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

R Sitorus, S M Ardjo, B Lorenz, M Preising

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. DNA samples were subjected to PCR to amplify the coding exons of the CYP1B1 gene using oligonucleotide primers as reported by Bejani et al. Subsequently, PCR products were analysed using single strand conformation polymorphism analysis (SSCP) as previously described. PCR products showing aberrant banding patterns in SSCP were sequenced directly by cycle sequencing using the fluorescent chain termination technique. Restriction endonuclease digestes 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 NalIII restriction endonuclease digest was 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

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%). 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, an 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 CYPB1 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.
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 diseasecausing 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 al15) 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 the enzyme.

The S215I mutation was confirmed by PCR using a 2% agarose/TBE gel for better resolution of fragments in a 6% polyacrylamide gel in a Multigel Long electrophoresis chamber (Biometra, 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 (Biometra, Göttingen). The gels were detected by silver staining according to Budowle et al.14
singly heterozygous for the V364M mutation. No further mutation could be identified in the \textit{CYP1B1} 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
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 CYP1B1 gene (case 5, fig 3B, C).

Figure 2  Mutation detection and confirmation of the V364M mutation. Nolll 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′ amplimer of exon 3 of CYP1B1 in case 5. (A) SSCP analysis showing aberrant migration of the 5′ amplimer 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′ amplimer of exon 3 in case 5 at nucleotide 1407. (C) Direct sequencing of the wild type amplimer indicating the amino acids Arg 355, Val 356, Gln 357, and Ala 358 abolished by the deletion.
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. 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. 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. 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 GTC 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 30 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. In Brazilian patients, a single truncating mutation g.4340delG (20.2%) was the most frequent mutation and was different from Turkish PCG families who showed an equal frequency of truncating and missense mutations in the tested samples. 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 (E281X1), 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 transmembrane 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. 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. In cases 4, 6, and 7 (table 2, fig 4) the second disease caus- ing mutation could not be identified, although the entire cod- ing 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, we also found that R48G and A119S were always co-inherited, indicating that these two SNPs are linked. 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 co-inherited, as previously reported.

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...
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Data access: GenBank: HSU56438

References

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