ONLINE MUTATION REPORT

CYP1B1 gene analysis in primary congenital glaucoma in Indonesian and European patients

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J Med Genet 2003;40:e9(<http://www.jmedgenet.com/cgi/content/full/40/1/e9>)

PPrimary congenital glaucoma (PCG) is an autosomal recessive disease, caused by unknown developmental defect(s) of the trabecular meshwork and anterior chamber angle. It manifests itself clinically during the neonatal or infantile period. The disease is characterised by high intraocular pressure (IOP), buphthalmos with corneal enlargement, and breaks in Descemet's membrane.¹

Two chromosomal locations for PCG on 2p21 (GLC3A, MIM 231300) and 1p36 (GLC3B) have been reported previously.² Recently, a third locus (GLC3C) has been reported that maps to chromosome 14q24.3.³ The GLC3A locus colocalises to the *CYP1B1* gene (MIM 601771) on chromosome 2p21. *CYP1B1* encodes a dioxin inducible member of subfamily I of the cytochrome p450 protein superfamily. The human *CYP1B1* gene consists of three exons of which the first is non-coding. The putative open reading frame starts in the second exon and is 1629 bp in length.⁴⁻⁶ Thirty-five distinctive mutations in *CYP1B1* have been described in different ethnic groups and populations (table 1).⁷⁻¹⁰ In ethnically mixed populations, mutations were found in 20-30% of patients with PCG,¹¹ whereas in consanguineous populations the prevalence increases to 85%.^{5,7,9} Ethnic differences and geographical variations may be associated with different mutation patterns. Descriptions of causative mutations of PCG in the Indonesian population have not been published so far. We therefore investigated the coding region of the *CYP1B1* gene in patients from Europe and Indonesia, in order to find molecular information on PCG, and compared the findings between these two different populations.

PATIENTS AND METHODS

Twenty-one index cases with a diagnosis of congenital glaucoma, 12 Indonesian (four Sundanese (fig 1), four Javanese, two Batak, two Indonesian-Chinese) and nine Europeans (five German, one Italian, one Hungarian, one English-American, one Turkish) were examined by either BL, RS, or SMA, and diagnosed with PCG. Age of onset was 0-3 years. Optic nerve cupping and ruptures of Descemet's membrane were determined whenever possible. No positive family history was obtained for any proband, although some had consanguineous parents (table 2).

Blood samples were taken from affected subjects and their relatives after informed consent according to the Declaration of Helsinki. DNA was extracted from peripheral blood lymphocytes according to a previously reported method.¹² DNA samples were subjected to PCR to amplify the coding exons of the *CYP1B1* gene using oligonucleotide primers as reported by Bejjani *et al.*⁷ Subsequently, PCR products were analysed using single strand conformation polymorphism analysis (SSCP) as previously described.¹³ PCR products showing aberrant banding patterns in SSCP were sequenced directly by cycle sequencing using the fluorescent chain termination technique. Restriction endonuclease digests were performed to confirm the sequencing result, using the PCR products of the patients, their relatives, and controls.

Two mutations could be confirmed by restriction endonuclease digest. An *Nla*III restriction endonuclease digest was

Key points

- Our work aimed to analyse *CYP1B1* gene mutations in patients with primary congenital glaucoma (GLC3A, OMIM 231300) in Indonesian and European (white) patients.
- Twenty-one index cases with a clinical diagnosis of primary congenital glaucoma were collected (12 Indonesian, nine European). Thirty-five DNA samples from the 21 index cases and their relatives were screened using SSCP analysis followed by direct sequencing and/or RFLP screening.
- Five distinct disease causing mutations were identified in six of the 21 index cases: V364M, S215I, E281X, and two deletions (1407del12bp, 1410del13bp). The V364M mutation was identified in three of 12 index cases originating from Indonesia (25.0%). Two of them were homozygous. By testing six short sequence repeat markers, a recent common founder for these patients was ruled out. A novel missense mutation (S215I) in exon 2 was identified in an Indonesian patient. A novel 12 bp deletion at nucleotide 1407 in exon 3 was identified in a patient of Turkish descent. In the second allele, a E281X mutation was identified. The 13 bp deletion at nucleotide 1410 was found in a patient of Italian descent. Five additional sequence variations, R48G, A119S, L432V, A453S, and IVS1-12t/c, which have been reported previously as single nucleotide polymorphisms, were identified. These variations were not distributed equally in the Indonesian and European patients.
- Our results support previous studies reporting that the *CYP1B1* gene is a major gene for primary congenital glaucoma (GLC3A). A different pattern of *CYP1B1* mutations and benign sequence variants appears to exist in Indonesian patients compared with European (white) patients. The latter patients showed predominantly truncating mutations, whereas the missense mutation V364M was found to be the most common disease causing mutation among Indonesian patients, especially in the Indonesian-Sundanese ethnic group. This V364M mutation was associated with a severe late stage phenotype, which probably results from a relative delay in health care because of geographical or social circumstances. Further studies in an extended set of samples are still needed to confirm these results.

performed in a 5 µl reaction volume including 3 µl of PCR product and 0.5 µl of reaction buffer and 1 µl of enzyme to confirm the M364V mutation. The enzyme was obtained from New England Biolabs, Frankfurt. The assay was performed overnight at 37°C and resulted in fragments of 169, 37, 84, and 50 bp for the wild type and 94, 75, 37, 84, and 50 bp for the

Table 1 Previously reported sequence changes of the *CYP1B1* gene

Nucleotide change	Predicted effect	Sequence change	Exon	Pathogenicity	Reference
Deletion of partial IVS2 and exon 3			IVS2	PCG	5
c.347A-T	M1T	ATG → ACG	2	Peters' anomaly	19
c.401C-T	Q19X	CAG → TAG	2	PCG	20
c.517G-C	W57C	TGC → TGG	2	PCG	10
c.528G-A	G61E	GGA → GAA	2	PCG	7
c.576T-C	L77P	CTG → CCG	2	PCG	8
c.779del10bp		CAG CGG GCG GCA GCC → CAG ____ _CC	2	PCG	8
c.847insT		GAG GGC → GAT GGG C	2	PCG	10
c.880delG		GTG GCG → GT_ GCG	2	PCG	20
c.921A-T	D192V	GAC → GTC	2	PCG	23
c.938G-A	V198I	GTC → ATC	2	PCG	23
c.1152del9bp		AGC AAC TTC ATC → A_ ____ _TC	2	PCG	8
c.1186C-A	C280X	TGA → TGA	2	PCG	23
c.1186G-T	E281X	GAA → TAA	2	PCG	10
c.1209insC		GCC CCC CGC → GCC CCC CCG C	2	PCG	10
c.1327insAT		ATC TTC → ATA TCT TC	2	PCG	23
c.1334GC-TT	A330F	GCC → TTC	2	PCG	23
c.1410del13bp		CGA GTG CAG GCA GAA → C_ ____ _A	3	PCG	10
c.1436G-A	V364M	GTG → ATG	3	PCG	23
c.1439G-T	G365W	GGG → TGG	3	PCG	10
c.1449G-A	R368H	CGT → CAT	3	PCG	8
c.1466G-A	D374N	GAC → AAC	3	PCG	7
c.1482C-T	P379L	CCC → CTC	3	PCG	10
c.1505G-A	E387K	GAA → AAA	3	PCG	10
c.1514C-A	R390S	CGC → AGC	3	PCG	8
c.1515G-A	R390H	CGC → CAC	3	PCG	10
c.1546dup10bp		CCT CAT GCC ACC ACT → CCT CAT GCC ACC TCA TGC CAC CAC T	3	PCG	10
c.1656C-T	P437L	CCG → CTG	3	PCG	10
c.1671C-G	P442R	CCA → CGA	3	PCG	20
c.1677G-A	R444Q	CGA-CAA	3	PCG	23
c.1690delG		AAG → GAC	3	PCG	10
c.1723delAG		AGA GTG → AG_ GTG	3	PCG	20
c.1749dup27		AGG CGG TGC ATT GGC GAA GAA CTT TCT AAG → A_ ____ _AG	3	PCG	10
c.1751C-T	R469W	CGG → TGG	3	PCG	2
c.1842A-G	E499G	GAG → GGG	3	PCG	23
IVS1-13c-t		ctctc → ctlttc	IVS1	Polymorphism	10
c.488C-G	R48G	CGG → GGG	2	Polymorphism	23
c.701G-T	A119S	GCC → TCC	2	Polymorphism	10
c.1075G-C	V243V	GTG → GTC	2	Polymorphism	23
c.1640C-G	L432P	CTG → GTG	3	Polymorphism	10
c.1693C-T	D449D	GAC → GAT	3	Polymorphism	10
c.1704A-G	N454S	AAC → AGC	3	Polymorphism	10

mutated amplicon, respectively. The assay was mixed with 2 µl of sucrose buffer (40% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol in 1 × TBE), loaded onto a 6% polyacrylamide/1 × TBE gel for better resolution of fragments smaller than 100 bp in a Mini Protean II electrophoresis chamber (BioRad, Munich), and resolved at 120 V for about one hour. Subsequently, the gel was silver stained.¹⁴

The S215I mutation was confirmed by *FokI* restriction endonuclease digest in a 10 µl reaction volume containing 8 µl of PCR product and 1 µl of enzyme and reaction buffer. The enzyme was obtained from MBI Fermentas, St Leon Roth. The assay was performed overnight at 37°C and resulted in fragments of 298 and 32 bp for the wild type and 127, 171, and 32 bp for the mutated amplicon. The assay was mixed with 2 µl of sucrose buffer, loaded onto a 2% agarose/1 × TBE gel in Horizon 58 electrophoresis chamber (Biomera, Göttingen) at 80 V for 75 minutes, and was subsequently stained with ethidium bromide.

Short sequence repeat markers D2S1788, D2S405, D2S1356, D2S1394, D2S1360, and D2S1352 from the Marshfield set of primers version 10 (<http://research.marshfieldclinic.org/genetics/sets/scrset10.htm>) in the region between 2p24.1- 2p12 were applied to test for a common founder in four Indonesian patients. The markers were amplified by PCR, mixed with loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 25 mmol/l EDTA, pH 8, in formamide) and resolved on a denaturing (6 mol/l urea) 6% polyacrylamide gel in a Multigel Long electrophoresis chamber (Biomera, Göttingen). The gels were detected by silver staining according to Budowle *et al.*¹⁴

RESULTS

Twenty-one DNA samples from index cases were screened using SSCP analysis followed by direct sequencing. The results were confirmed in 14 relatives by SSCP and/or RFLP screening.

Mutational screening of the *CYP1B1* gene detected five distinct disease causing mutations in six of 21 index cases. The mutations and respective genotype-phenotype correlations are summarised in table 2. We identified a G→A transition at the first nucleotide of codon 364 in exon 3, which predicts the substitution of Met for Val (fig 2A). The mutation creates a *NlaIII* restriction site which is useful for population screening (fig 2B). The V364M mutation (c.1346G→A, according to GenBank entry HSU56438 and the sequence given by Sutter *et al.*¹⁵ and Stoilov *et al.*¹⁶) is a missense mutation which has been reported previously in Japanese patients in the compound heterozygous state.¹⁶ This mutation may affect the highly conserved positions at the end of helix J of the protein, which determine the proper folding and haem binding ability of P450 molecules. In our study, the V364M mutation was identified in three of 12 Indonesian index cases (25%) and is therefore the most common mutation in them. The mutation was present in the homozygous state in two index cases (case 1 (fig 1) and case 3) and the brother of case 1 (case 2 (fig 1)), whose parents were known to be consanguineous. Unfortunately, no DNA from the parents was available for further screening. Case 4, whose parents were not consanguineous, is

Figure 1 (A) Case 1 and (B) case 2 (aged 19 and 17 years, respectively). (C) Case 4 (aged 16 years) with a severe and advanced stage of the disease. (D) Pedigree of cases 1 and 2 showing consanguinity.

singly heterozygous for the V364M mutation. No further mutation could be identified in the *CYP11B1* gene by direct sequencing of the coding region in this patient. Interestingly, all index cases who carried the V364M mutation originate in the Sundanese population. This ethnic group, one among many in Indonesia, live in west Java, and show a high degree of consanguinity. Therefore, we looked for the presence of a common founder in cases 1-4. For all markers investigated, a recent common founder could be ruled out since at least one allele varied among the index cases. Even the related cases 1 and 2 (sibs) shared only one allele for markers D2S1352, D2S1394, and D2S1788 (data not shown).

A second novel disease causing mutation (S215I (c.992G→T)) was identified in another Sundanese patient in the heterozygous state. His phenotype was similar to the phenotype of the other Sundanese patients (table 1). The clinical findings of the Indonesian-Sundanese patients showed severe and advanced stages of disease, which is most likely because

Table 2 CYP11B1 gene: genotype-phenotype correlations on mutations reported in this study											
Case	Gender	Sequence change allele 1/allele 2	Predicted effect	Exon	Ethnic background	Consanguinity	Age at diagnosis	Age at onset	Therapy, started at age	IOP at diagnosis (mmHg) RE/LE	Visual acuity RE/LE
1	F	c.1436G-A Homozygous	V364M Homozygous	3	Indonesian-Sundanese	Yes	19 y	<3 y	M, 19 y	High/30–40	HM/LP*
2	M	c.1436G-A Homozygous	V364M Homozygous	3	Indonesian-Sundanese	Yes	15 y	<3 y	M, 15 y	High/30–40	LP/LP*
3	M	c.1436G-A Homozygous	V364M Homozygous	3	Indonesian-Sundanese	Yes	15 y	<3 y	M, 15 y	High/30–40	LP/LP*
4	M	c.1436G-A/ND	V364M Homozygous	3	Indonesian-Sundanese	No	16 y	<3 y	M, 16 y	High/30–40	LP/LP*
5	F	c.1189G-T/1407del12bp	E281X/del355RVGA	2/3	Turkish	No	3 mth	Congenital	TET, 3 mth CPC, 11 mth	25/25	1,0/1,0
6	F	c.1410del13bp/ND	Frameshift/ND	3	Italian	No	2 mth	4 d	CPC, 3 y	17/20	1,6/2,4 cyc/degree†
7	M	c.992G-T/ND	S215I/ND	2	Indonesian-Sundanese	No	24 y	<3 y	T, 9 mth M, 24 y	High/30–40	LP/LP*

*HM = hand movement; LP = light perception. †By PL + TAC procedure; M, pilocarpine + azetazolamide, CPC: cyclophotocoagulation, TET: trabeculectomy, T: trabeculectomy, ND: the compound mutation could not be determined. Sequence data refer to Genbank entry HSU56438 and the sequence given by Sutter *et al*⁵ and Stoilov *et al*.⁵

Figure 2 Mutation detection and confirmation of the V364M mutation. *N/AIII* restriction endonuclease digest. Lane 1: case 1, lane 2: case 2, lane 3: case 4, lane 4: case 3, lanes 5 and 6: controls, lane M1: marker 25 bp ladder, lane M2: marker 100 bp ladder).

Figure 3 SSCP and direct sequencing of the 5' amplicon of exon 3 of *CYP11B1* in case 5. (A) SSCP analysis showing aberrant migration of the 5' amplicon of exon 3 in case 5 in lane 2 (lanes 1 and 3: controls, WT: wild type bands, Mu: mutant bands). (B) A 12 bp deletion could be shown by direct sequencing of the 5' amplicon of exon 3 in case 5 at nucleotide 1407. (C) Direct sequencing of the wild type amplicon indicating the amino acids Arg 355, Val 356, Gln 357, and Ala 358 abolished by the deletion.

of a delay in health care and treatment related to social circumstances (fig 1A-C).

In the European patients, two deletions and one nonsense mutation were detected in two unrelated index cases. The first mutation is a 12 bp deletion at nucleotide c.1407 (fig 3). This is a novel in frame deletion identified in a 3 year old girl of Turkish descent. The patient was referred to our department

for genetic analysis from a collaborating human genetics unit. The first diagnosis was congenital glaucoma but was changed later to Peters' anomaly as the patient developed. The patient underwent trabeculectomy and cyclophotocoagulation. The mutation abolishes four amino acids downstream from amino acid Thr 354 inside the highly conserved region of Helix J of the *CYP11B1* gene (case 5, fig 3B, C). In the second allele, a G→T

Table 3 Polymorphisms in the *CYP1B1* gene identified in this study

Location	Codon	Nucleotide change	Predicted effect	Allele frequency Indonesian/European (%)
Intron 1		cctctctctg→cctcttctctg	-13c/t*	15.8/50.0
Exon 2	48	CGG→GGG	R48G	21.0/33.3
Exon 2	119	GCC→TCC	A119S	5.2/6.6
Exon 3	453	AAC→AGC	N453S	0.0/33.3
Exon 3	432	GTG→CTG	L432V	0.0/13.3

*Upstream from ATG. Sequence data refer to GenBank entry HSU56438 and the sequence given by Sutter *et al*⁵ and Stoilov *et al*.⁵

transition at nucleotide position c.1087 in exon 2 results in the substitution of Glu by a premature stop codon, which truncates the *CYP1B1* open reading frame. This mutation has been reported previously.¹⁰ No genomic DNA from the unaffected parents was provided to screen the mutations. Neither mutation was present in 40 chromosomes from controls.

The third mutation in the European samples was a 13 bp deletion at nucleotide c.1410 (data not shown) and was found in a 3 year old girl of Italian descent (case 6). Her disease was detected at the age of 2 months. Gonioscopy showed a dysplastic anterior chamber angle. At the age of 6 months a trabeculotomy was performed in the left eye and at 9 months in the right eye. Because of insufficient control of the intraocular pressure, a second trabeculotomy was performed in the left eye at 9 months. At the last follow up at 3.5 years, the intraocular pressure was well controlled in both eyes. Visual acuity was 1.0 in the right eye and 0.63 in the left eye (Cardiff cards at 1 m). The deletion introduces a frameshift in the remaining downstream codon and probably results in a functional null allele. No additional disease causing mutation but two benign variants (L432V and IVS1-12t/c) could be identified in the second allele.

In addition to L435V and IVS1-12t/c, three other distinct benign variants, R48G, A119S, and N453S, were identified in the European samples, which have been previously reported elsewhere (table 3).

DISCUSSION

We screened the coding region of the *CYP1B1* gene in 21 patients suffering from congenital glaucoma. Twelve of them were of Indonesian and nine of European descent. This is the first report on the role of the *CYP1B1* gene in PCG in Indonesian patients. Ethnic differences in the occurrence of *CYP1B1* mutations appear to exist.^{7,9,17} In our study we identified *CYP1B1* mutations in 33.3% and 22.2% of subjects screened from Indonesia and Europe, respectively. In the Indonesian patients the missense mutations V364M (3/12 patients) and S215I (1/12 patients) were identified. Mutations were found exclusively in Indonesian-Sundanese patients. These data suggest that V364M is the most common mutation in this ethnic group. We ruled out that V364M is a recent founder mutation in this ethnic group by testing six short sequence repeat markers at 2p24-12. Alleles varied among all tested patients for the informative markers. The V364M mutation has been reported in a Japanese study in the compound heterozygous state in one of 11 families.¹⁷ This mutation, however, has never been reported in white populations and we also did not find this mutation in our European patients. The possibility exists that the mutation originated from an ancient founder and then spread throughout Asia, since even cases 1 and 2 (sibs) shared only one allele for some markers, despite being the product of a consanguineous marriage. The V364M mutation might also have occurred independently owing to a mutational hot spot caused by the surrounding sequence (GTC GTG GGG).

A serine to isoleucine transition affecting the second nucleotide of codon 215 in exon 2 was identified in an Indonesian

patient and is a novel mutation. It was singly heterozygous (only one mutant allele was detected). No genomic DNA from the unaffected parents was provided for segregation analysis; however, this missense mutation was not present in 50 chromosomes from randomly selected normal subjects.

In a Saudi Arabian PCG study, other distinct missense mutations G61E, R468W, and D374N were reported as the most common mutations, accounting for 72%, 12%, and 7% of the tested alleles, respectively.⁷ In Brazilian patients, a single truncating mutation g.4340delG (20.2%) was the most frequent mutation³ and was different from Turkish PCG families who showed an equal frequency of truncating and missense mutations in the tested samples.^{2,10}

In contrast to the Indonesian PCG families, the truncating mutations in the European PCG families account for the majority of the *CYP1B1* mutations identified in this population. All of the mutations identified probably truncate the open reading frame. They include one nonsense mutation (E281X), one frameshift mutation (c.1410del13bp), and one in frame mutation (c.1407del12bp). These mutations are expected to eliminate amino acids 79-377 from the carboxy terminus of the *CYP1B1* polypeptide. Thus, if a stable protein is synthesised, every mutant molecule would lack at least the haem binding region, which is essential for the function of the cytochrome P450 molecule. Therefore, it is expected that these mutations will result in functional null alleles.

In case 5, Peters' anomaly was diagnosed, which has been correlated previously with mutations in *CYP1B1*.^{18,19} It appears that this entity does not result from a single specific mutation or type of mutation since in our case the combination of a stop mutation and a deletion shows a similar phenotype to a stop mutation (W57X) and a missense change (M1T), which by themselves do not predict the phenotype of Peters' anomaly.²⁰

In cases 4, 6, and 7 (table 2, fig 4) the second disease causing mutation could not be identified, although the entire coding sequence of *CYP1B1* was analysed. However, we could not rule out a mutation in the promoter or other non-coding regions of *CYP1B1*.

In our study, a different pattern of single nucleotide polymorphisms (SNPs) appears to exist in the Indonesian population compared to European (white) patients (table 3). Benign sequence variants such as A453S and L432V have been identified in European patients only. Additionally, the allele frequency of the non-coding variant IVS1-12t/c was much higher in European than in Indonesian patients (60% compared to 15.8%).

However, as in other reported studies,^{10,21} we also found that R48G and A119S were always coinherited, indicating that these two SNPs are linked.²¹ In addition we could not confirm the hypothesis that the combination of four well known polymorphisms, R48G, A119S, L432V, and N453S, establish a pathogenic allele when coinherited, as previously reported.²³

In conclusion, our data indicate that mutations identified in this study support the previous findings that mutations in the *CYP1B1* gene are responsible for the PCG phenotype that is associated with the GLC3A locus. A different pattern of *CYP1B1* disease causing mutations and benign variants appears to exist in Indonesian patients when compared to

Figure 4 Direct sequencing of the 5' amplicon of exon 2 in case 7. (A) Direct sequencing representing a novel mutation S215I. A G→T transition at the second nucleotide in codon 215 in exon 2 leads to the substitution of a Ile residue for a Ser residue. (B) *FokI* restriction endonuclease digest (lanes 1, 3: controls, lane 2: case 7).

patients from other ethnic backgrounds. In contrast to European patients who showed truncating mutations, Indonesian patients from the Sundanese ethnic group exhibited the missense V364M mutation as the most common mutation in our screen. Further studies in a larger number of families are still required to confirm these results.

ACKNOWLEDGEMENTS

RS spent periods of research in Germany, supported by a Grant of the Georg Forster Research Fellowship Programme (The Alexander von Humboldt Stiftung Special Programme). We thank Professor Horst, Ms Sukaesih and Ms Hermin at The School Blindness, Bandung, Jean Paul op de Laak, Dr E da Silva, Dr Susanti Sirait, and especially the patients and their families who participated in this study.

Data access: GenBank: HSU56438

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