Novel Cytochrome P4501B1 (CYP1B1) Gene Mutations in Japanese Patients with Primary Congenital Glaucoma

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PURPOSE. To investigate CYP1B1 gene mutations in Japanese patients with primary congenital glaucoma (PCG).

METHODS. Sixty-five unrelated Japanese patients with PCG were screened by PCR-single-strand conformational polymorphism (SSCP) analysis followed by direct sequencing. No patients were offspring of consanguineous marriages, a common occurrence among patients in previous reports. PCG haplotypes were constructed with intragenic polymorphisms in affected individuals. Three-dimensional atomic structures of human CYP1B1 and four mutant CYP1B1 sequences representing missense mutations were assembled using homology modeling and were regularized by an energy-minimization procedure.

RESULTS. Eleven novel mutations, including seven definite and four probable mutations, were detected in 15 (20%) of the 65 unrelated patients. Of the seven definite mutations, three were predicted to truncate the CYP1B1 open reading frame. The other four were missense mutations (Asp192Val, Ala330Phe, Val364Met, and Arg444Gln), all located in conserved core structures determining proper folding and heme-binding ability of cytochrome P450 molecules. Molecular modeling demonstrated that two of four mutations in positions 330 and 364 were structurally neutral, but Arg444Gln caused significant structural change. Of the four probable mutations, three were missense (Val198Ile, Val230Leu, and Gly499Gly), the other was a base substitution in the noncoding region of exon 1.

CONCLUSIONS. The 11 varied CYP1B1 mutations found in 13 unrelated Japanese patients with sporadic occurrence of PCG represent an allelic heterogeneity and may be unique to a specific population. (Invest Ophthalmol Vis Sci. 2001;42: 2211-2216)

The glaucomas are a heterogeneous group of insidious diseases associated with elevated intraocular pressure (IOP) and optic nerve atrophy. Primary congenital (infantile) glaucoma (PCG; gene symbol, GLC3) is usually diagnosed during the first year of life and is more severe and difficult to manage than other types. Clinical features of PCG typically include tearing, photophobia, and clouding of the cornea. PCG occurs as a result of developmental anomalies of the chamber angle that prevent drainage of aqueous humor, thereby elevating IOP. Because the coating of the infantile eye is elastic, it stretches in response to elevated pressure, resulting in an enlarged globe (buphthalmos).

The incidence of PCG varies geographically, reported to be 1 in 5,000 to 1 in 22,000 newborns in Western countries, 1 in 2,500 in the Middle East, and 1 in 1,250 in the Rom (gypsy) population of Slovakia, in whom PCG is the major cause of blindness. The incidence of this disease is unclear in Japan. Most cases of PCG are sporadic in occurrence. In approximately 10% of cases in which a hereditary pattern is evident, inheritance is usually believed to be autosomal recessive. However, boys are affected more often than girls, and cases of incomplete penetrance have been documented. In some PCG-affected families, the inheritance pattern may be multifactorial or polygenic.

Recently, a putative PCG locus, GLC3A, was linked to markers on the short arm of chromosome 2p21.11 An additional PCG locus, GLC3B, has been localized to chromosome 1p36.12 Thus, PCG is a genetically heterogeneous disease with at least two loci. Furthermore, Stoilov et al.15 identified three different mutations in the cytochrome P4501B1 gene, CYP1B1, in five Turkish families. Later, three mutations in the same gene were identified in 24 of 25 families with PCG in Saudi Arabia,14 and 16 mutations were found in 22 families with PCG in Turkey, the United States, Canada, and the United Kingdom. These data strongly suggest that mutations affecting CYP1B1 are responsible for the PCG phenotype associated with the GLC3A locus. CYP1B1 gene mutations were associated with over 85% of families with PCG in Saudi Arabia, Turkey, and Slovakia. In the present study, we screened the CYP1B1 gene in 65 unrelated Japanese probands with PCG and identified 11 novel mutations in 13 probands (20%).

METHODS

Subjects

Blood samples were collected from 65 patients with a diagnosis of PCG at the following hospitals: 28 at Keio University Hospital, 15 at Tokyo University Hospital, 15 at the National Children's Hospital, 4 at Kyoto University Hospital, and 3 at Tenri Yorozu Hospital. These 65 Japanese probands were unrelated, and none was the offspring of consanguineous marriages. All patients included in the present study had an aggressive form of glaucoma, with time of onset ranging from less than 1 year to 3 years. Disease had manifested as symptoms of elevated IOP associated with corneal edema, rupture of Descemet's membrane, or buphthalmos. Patients with elevated IOP associated with other ocular or systemic anomalies were excluded. Blood samples were also col-

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selected from parents of patients with CYP1B1 mutations. Informed consent was obtained from the parents of each subject for their child’s as well as their own participation in the study. This investigation was performed according to the guidelines of the Declaration of Helsinki.

**Mutation Screening**

Genomic DNAs were prepared from leukocytes by the methods of proteinase K-phenol-chloroform extraction. First, we screened the CYP1B1 gene (GenBank accession number U56438) by PCR-single strand conformaton polymorphism (SSCP) analysis in a standard protocol, using nine specific sets of primers in three exonic regions. The putative open reading frame begins in the second exon. Samples showing abnormal mobility in this analysis were further analyzed by direct sequencing of the PCR products with an automated DNA sequencing kit (Thermo Sequenase II Dye Terminator Cycle Sequencing kit; Amersham Pharmacia Biotech, Uppsala, Sweden). Sixty unaffected Japanese subjects (20 males and 40 females) were examined as well as their own participation in the study. This investigation was performed according to the guidelines of the Declaration of Helsinki.

**Analysis of the Primary Structure of CYP1B1 by Comparative Sequence Alignment**

Amino acid sequences of CYP1B1, CYP1A1, and CYP1A2 obtained from SwissProt (Swiss Institute of Bioinformatics, Geneva, Switzerland) were compared between human, mouse, and rat using a computer sequence alignment program (Omiga; ver. 2.0; Oxford Molecular Ltd., Oxford, UK). Analysis was performed according to the manufacturer’s default setting.

**Molecular Modeling of the Conserved Terminal Half of the CYP1B1 Protein**

Structural coordinates of cytochromes P450 (2hpdp and 2c17) were obtained from the Brookhaven Protein Data Bank (Brookhaven, NY).17 The structures of both cytochromes were used as templates to construct two separate models of human CYP1B1. Multiple sequence alignment was performed by the method of Needleman and Wunsch,18 as incorporated in a software program (Look, ver. 3.5.2; Molecular Applications Group, Palo Alto, CA). The human CYP1B1 structure was obtained by using the automatic segment-matching method available in the computer program.19 Superposition of two predicted models of human CYP1B1 demonstrated high similarity for the conserved portion of both structures. However, the model based on the structure of 2c17 was more complete and therefore was used for analysis of mutations. In each mutant structure, the conformation of the replaced and neighboring residues was refined by self-consistent ensemble optimization,20 which uses a statistical mean force field approximation to obtain the structure with minimum global energy. Four models of mutant CYP1B1 corresponding to each of four missense mutations (Asp192Val, Ala330Phe, Val364Met, and Arg444Gln) were prepared by this method. Finally, the predicted structures were tested by computer (Procheck; Roman Laskowsky, University College, London, United Kingdom; information on availability is found at http://www.biochem.ucl.ac.uk).21

**RESULTS**

**Mutations in the CYP1B1 Gene**

DNA from affected individuals was analyzed by PCR-SSCP assay of the exonic regions of CYP1B1, followed by direct sequencing of amplification products. Seven novel mutations (bold letters in Fig. 1) were detected in 9 of the 65 unrelated Japanese individuals (the first nine families in Table 1). Three of the seven mutations were predicted to truncate the CYP1B1 open reading frame. One represented a nonsense mutation (Cys280stop), whereas two resulted in frameshift mutations (3964delC at codon 53, and 4776insAT at codon 324). We detected four missense mutations: Asp192Val, Ala330Phe, Val364Met, and Arg444Gln. Sequencing analysis of 10 clones obtained from the PCR products of the exon 2 region including codon 330, revealed that four clones had substitutions of the mutant allele, the PCR products of this region were subcloned into a vector (pT7 Blue T-Vector; Takara, Tokyo, Japan). This was followed by DNA sequencing, using the kit and DNA sequencer. Primers M13 and M13 RV (Takara) were used for sequencing.

Sequence variations detected in control subjects and affected individuals were recorded for analysis. PCR haplotypes were constructed from SwissProt (Swiss Institute of Bioinformatics, Geneva, Switzerland) and also in their parents.

**Figure 1.** GenOMIC STRUCTURE OF CYP1B1, OBSERVED AMINO ACID CHANGES, AND MULTIPLE-SEQUENCE ALIGNMENT OF MUTANT AMINO ACIDS. Seven missense mutations are described below the schematic gene structure. Affected positions are conserved among nine members of the cytochrome P450 gene superfamily. **Boldface** and **standard letters** indicate seven definite or four probable mutations, respectively.
Individuals in 11 Japanese Families with Primary Congenital Glaucoma

<table>
<thead>
<tr>
<th>Number of Families</th>
<th>Mutation DNA Change (Predicted Effect)</th>
<th>Mutation DNA Change (Predicted Effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4776insAT (frameshift)</td>
<td>G7927A (Val364Met)</td>
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<tr>
<td>1</td>
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</tr>
<tr>
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<td>A4380T (Asp192Val)</td>
<td>G7927A (Val364Met)</td>
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</tr>
<tr>
<td>1</td>
<td>G7927A (Val364Met)</td>
<td>G8168A (Arg444Gln)</td>
</tr>
<tr>
<td>2</td>
<td>C4645A (Cys280stop)</td>
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</tr>
<tr>
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<td>3964delC (frameshift)</td>
<td>G7927A (Val364Met)</td>
</tr>
<tr>
<td>1</td>
<td>G4397A (Glu99Gly)‡</td>
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</tr>
<tr>
<td>1</td>
<td>A8335G (Val320Leu)‡</td>
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</tr>
</tbody>
</table>

† Homozygous.  
‡ Probable mutation found in one of two alleles.

Only one patient showed a homozygous mutation of Asp192Val (Table 1). Three patients showed compound heterozygous mutations resulting in a frameshift due to a 2bp insertion at codon 324 and an amino acid substitution as a result of a missense mutation (Val364Met). The remaining five patients had compound heterozygous mutations, including Asp192Val and Val364Met, Ala350Phe and Val364Met, Val364Met and Arg444Gln, and Cys280stop and Arg444Gln, or a frameshift due to a 1-bp deletion and Arg444Gln. A Val364Met mutation was detected in six of the nine patients (6 of 18 chromosomes) with CYP1B1 mutations. The families with the Val364Met mutation were all descended from inhabitants of an eastern region of Japan.

In the present study, two patients with PCG had a heterozygous mutation of Val364Met (Table 1). Three patients showed compound heterozygous mutations resulting in a frameshift due to a 2bp insertion at codon 324 and an amino acid substitution as a result of a missense mutation (Val364Met). The remaining five patients had compound heterozygous mutations, including Asp192Val and Val364Met, Ala350Phe and Val364Met, Val364Met and Arg444Gln, and Cys280stop and Arg444Gln, or a frameshift due to a 1-bp deletion and Arg444Gln. A Val364Met mutation was detected in six of the nine patients (6 of 18 chromosomes) with CYP1B1 mutations. The families with the Val364Met mutation were all descended from inhabitants of an eastern region of Japan.

Polymorphisms in CYP1B1 Gene and Haplotype Analysis

PCR-SSCP or sequence analysis of the three exons in the CYP1B1 gene in the 65 affected individuals resulted in identification of five single-nucleotide polymorphisms. Four of these, Arg48Gly (C3947), Ala119Ser (G4160T), Val432Leu (G8131C), and Asp449Asp (T8184C), have been reported previously in patients with PCG.10,15 Arg48Gly and Ala119Ser are always found together in normal Japanese subjects. This linked amino acid substitution has been reported not to alter CYP1B1 function.22 One polymorphism, Val243Val(G4534C), was novel and was not detected in the 60 unaffected Japanese individuals.

Analysis of Missense Mutations in the CYP1B1 Structure

Four missense mutations (Asp192Val, Ala330Phe, Val364Met, and Arg444Gln) were analyzed based on the atomic structure of CYP1B1 predicted by homology modeling as described in the Methods section. Mutations in positions 330 and 364 did not produce significant structural changes. Only a small recognizable conformational change was observed in Ala330Phe, because of the replacement of the small Ala side chain by the large hydrophobic Phe. Another missense mutation (Asp192Val) was predicted to be located ahead of the conserved residue Pro193. The Asp192 position was occupied by a hydrophobic residue, specifically Asp (D), Glu (E), or Asn (N), in the nine CYP1 gene families analyzed. The Asp192Val mutation would result in substitution of the hydrophobic Asp residue for the hydrophobic Val amino acid. The Val364 position was occupied by a hydrophobic residue with Val (V) or Ile (I) in the nine CYP1 gene families analyzed. This position was located in the conserved region of the K helix.

Evaluation of Mutated Positions by Multiple Sequence Alignment

Comparison of amino acid sequence alignment in nine different cytochrome P450 proteins revealed that all missense mutations had occurred at highly conserved positions (Fig. 1). Ala330Phe and Arg444Gln affected positions that were conserved among the nine different CYP1 gene families analyzed and were located in highly conserved regions of the I helix and of the meander, respectively. The meander, which was just C-terminal of the K’ helix, was so named because at first glance this region appears to make a random walk from the K’ helix to the heme-binding region; actually, this was not the case. Asp192Val affected a position that was conserved in six of the nine different CYP1 gene families analyzed and immediately preceded the invariant residue Pro193. The Asp192 position was occupied by a hydrophobic residue, specifically Asp (D), Glu (E), or Asn (N), in the nine CYP1 gene families analyzed. The Asp192Val mutation would result in substitution of the hydrophobic Asp residue for the hydrophobic Val amino acid.

Summary of Mutations in the CYP1B1 Gene

<table>
<thead>
<tr>
<th>Family</th>
<th>DNA Change</th>
<th>Mutation</th>
<th>Predicted Effect</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>G4793T</td>
<td>C4794T</td>
<td>.unknown</td>
</tr>
<tr>
<td>2</td>
<td>1 A4380T</td>
<td>(Asp192Val)</td>
<td>† Unidentified</td>
</tr>
<tr>
<td>3</td>
<td>G4776insAT</td>
<td>(frameshift)</td>
<td>G7927A (Val364Met)</td>
</tr>
<tr>
<td>4</td>
<td>1 A8333G</td>
<td>(Glu499Gly)</td>
<td>‡ Unidentified</td>
</tr>
<tr>
<td>5</td>
<td>1 G4397A</td>
<td>(Val198Ile)</td>
<td>‡ Unidentified</td>
</tr>
<tr>
<td>6</td>
<td>1 A8335G</td>
<td>(Val320Leu)</td>
<td>‡ Unidentified</td>
</tr>
<tr>
<td>7</td>
<td>1 G7927A</td>
<td>(Val364Met)</td>
<td>‡ Unidentified</td>
</tr>
<tr>
<td>8</td>
<td>1 G4763T</td>
<td>(Val320Leu)</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 2. Structural model of the human CYP1B1 gene constructed by homology modeling. Images of superimposed native and mutant structural fragments containing the missense mutations Asp192Val, Ala330Phe, Val364Met, and Arg444Gln are presented in (A), (B), (C), and (D), respectively. Native and mutant CYP1B1 structures are shown in green and red, respectively. Interatomic distances corresponding to hydrogen bonds are shown by thin green lines. In the vicinity of Arg444, two hydrogen bonds are formed between Arg444 side-chain nitrogens and carbonyl oxygens of the main chain.
showed that replacement of the negatively charged Asp192 residue by a hydrophobic Val affects conformations of 11 neighbor residues. As a result, the Leu343 residue changed conformation and lost the hydrogen bond between the side chain of Arg194 and the main chain of Asn228 (Fig. 2A).

The most significant structural changes were observed in the Arg444Gln mutation that was located in the long fragment of the polypeptide chain (residues 400–460) involving β-residues related to β-sheets 1 and 2 (β-strands 11, 12, and 13). The fragment of the polypeptide chain in the mutant protein that contains the Arg444Gln mutation is shown in red in Figure 2B. The residue Arg444 was predicted to be located in the structural fragment extending from Pro435 to Lys448 (i.e., related to meander). Arg444 stabilizes the meander structure by the contact of nonpolar atoms of the Arg444 side chain and also by forming two hydrogen bonds between NH1 and NH2 atoms of Arg444 and the carbonyl oxygens of residues Trp434 and Asn439, respectively. Replacement of the Arg side chain by the residue Arg444 stabilizes the flap between the heme-binding domain and meander and may help to form the redox-partner binding site.

**DISCUSSION**

PCG shows a particularly high incidence in the Rom population of Slovakia and in Saudi Arabians. In these two populations, most patients with PCG showed the homozygous mutations Glu387Lys and Gly61Glu. The homozgyosity resulted from a prevalence of consanguinity in these groups. The Glu387Lys was also detected in three families in Canada, one of which originated from a consanguineous Amish community.

CYP1B1 gene mutations were identified in more than 85% of PCG-affected families with a minimum of two affected subjects in Saudi Arabia, Turkey, and Slovakia. In contrast, only 20% of our 65 unrelated Japanese patients with sporadic PCG harbored a CYP1B1 gene mutation. Similarly, Kakuuchi et al. reported a truncation mutation (1620insG) in the CYP1B1 gene in only one (17%) of six Japanese families with PCG. In the present study, 11 novel mutations were detected in 13 unrelated families. An insertion of two extra base pairs (AT) after the second nucleotide of codon 324 (1318insAT) created a stop codon 104 amino acids downstream from the original amino acid Ile324. This insertion in codon 324 did not alter the amino acid at codon 324, Ile324Ile (ATC→ATA). Deletion of one of the three base pairs of C at the third nucleotide (3964delC) of codon 53, at the first nucleotide (3965delC) of codon 54, or at the second nucleotide (3966delC) of codon 54 created a stop codon 59 amino acids downstream from the original amino acid Gly53. This deletion in codon 53 or 54 did not alter the amino acid in codon 53, Gly324Gly (GGC→GGG).

A hydrophilic Asp residue occupied position 192 in the nine CYP1 gene families analyzed. Replacement of the hydrophilic Asp192 by nonpolar Val (Asp192Val) increases the hydrophobic potential of this residue and may affect proper protein folding of native structure.

Ala330Phe is located in the conserved helix I and represents the first missense mutation to be detected in this domain. This position was conserved in the nine different CYP1 gene families analyzed. The mutation is unique, because the two nucleotides at codon 330 were changed in only one allele. The Val330 residue is located at the end of helix J, a highly conserved core structural element. Here, Val is present in the CYP1B1 gene of human, mouse, and rat. However, the predicted three-dimensional structure models of mutant CYP1B1 corresponding to these two missense mutations did not demonstrate significant structural changes, probably because these mutations did not alter hydrophobic properties at their sites.

The Arg residue at position 444 was conserved among the nine different CYP1 gene families analyzed. Residue Arg444 is involved in nonpolar interactions, forms two hydrogen bonds in the predicted CYP1B1 structure, and stabilizes the structure of meander, as indicated in the Results section. In addition, Glu387 lost two hydrogen bonds with the side chain of Arg390 and the carbonyl oxygen of Asp441. Residues Glu587, Arg350, and Arg444 are involved in the so-called ERR triad, invariant in most cytochrome P450 structures. Because the ERR triad may maintain heme-binding, mutations in this region could destabilize this important protein function. Indeed, in cytochrome P450, a mutation of arginine involving the meander that was located in a position similar to that of Arg444 in the human CYP1B1 protein, resulted in a completely inactive protein. Further, the Phe445 residue, located next to Arg444, stabilizes the flap between the heme-binding domain and meander and may help to form the redox-partner binding site. Therefore, destabilization of the meander structure by the Arg444Gln mutation may influence the heme-binding and redox-partner functions of CYP1B1.

In the present study, four probable mutations, Val198Ile, Val320Leu, and Glu499Gly, and a C3130T alteration in the noncoding region of exon 1, were detected. These four alterations were not present in 120 chromosomes isolated from unaffected Japanese control individuals. Mutations in the 5′-untranslated regions have been shown to affect mRNA stability, although we could not confirm that this occurred in C3130T. Two unrelated patients with PCG were compound heterozygotes with both C3130T and Val320Leu. However, two amino acid changes, Val198Ile and Glu499Gly, were detected on only one allele in two patients with PCG. No second mutation was identified in these individuals, possibly because of a change in the promoter region or because of limited sensitivity of our PCR-SSCP screening analysis.

In conclusion, 11 novel mutations, including 7 definite and 4 probable mutations, were detected in the CYP1B1 gene in 13 unrelated sporadic Japanese subjects with PCG. Considerable molecular heterogeneity in this gene was seen in nonconsanguineous Japanese families with sporadic disease occurrence. The mutations appear to be unique to Japanese patients.

**References**

10. Bejani BA, Stockton DW, Lewis RA, et al. Multiple CYP1B1 mutations and incomplete penetrance in an inbred population segregating primary congenital glaucoma suggest frequent de novo


