JP, Melo MB, Betinjane AJ, Carani JC, Oltrogge EV, Sarfarazi M. Molecular genetics of primary congenital glaucoma in Brazil. Invest Ophthalmol Vis Sci 2002; 43:1820-7. [PMID: 12036985]
- Bagiyeva S, Marfany G, Gonzalez-Angulo O, Gonzalez-Duarte R. Mutational screening of CYP1B1 in Turkish PCG families and functional analyses of newly detected mutations. Mol Vis 2007; 13:1458-68. [PMID: 17893647]
- Hilal L, Boutayeb S, Serrou A, Refass-Buret L, Shisseh H, Bencherifa F, El Mzibri M, Benazzouz B, Berraho A. Screening of CYP1B1 and MYOC in Moroccan families with primary congenital glaucoma: three novel mutations in CYP1B1. Mol Vis 2010; 16:1215-26. [PMID: 20664688]
- Dimasi DP, Hewitt AW, Straga T, Pater J, MacKinnon JR, Elder JE, Casey T, Mackey DA, Craig JE. Prevalence of CYP1B1 mutations in Australian patients with primary congenital glaucoma. Clin Genet 2007; 72:255-60. [PMID: 17718864]
- Colomb E, Kaplan J, Garchon HJ. Novel cytochrome P450 1B1 (CYP1B1) mutations in patients with primary congenital glaucoma in France. Hum Mutat 2003; 22:496. [PMID: 14635112]
- Campos-Mollo E, Lopez-Garrido MP, Blanco-Marchite C, Garcia-Feijoo J, Peralta J, Belmonte-Martinez J, Ayuso C, Escribano J. CYP1B1 mutations in Spanish patients with primary congenital glaucoma: phenotypic and functional variability. Mol Vis 2009; 15:417-31. [PMID: 19234632]
- 31. Chitsazian F, Tusi BK, Elahi E, Saroei HA, Sanati MH, Yazdani S, Pakravan M, Nilforooshan N, Eslami Y, Mehrjerdi MA, Zareei R, Jabbarvand M, Abdolahi A, Lasheyee AR, Etemadi A, Bayat B, Sadeghi M, Banoei MM, Ghafarzadeh B, Rohani MR, Rismanchian A, Thorstenson Y, Sarfarazi M. CYP1B1 mutation profile of Iranian primary congenital glaucoma patients and associated haplotypes. The Journal of molecular diagnostics. J Mol Diagn 2007; 9:382-93. [PMID: 17591938]
- Alfadhli S, Behbehani A, Elshafey A, Abdelmoaty S, Al-Awadi S. Molecular and clinical evaluation of primary congenital glaucoma in Kuwait. Am J Ophthalmol 2006; 141:512-6. [PMID: 16490498]
- Plásilová M, Stoilov I, Sarfarazi M, Kadasi L, Ferakova E, Ferak V. Identification of a single ancestral CYP1B1 mutation in Slovak Gypsies (Roms) affected with primary congenital glaucoma. J Med Genet 1999; 36:290-4. [PMID: 10227395]
- 34. Narooie-Nejad M, Paylakhi SH, Shojaee S, Fazlali Z, Rezaei Kanavi M, Nilforushan N, Yazdani S, Babrzadeh F, Suri F, Ronaghi M, Elahi E, Paisan-Ruiz C. Loss of function mutations in the gene encoding latent transforming growth factor beta binding protein 2, LTBP2, cause primary

congenital glaucoma. Hum Mol Genet 2009; 18:3969-77. [PMID: 19656777]

- 35. Azmanov DN, Dimitrova S, Florez L, Cherninkova S, Draganov D, Morar B, Saat R, Juan M, Arostegui JI, Ganguly S, Soodyall H, Chakrabarti S, Padh H, Lopez-Nevot MA, Chernodrinska V, Anguelov B, Majumder P, Angelova L, Kaneva R, Mackey DA, Tournev I, Kalaydjieva L. LTBP2 and CYP1B1 mutations and associated ocular phenotypes in the Roma/Gypsy founder population. Eur J Hum Genet 2011; 19:326-33. [PMID: 21081970]
- Hollander DA, Sarfarazi M, Stoilov I, Wood IS, Fredrick DR, Alvarado JA. Genotype and phenotype correlations in congenital glaucoma. Trans Am Ophthalmol Soc 2006; 104:183-95. [PMID: 17471339]
- Hollander DA, Sarfarazi M, Stoilov I, Wood IS, Fredrick DR, Alvarado JA. Genotype and phenotype correlations in congenital glaucoma: CYP1B1 mutations, goniodysgenesis, and clinical characteristics. Am J Ophthalmol 2006; 142:993-1004. [PMID: 17157584]
- Walton DS, Katsavounidou G. Newborn primary congenital glaucoma: 2005 update. J Pediatr Ophthalmol Strabismus 2005; 42:333-41. [PMID: 16382557]
- Della Paolera M, de Vasconcellos JP, Umbelino CC, Kasahara N, Rocha MN, Richeti F, Costa VP, Tavares A, de Melo MB. CYP1B1 gene analysis in primary congenital glaucoma Brazilian patients: novel mutations and association with poor prognosis. J Glaucoma 2010; 19:176-82. [PMID: 19528825]
- 40. Stoilov I, Akarsu AN, Alozie I, Child A, Barsoum-Homsy M, Turacli ME, Or M, Lewis RA, Ozdemir N, Brice G, Aktan SG, Chevrette L, Coca-Prados M, Sarfarazi M. Sequence analysis and homology modeling suggest that primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. Am J Hum Genet 1998; 62:573-84. [PMID: 9497261]
- López-Garrido MP, Sanchez-Sanchez F, Lopez-Martinez F, Aroca-Aguilar JD, Blanco-Marchite C, Coca-Prados M, Escribano J. Heterozygous CYP1B1 gene mutations in Spanish patients with primary open-angle glaucoma. Mol Vis 2006; 12:748-55. [PMID: 16862072]
- Mashima Y, Suzuki Y, Sergeev Y, Ohtake Y, Tanino T, Kimura I, Miyata H, Aihara M, Tanihara H, Inatani M, Azuma N, Iwata T, Araie M. Novel cytochrome P4501B1 (CYP1B1) gene mutations in Japanese patients with primary congenital glaucoma. Invest Ophthalmol Vis Sci 2001; 42:2211-6. [PMID: 11527932]
- Melki R, Colomb E, Lefort N, Brezin AP, Garchon HJ. CYP1B1 mutations in French patients with early-onset primary openangle glaucoma. J Med Genet 2004; 41:647-51. [PMID: 15342693]

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