profile from the column most likely contain either a different or an additional mutation and should be sequenced for confirmation.

Acknowledgments

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[6] Genotyping Human Cytochrome: P450 1B1 Variants

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Introduction

Human cytochrome P450 1B1 (CYP1B1) was first isolated by differential hybridization as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-responsive cDNA clone from a human keratinocyte cell line treated with TCDD.[1] Analysis of the complete cDNA sequence of this mRNA identified a new gene subfamily of cytochrome P450, CYP1B1, based on 40% sequence homology to other polycyclic aromatic hydrocarbon (PAH)-inducible isoforms, CYP1A1 and CYP1A2.[2]

Initial characterization of the human CYP1B1 gene[3] described the DNA sequence of a 12-kb genomic clone corresponding to the entire 5.1-kb CYP1B1 cDNA and containing 3.0 kb of upstream DNA. Comparison of these sequences revealed the location of three exons (371, 1044, and 3707 bp) and two introns (390 and 3032 bp), with the CYP1B1 open reading frame spanning exons 2 and 3. High-resolution chromosome mapping confirmed the previous somatic cell hybrid analysis[2] and placed the CYP1B1 gene at 2p21–22 of human chromosome 2.[3] Comparison of the human CYP1B1 genomic and cDNA sequences, obtained independently from two cell lines derived from different individuals, revealed three sequence differences, including an amino acid change at valine-432. Concurrent human genetic studies to identify one of two loci for primary congenital glaucoma

[1] T. R. Sutter, K. Guzman, K. M. Dold, and W. F. Greenlee, *Science* **254,** 415 (1991).

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0076-6879/02 $35.00

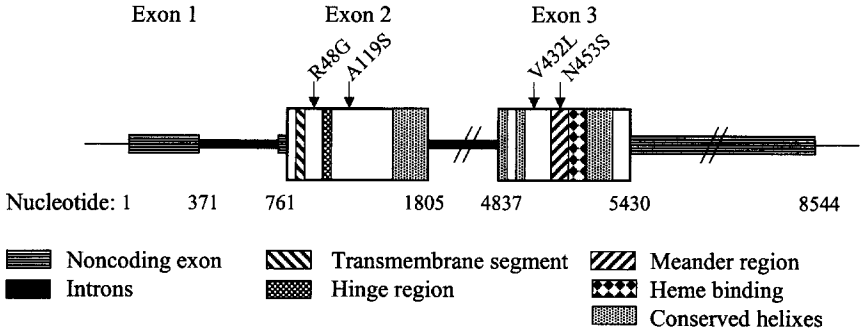

FIG. 1. Schematic of the human CYP1B1 gene. The four coding region single nucleotide polymorphisms are identified by arrows and indicate the corresponding change in the amino acid sequence. The nucleotide numbered 1 is the reported transcription start site[3] and corresponds to nucleotide 3045 of the GenBank/EBI Data Bank sequence with the accession number U56438. Other characteristics, including five conserved protein regions, are indicated.

(PCG) led to the mapping of the PCG locus GCL3A to human chromosome 2 at the 2p21 region.[4] Subsequent investigations have led to independent reports that identify distinct CYP1B1 gene mutations that segregate with the GCL3A phenotype in PCG families.[4–7]

Based on extensive DNA sequence analysis of the translated regions of the CYP1B1 gene in 22 PCG families and 100 randomly selected normal individuals,[6] the Val432-Leu CYP1B1 polymorphism was confirmed. Three additional polymorphisms predicting variant amino acid sequences were identified: Arg48-Gly, Ala119-Ser, and Asn453-Ser. The frequency of each wild-type allele, calculated for the reported sample of 100 normal individuals,[6] is as follows: Arg-48, 0.71; Ala-119, 0.71; Val-432, 0.28; and Asn-453, 0.76. The identification of several frequently occurring polymorphisms of the human CYP1B1 gene demonstrates the strength of direct genomic sequence analysis as a method to identify single nucleotide polymorphisms.

This article describes the methods used in our laboratory to genotype the four common single nucleotide polymorphisms found in the coding region of CYP1B1 (Fig. 1).

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[5] M. Sarfarazi, *Hum. Mol. Genet.* **6,** 1667 (1997).

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Genotyping CYP1B1

Isolation of Genomic DNA

Human genomic DNA can be isolated from blood using the Qiagen Genomic-tip System (Qiagen, Valencia, CA). This purification system uses an anion-exchange resin, which isolates high molecular weight DNA conveniently and quickly. Leukocytes are the only cells in the blood that contain nuclei and can be separated from other components of blood by centrifugation.

Collect at least 1 ml of blood in a heparinized Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Keep samples at 4° and fractionate by centrifugation within 6 hr of collection. Fractionate whole blood by centrifugation for 10 min at 3300*g* at room temperature using a swinging bucket rotor. The blood is separated into three layers. The middle layer, known as the buffy coat, contains leukocytes; the upper layer is plasma; and the lower level contains erythrocytes. Isolate the buffy coat and keep frozen until DNA is purified. At time of purification, adjust the leukocyte concentration to the recommended concentration for the appropriate Qiagen Genomic tip. Following the protocol of the Qiagen Genomic-tip system, lyse the cells, bind DNA to the resin, wash the resin, and elute and precipitate the DNA. Finally, resuspend the DNA in TE (10 m*M* Tris, 1 m*M* EDTA, pH 8) and store at 4° until genotype analysis. Yields from this procedure should be approximately 15 μg DNA/ml of whole blood. Check the quality of the DNA by agarose gel electrophoresis; it should be 25–50 kb in size.

Amplification of CYP1B1 Fragments from Genomic DNA

For polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assays[8–11] of exon 2 and 3 of the CYP1B1 gene, use three sets of PCR primers (Table I). Make or purchase (Integrated DNA Technologies, Inc., Coralville, IA) the following primers.

Primer set 1: forward 5′-tctctgcacccctgagtgtc-3′, reverse complement 5′-tagtg gccggtacgttctcc-3′
Primer set 2: forward 5′-aattggatcaggtcgtgg-3′, reverse complement 5′-atttcag cttgcctcttgc-3′
Primer set 3: forward 5′-cacctctgtcttgggctacc-3′, reverse complement 5′-atttca gcttgcctcttgcttc-3′.

[8] K. B. Mullis, F. Faloona, S. J. Scharf, R. K. Saiki, G. T. Horn, and H. A. Erlich, *Cold Spring Harb. Symp. Quant. Biol.* **51,** 263 (1986).
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TABLE I
LOCATIONS OF KNOWN CODING POLYMORPHISMS[a] IN HUMAN CYP1B1

Location	Codon	Primer	Enzyme and recognition sequence	Allele		Base pairs (total, fragments) and amplified regions
				Wild type	New	
Exon 2	48	F[b]: 5′-tctctgcacccctgagtgtc-3′	*Csp*I	CGG[c]	GGG	1015, 829/186
		RC: 5′-tagtggccggtacgttctcc-3′	cg▾g(a/t)ccg			nt 718–1732
Exon 2	119	F: 5′-tctctgcacccctgagtgtc-3′	*Ngo*MIV	GCC[c]	TCC	1015, 616/399
		RC: 5′-tagtggccggtacgttctcc-3′	g▾ccggc			nt 718–1732
Exon 3	432	F: 5′-aattggatcaggtcgtgg-3′	*Eco*57I	GTG	CTG[c]	579, 341/238
		RC: 5′-atttcagcttgcctcttgc-3′	ctgaag(n)$_{16}$▾			nt 4870–5448
Exon 3	453	F: 5′-cacctctgtcttgggctacc-3′	*Mwo*I	AAC	AGC[c]	437, 297/140
		RC: 5′-atttcagcttgcctcttgcttc-3′	gcnnnnn▾nngc			nt 5012–5448

[a] There is also a silent polymorphism at codon 449 (GAT → GAC).
[b] F, Forward primer; RC, reverse complement primer.
[c] Codon present in the nucleotide sequence that is recognized by the indicated restriction endonuclease.

Use the *Taq* PCR Core Kit (Qiagen) to amplify genomic DNA. Place a 50-μl reaction containing 100 ng of genomic DNA, 200 μM of each of the four deoxynucleotide triphosphates, 1× reaction buffer with 1.5 mM $MgCl_2$, 1× Q-solution, 20 pmol of each primer, and 2.5 units *Taq* polymerase into a GeneAmp PCR System 9600 thermocycler (PerkinElmer, Foster City, CA). Alternatively, we have found that the Q solution can be replaced with 2 mM $MgCl_2$ in the reaction mixture. For genotype analysis, we recommend a PCR unit with a heated cover so that the use of an oil overlay is avoided. After an initial denaturing step for 3 min at 94°, repeat the following cycle 30 times: 94° for 1 min, 60° for 30 sec, and 72° for 1 min, followed by a final 10 min elongation cycle at 72° and storage at 4°. To check for laboratory template contamination, include a negative control reaction (without DNA) in each set of PCR reactions. Analyze 9 μl of each PCR product, including the control, by gel electrophoresis (1.5% agarose gel) in order to confirm the expected size of the product. Remove amplification primers and primer dimers from the PCR reaction using the Wizard PCR Preps DNA purification system (Promega, Madison, WI). This step allows visualization of low molecular weight fragments without the possible interference by primer dimers.

Although the conditions described earlier have repeatedly amplified the target CYP1B1 sequence in our laboratory, successful PCR conditions will vary and are dependent on the length and nucleotide composition of the template target sequence, as well as on the length and nucleotide composition of the primer sequences. Conditions can be optimized by varying the concentrations of $MgCl_2$, primers, and template, as well as by varying annealing temperatures. In addition, adding DNA denaturants such as formamide, DMSO, and Q solution (Qiagen) can improve amplification by modifying the melting temperature of DNA.[12]

[12] Alkami Biosystems, Inc., www.alkami.com (1999).

Digestion of CYP1B1 PCR Fragments with Restriction Endonucleases

Analysis of the reported CYP1B1 gene sequence revealed that each of the four polymorphisms in the coding region of CYP1B1 could be evaluated using four different restriction endonucleases: *Csp*I (Promega), *Ngo*MIV (New England Biolabs, Beverly, MA), *Eco*57I (MBI Fermentas, Hanover, MD), and *Mwo*I (New England Biolabs). To visualize DNA fragments, combine the restriction endonuclease reaction solutions with 1× loading buffer (10% Ficoll and 0.25% xylene cyanol) and separate the DNA by electrophoresis through a 2% NuSieve 3 : 1 agarose gel (FMC Bioproducts, Rockland, ME) in 1× TAE buffer (40 m*M* Tris–acetate, 1 m*M* EDTA, pH 8.5) at 5 V per cm length of the gel. Stain the gels with the fluorescent SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR), visualize the stained DNA by UV transillumination, and document by photography using a SYBR Green/Gold gel stain photographic filter (Molecular Probes). SYBR Gold gel stain is more sensitive than ethidium bromide staining, allowing for visualization of small DNA fragments that are often difficult to detect. Each sample is genotyped based on the relative size of the observed fragment(s), compared to the base pair standard (Fig. 2).

Digest 20 μl of each PCR product with the indicated restriction endonucleases (Table I). Digestions of samples with 5 units of *Csp*I reveal the presence of a polymorphism in exon 2 (codon 48). This restriction endonuclease site is present in a homozygous wild-type sample, and two bands corresponding approximately to 829 and 186 bp are seen on the gel (Fig. 2A, lane 1). A homozygous polymorphic sample does not have the restriction site and thus is not digested, and a

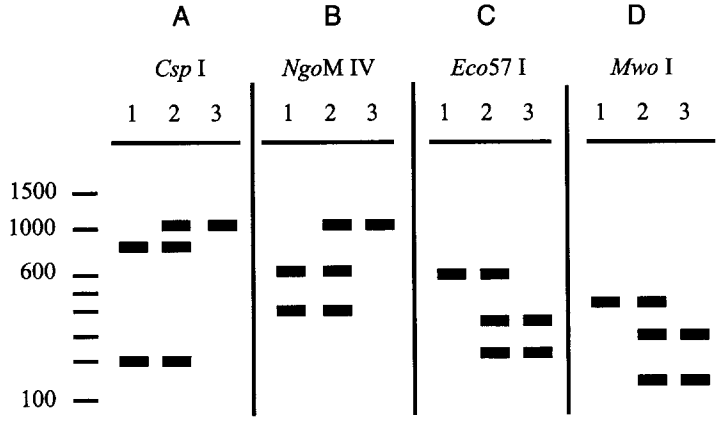

FIG. 2. Depiction of restriction fragments from PCR products in agarose gels. Fragments were generated by the digestion of PCR products with the indicated restriction endonuclease (see text for detailed explanation). Lane 1, homozygous wild-type individual; lane 2, heterozygous individual; and lane 3, homozygous polymorphic individual. DNA size standards (bp) are on the left-hand side.

single band appears at approximately 1015 kb (Fig. 2A, lane 3). A heterozygote sample, which has both a wild-type and a polymorphic allele, contains all three bands, corresponding to 1015, 829, and 186 bp (Fig. 2A, lane 2). Digestions of samples with 5 units of *Ngo*MIV reveal the presence of a polymorphism in exon 2 (codon 119). This restriction endonuclease site is present in a homozygous wild-type sample, and two bands corresponding approximately to 616 and 399 bp are seen on the gel (Fig. 2B, lane 1). A homozygous polymorphic sample does not have the restriction site and thus is not digested, and a single band appears at approximately 1015 kb (Fig. 2B, lane 3). A heterozygote sample, which has both a wild-type and a polymorphic allele, contains all three bands, corresponding to 1015, 616, and 399 bp (Fig. 2B, lane 2). Digestions of samples with 2 units of *Eco*57I reveal the presence of a polymorphism in exon 3 (codon 432). This restriction endonuclease site is absent in a homozygous wild-type sample, and a single band corresponding to 579 bp is seen on the gel (Fig. 2C, lane 1). A homozygous polymorphic sample has the restriction site, and two bands appear at approximately 341 and 238 bp (Fig. 2C, lane 3). A heterozygote sample, which has both a wild-type and a polymorphic allele, contains all three bands, corresponding to 579, 341, and 238 bp (Fig. 2C, lane 2). Digestions of samples with 5 units of *Mwo*I reveal the presence of a polymorphism in exon 3 (codon 453). This restriction endonuclease site is absent in a homozygous wild-type sample, and a single band corresponding to 437 bp is seen on the gel (Fig. 2D, lane 1). A homozygous polymorphic sample has the restriction site, and two bands appear at approximately 297 and 140 bp (Fig. 2D, lane 3). A heterozygote sample, which has both a wild-type and a polymorphic allele, contains all three bands, corresponding to 437, 297, and 140 bp (Fig. 2D, lane 2).

As a positive control for the activity of the restriction endonucleases, digest amplified genomic DNA that has previously been determined by DNA sequence analysis to be homozygous for the restriction site under investigation.

Conclusions

Genotyping by RFLP analysis will soon be replaced by more rapid, high-throughput assays based on electrochemical or fluorescent detection methods, including oligonucleotide microarrays. In the interim, the procedures described here should facilitate CYP1B1 genotyping of banked DNA samples.

Acknowledgments

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