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# Functional Analysis of Six Different Polymorphic CYP1B1 Enzyme Variants Found in an Ethiopian Population

ELENI AKLILLU, MIKAEL OSCARSON, MATS HIDESTRAND, BRITH LEIDVIK, CHARLOTTA OTTER, and MAGNUS INGELMAN-SUNDBERG

*Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden (E.A., M.O., M.H., M.I.-S.); and AstraZeneca Mölndal R&D, Molecular Biology, Mölndal, Sweden (B.T., C.O.)*

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## ABSTRACT

Cytochrome P450 1B1 (CYP1B1) is an extrahepatic enzyme of potential importance for the metabolism of estrogen and for metabolic activation of environmental carcinogens. We investigated an Ethiopian population for functional polymorphisms in the *CYP1B1* gene using genomic DNA sequencing and detected three novel single nucleotide polymorphisms (SNPs). One of these (4360C→G in exon 3) is present at a frequency of 7% and causes an Ala443Gly amino acid substitution. In addition, the four described previously missense mutations Arg48Gly, Ala119Ser, Leu432Val, and Asn453Ser were found with frequencies of 51, 50, 53, and 2%, respectively, yielding a total of 32 possible *CYP1B1* haplotypes. Allele-specific PCR methods for haplotype analysis were developed and seven different *CYP1B1* alleles were found: *CYP1B1*1, *2, *3, *4, *5, *6,* and **7* with frequencies of 8, 37, 39, 2, 0.7, 6, and 7%, respectively. The functional properties of different forms of CYP1B1, as well as of the Leu432Val + Asn453Ser and Leu432Val + Ala443Gly variants, were evaluated after heterologous expression of the corresponding cDNAs in *Saccaromyces cerevisiae*. The results revealed that CYP1B1.6 and CYP1B1.7, having the amino acid substitutions Arg48Gly, Ala119Ser, and Leu432Val in common, exhibited altered kinetics with significantly increased apparent $K_m$ and lowered $V_{max}$ values for both the 2- and 4-hydroxylation of 17 $\beta$-estradiol, whereas the other constructs were indistinguishable from the CYP1B1.1 enzyme. The results emphasize the necessity of a complete haplotype analysis of enzyme variants for evaluation of functional consequences in vivo and for analyses of genetic polymorphisms in relation to, for example, cancer incidence.

Human cytochrome P450 1B1 (CYP1B1) has been shown to be an important enzyme in the activation of diverse procarcinogens, such as arylarenes, nitroarenes, polycyclic aromatic hydrocarbons, and arylamines, to reactive metabolites that cause DNA damage (Shimada et al., 1996). CYP1B1 is constitutively expressed in steroidogenic tissues such as uterus, breast, ovary, testis, prostate, and the adrenal gland (Shimada et al., 1996) and is active in the metabolism of estradiol (Hayes et al., 1996), as well as of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene (7,12-DMBA), known procarcinogens. The enzyme is also present in many other extrahepatic tissues, including kidney, thymus, spleen, brain, heart, lung, colon, and intestine (Sutter et al., 1994; Shimada et al., 1996). A nuclear localization of the enzyme has been proposed based upon immunohistochemistry experiments (Muskhelishvili et al., 2001). CYP1B1 is expressed at a higher level in a wide range of human cancers, including cancers of the skin, brain, testis (Murray et al., 1997), and breast (McKay et al., 1995) compared with the nontransformed tissue.

The human *CYP1B1* gene has been mapped to chromosome 2 (Sutter et al., 1994) and encompasses three exons with the coding region starting in exon 2 (Tang et al., 1996). The mRNA is 5.2 kilobases and encodes a protein of 543 amino acids (Sutter et al., 1994). Six different single nucleotide polymorphic sites (SNPs) have previously been reported in the human *CYP1B1* gene (Stoilov et al., 1998), of which four cause amino acid substitutions. Three common *CYP1B1* alleles have been identified so far. *CYP1B1*2* has two linked polymorphisms in exon 2, 142C→G (m1) and 355G→T (m2), resulting in the Arg48Gly and Ala119Ser amino acid substitutions, respectively. *CYP1B1*3*, has 4326C→G (m3), causing a Leu432Val exchange, and *CYP1B1*4* has 4390A→G (m4), leading to an Asn453Ser substitution (see http://www.imm.ki.se/CYPalleles/cyp1b1.htm).

Human CYP1B1 heterologously expressed in yeast shows higher specific activity toward 4-hydroxylation than 2-hy-

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**ABBREVIATIONS:** SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; 7,12-DMBA, 7,12-dimethylbenz[*a*]anthracene; m1, 142C→G causing Arg48Gly; m2, 355G→T causing Ala119Ser; m3, 4326C→G causing Leu432Val; m4, 4390A→G causing Asn453Ser; m5, 4360C→G causing Ala443Gly; bp, base pair(s); wt, wild-type; P450, cytochrome P450.



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droxylation of 17β-estradiol (Hayes et al., 1996) compared with CYP1A1 and CYP1A2, which preferentially oxidize the steroid at the 2-position (Yamazaki et al., 1998). The finding of a considerably higher rate of 4-hydroxylation of 17β-estradiol in breast cancer microsomes, compared with a very low level of 4-hydroxylation in normal breast tissue (Liehr and Ricci, 1996), has received attention because 4-hydroxyestradiol has been suggested to be carcinogenic in both human myometrium (Liehr et al., 1995) and breast tissue (Liehr and Ricci, 1996). Increased CYP1B1 expression and elevated rates of 4-hydroxylation of estradiol in transformed tissue may be markers of a malignant phenotype and may also provide a basis for targeting of anticancer drugs that are metabolized by this enzyme.

The importance of CYP1B1 in chemical carcinogenesis has been illustrated using *Cyp1b1*-null mice. Embryonic fibroblast cells derived from *Cyp1b1* null mice were found to be resistant to 7,12-DMBA mediated toxicity in contrast to cells from the wild-type animals (Buters et al., 1999). The *Cyp1b1*-null mice were also protected from 7,12-DMBA-induced malignant lymphomas (Buters et al., 1999), as well as bone marrow cytotoxicity and preleukemia (Heidel et al., 2000), compared with their wild-type counterparts.

Several studies have been devoted to the investigation of a potential relationship between SNPs present in the human *CYP1B1* gene and incidence of various cancer forms (Bailey et al., 1998; Tang et al., 2000; Watanabe et al., 2000; Zheng et al., 2000). Some studies have revealed a significant relationship between the presence of the Ala119Ser variant and the incidence of breast cancer as well as squamous cell carcinoma in the lung (Watanabe et al., 2000). An increased risk for prostate cancer was also reported in subjects homozygous for the Leu432Val form (Tang et al., 2000). It is anticipated that these polymorphisms would cause an altered function of the enzyme to provide a role for the CYP1B1 polymorphism in determining interindividual differences in susceptibility for carcinogenesis.

The aim of the present study was to analyze for *CYP1B1* missense mutations in an African population, develop methods for detection of the different possible haplotypes and evaluate their functional properties after expression of the enzyme variants in vitro. The results show altered kinetic properties for two of six CYP1B1 enzyme variants occurring in an Ethiopian population and emphasize the necessity of relating haplotypes rather than SNPs to disease when conducting molecular epidemiological studies.

## Materials and Methods

**Genomic Sequencing of the Human *CYP1B1* Gene.** The open reading frame of *CYP1B1* and exon-intron boundaries were amplified by PCR in three different fragments (Stoilov et al., 1998) from five genomic DNA samples, previously genotyped for m1, m2, m3, and m4 (see below). The PCR products were purified using the Wizard PCR Preps DNA Purification kit (Promega, Madison, WI). Fragments one and two, which consist of overlapping regions in exon two, were sequenced in both directions using primers seq1F and seq1R and primers seq2F and seq2R, respectively. Fragment three, which comprises the whole coding region of exon three, was sequenced using primers seq3F and seq3R, respectively. Primer sequences are listed in Table 1. DNA sequencing was performed using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI Prism 377 DNA sequencer.

**Genotyping of the CYP1B1 Missense Mutations.** The genomic DNA used was from 150 healthy unrelated Ethiopians who previously had participated in a study about genetic polymorphism in the *CYP2D6* gene (Aklillu et al., 1996). The ethical committee at Karolinska Institutet approved the use of these samples for the current investigation.

A two-step, allele-specific PCR method was developed to analyze the four missense mutations described previously: 142C→G (m1), 355G→T (m2), 4326C→G (m3), and 4390A→G (m4) as well as the newly identified missense mutation, 4360C→G (m5). Primer sequences are given in Table 1. Primers, optimal PCR condition, number of reaction cycles, and expected fragment length for analysis of each SNP site are summarized in Table 2. A region covering the polymorphic sites in each exon was amplified in the first PCR (PCR 1) followed by a second SNP specific PCR (PCR 2). In brief, part of exon two covering both the m1 and m2 sites was amplified in PCR 1 as follows: PCR amplification was performed in a total volume of 25 μl containing about 100 ng of genomic DNA, 1.3 mM $MgCl_2$, 0.25 μM concentrations of each primer, 10% dimethyl sulfoxide (Sigma, St. Louis, MO), 0.25 mM concentrations of each dNTP, 0.75 U of *Taq* polymerase (Advanced Biotechnologies, Epsom, UK), in a 1× reaction buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl) using a GeneAmp PCR System 9700 (PerkinElmer Life Sciences, Boston, MA). After initial denaturation at 95°C for 1 min, 35 cycles, each consisting of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 1 min, were carried out, followed by final extension at 72°C for 7 min. The PCR 1 product was subsequently used as a template in PCR 2, which contained 1 μl of PCR 1 product (diluted 10-fold in water), 0.125 μM concentrations of each primer, 0.125 mM concentrations of each dNTP, and 0.375 U of *Taq* polymerase and was carried out in a 1× reaction buffer (see above) in a total volume of 25 μl. The reaction was performed with initial denaturation at 95°C for 1 min, followed by reaction cycles with denaturation at 95°C for 15 s, annealing at the temperature shown

TABLE 1
Sequences of the primers used in this study

| Primer Name | Location[a] | Primer Sequence (5′ to 3′) |
|---|---|---|
| ex2F | 3828–3847 | ACG ACC CTT GGC CGC TAA AC |
| ex2R | 4741–4724 | GCC ACC ACC GTG CGA GTC |
| Fm1wt | 3933–3947 | GGA GGC GGC AGC TCG |
| Fm1mt | 3933–3947 | GGA GGC GGC AGC TCG |
| Fm2wt | 4146–4160 | TCG CCG ACC GGC CGG |
| Fm2mt | 4146–4160 | TCG CCG ACC GGC CGT |
| 1/2mutR | 4348–4331 | CAG CAG CGC CAC CAG CTC |
| ex3F | 7993–8012 | TAT GAA GCC ATG CGC TTC TC |
| ex3R | 8262–8243 | AAG TTC TTC GCC AAT GCA CC |
| m3Rwt | 8145–8131 | GTT AGG CCA CTT CAG |
| m3Rmt | 8145–8131 | GTT AGG CCA CTT CAC |
| Rm4wt | 8209–8195 | TGG TCA GGT CCT TGT |
| Rm4mt | 8209–8195 | TGG TCA GGT CCT TGC |
| Rm5wt | 8179–8165 | TGT CCA AGA ATC GAG |
| Rm5mt | 8179–8165 | TGT CCA AGA ATC GAC |
| m1Fwt | 3930–3947 | AAC GGA GGC GGC AGC TCC |
| m1Fmt | 3930–3947 | AAC GGA GGC GGC AGC TCG |
| Rm2wt | 4174–4160 | GAA GGA GCC GAA GGC |
| Rm2mt | 4174–4160 | GAA GGA GGC GAA GGA |
| Fm3wt | 8117–8131 | TGA ATC ATG ACC CAC |
| Fm3mt | 8117–8131 | TGA ATC ATG ACC CAG |
| 1B13R | 8624–8605 | TAT GGA GCA CAC CTC ACC TG |
| seq1F | 3701–3720 | TCC ACC CAA CGG CAC TCA GT |
| seq1R | 4432–4413 | ACA CAC GGC ACT CAT GAC GT |
| seq2F | 4214–4233 | TAC TCG GAG CAC TGG AAG GT |
| seq2R | 4934–4915 | CCT GCT TGC AAA CTC AGC AT |
| seq3F | 7759–7778 | GTC ACT GAG CTA GAT AGC CT |
| seq3R | 8612–8593 | CTC ACC TGA TGG ACA GTT GA |

[a] Location of primers is numbered according to GenBank accession number U56438.

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in Table 2 for 20 s, and elongation at 72°C for 45 s, followed by a final extension at 72°C for 7 min. Analysis of m3, m4, and m5 was carried out by first amplifying part of exon 3, which spans the three polymorphic sites in a PCR 1 reaction (as described above but without dimethyl sulfoxide), followed by PCR 2 using the primers described in Table 2.

**CYP1B1 Haplotype Analysis.** Subjects heterozygous for m1 and m2 and for m3 and m4 were further analyzed for linkage disequilibrium of the polymorphisms as outlined in Fig. 1. Part of each exon covering the polymorphic sites amplified in PCR 1 (see above) was used as a template in PCR 2 after a 10-fold dilution in water. In PCR 2, the haplotypes were determined using two allele-specific primers in both forward and reverse directions in all four possible combinations. Reaction mixtures were as describe above, and primer combinations and optimal PCR conditions are summarized in Table 2.

In subjects heterozygous for both m3 and m5, an allele-specific forward primer (Fm3wt or Fm3 mt) with a common reverse primer (ex3R) were used in PCR 2, as described above, to separate the two alleles with respect to the presence of m3 (for reaction conditions see Table 2). The 4360C→G (m5) polymorphism introduces a *Bst*NI restriction site (Fig. 3). Fourteen microliters of the PCR 2 product was therefore analyzed for the presence of m5 by digestion with 8 U of *Bst*NI (New England Biolabs) at 60°C overnight in a 20-$\mu$l total reaction mixture containing 1× New England Biolabs buffer 2 and 2 $\mu$g of bovine serum albumin. The products were subsequently separated on a 3% agarose gel.

All subjects heterozygous for SNPs in both exon 2 (m1 or m2) and exon 3 (m3, m4, or m5) were further re-analyzed for linkage disequilibrium in the following way: The two alleles were first separated by allele-specific long PCR with respect to the presence or the absence of the m1 SNP (see Fig. 1) followed by genotyping of each individual allele for the SNPs in exon 3. In brief, a 4695-bp fragment containing part of exon 2 and the entire coding regions of exon 3 was amplified from genomic DNA by long PCR using the forward primers m1Fwt and m1Fmt, respectively, and the common reverse primer 1B13R (Table 1). The PCR mix contained about 50 ng of genomic DNA, 0.2 $\mu$M concentrations of each primer, 0.2 mM concentrations of each dNTP, 0.8–1.0 mM $Mg(OAc)_2$, and 0.5 U of rTth DNA polymerase XL (PerkinElmer) in 1× buffer XL II in 0.2-ml, thin-walled, hot-start micro 20 tubes and was amplified using a PerkinElmer GeneAmp PCR System 9700. The amplification involved 30 cycles of denaturation at 94°C for 12 s and annealing/extension at 68°C for 5 min. Eight microliters of PCR product was subsequently analyzed on 0.8% agarose gel (Fig. 4A). DNA samples being homozygous wt and homozygous mutated for m1 were used as controls to ensure the absence of nonspecific amplification. The long PCR products were diluted 10-fold in water and 1 $\mu$l was used as a template in a PCR 2 reaction, where the corresponding SNP in exon 3 to which the subject was heterozygous was analyzed as described above for PCR 2 (Fig. 4B).

**Evaluation of the Haplotype Method.** A 4949-bp fragment (from 3676–8624 in GenBank accession number U56438), containing the whole coding regions of exon 2 and exon 3 as well as the entire intron 2 were amplified by long PCR using primers clone 1B1-F (5′-TGC AAA GCT TTC AGA GAG TCA GCT CCG-3′) and clone 1B1-R (5′-CTA GCC GCG GTA TGG AGC ACA CCT CAC CTG-3′) from seven subjects assigned as having different *CYP1B1* haplotypes by the above described haplotyping method. The *Hin*dIII and *Sac*II restriction sites in primer clone 1B1-F and 1B1-R, respectively, are underlined. The conditions for long PCR were as described above except using 1.0 mM $MgO(Ac)_2$ and 35 reaction cycles. The long PCR fragments were purified using the Wizard PCR Preps DNA Purification kit (Promega) and cloned into pBluescript SK after digestion with *Hin*dIII and *Sac*II (New England Biolabs, Beverly, MA). The whole coding region and exon-intron boundaries were sequenced in three fragments as described above using the ABI PRISM BigDye Terminator cycle sequencing Ready reaction kit and analyzed on ABI PRISM 377 DNA sequencer.

**Construction of pYeDP60-CYP1B1 Expression Plasmids.** The various CYP1B1 cDNAs were generated by site-directed mutagenesis and cloned into the pYeDP60 expression plasmid (Urban et al., 1990). The *CYP1B1*1* and *CYP1B1*2* expression plasmids are as described in (McLellan et al., 2000). The *CYP1B1*1* plasmid was used as a template to generate the m3 SNP using the forward primer 5′-GGT CTG TGA ATC ATG ACC CA**G** TGA AGT GGC CTA ACC CGG-3′, the reverse primer 5′-CCG GGT TAG GCC ACT TCA **C**TG GGT CAT GAT TCA CAG ACC- 3′ and the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the

TABLE 2

Primer combination and optimal PCR conditions used to genotype 142C→G (m1), 355G→T (m2), 4326C→G (m3), 4390A→G (m4), and 4390C→G (m5), as well as linkage disequilibrium analysis (see *Materials and Methods*)

| SNP | PCR Type | Primers Used | Annealing Temp | $MgCl_2$ | No. of cycles | Fragment length |
|---|---|---|---|---|---|---|
| | | | *°C* | *mM* | | *bp* |
| m1 | PCR 1 | ex2F + ex2R | 60 | 1.3 | 35 | 914 |
| | PCR 2 | Fm1wt/Fm1mt + 1/2mutR | 54 | 1.3 | 15 | 416 |
| m2 | PCR 1 | ex2F + ex2R | 60 | 1.3 | 35 | 914 |
| | PCR 2 | Fm2wt/Fm2mt + 1/2mutR | 54 | 1.3 | 15 | 203 |
| m3 | PCR 1 | ex3F + ex3R | 60 | 1.3 | 35 | 270 |
| | PCR 2 | ex3F + m3Rwt/m3Rmt | 54 | 1.3 | 20 | 153 |
| m4 | PCR 1 | ex3F + ex3R | 60 | 1.3 | 35 | 270 |
| | PCR 2 | ex3F + Rm4wt/Rm4mt | 54 | 1.3 | 20 | 217 |
| m5 | PCR 1 | ex3F + ex3R | 60 | 1.3 | 35 | 270 |
| | PCR 2 | ex3F + Rm5wt/Rm5mt | 54 | 1.3 | 20 | 186 |
| m1 vs. m2 | PCR 1 | ex2F + ex2R | 60 | 1.3 | 35 | 914 |
| | PCR 2 | Fm1wt + Rm2wt<br>Fm1wt + Rm2mt<br>Fm1mt + Rm2wt<br>Fm1mt + Rm2mt | 56 | 0.8 | 20 | 242 |
| m3 vs. m4 | PCR 1 | ex3F + ex3R | 60 | 1.3 | 35 | 270 |
| | PCR 2 | Fm3wt + Rm4wt<br>Fm3wt + Rm4mt<br>Fm3mt + Rm4wt<br>Fm3mt + Rm4mt | 54 | 0.9 | 20 | 93 |
| m3 vs. m5 | PCR 1 | ex3F + ex3R | 60 | 1.3 | 35 | 270 |
| | PCR 2 | Fm3wt/Fm3mt + ex3R followed by BstNI RFLP | 54 | 1.3 | 20 | 146 |
| m1 vs. m3, m4 or m5 | PCR 1 | m1Fwt/m1Fmt + 1B13R (allele-specific long PCR) followed by genotyping for m3, m4 or m5 as described above in PCR II | 68 | 0.8–1.0 | 30 | 4695 |

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manufacturer's instructions. The m1+m2+m3 variant, *CYP1B1*6*, was made using the *Not*I-*Sac*I fragment from the *CYP1B1*3* plasmid to replace the corresponding part in the *CYP1B1*2* plasmid. The other expression plasmids were generated using the same principle as described for the *CYP1B1*3* plasmid. The m4 SNP was introduced into the *CYP1B1*1* plasmid using the forward primer 5′-GGA CGG CCT CAT CA**G** CAA GGA CCT GAC CAG C-3′ and the reverse primer 5′-GCT GGT CAG GTC CTT G**C**T GAT GAG GCC GTC C-3′. The m1+m2+m3+m5 variant, *CYP1B1*7*, was generated by introducing the m5 polymorphism into the *CYP1B1*6* plasmid using the forward primer 5′-GGA GAA CTT TGA TCC AG**G** TCG ATT CTT GGA CAA GG-3′ and the reverse primer 5′-CCT TGT CCA AGA ATC GA**C** CTG GAT CAA AGT TCT CC-3′. The m3+m5 variant was generated by introducing the m5 polymorphism into the *CYP1B1*3* plasmid. The forward primer was 5′-GGA GAA CTT TGA TCC AG**G** TCG ATT CTT GGA CAA GG-3′ and the reverse primer 5′-CCT TGT CCA AGA ATC GA**C** CTG GAT CAA AGT TCT CC-3′. For production of the m3+m4 variant, the m4 polymorphism was introduced into the *CYP1B1*3* plasmid. The forward primer was 5′- GGA CGG CCT CAT CA**G** CAA GGA CCT GAC CAG C-3′ and the reverse primer 5′-GCT GGT CAG GTC CTT G**C**T GAT GAG GCC GTC C-3′. The SNP, intended to be introduced in the corresponding cDNA, is shown in bold. All plasmids were sequenced using the ABI Prism Big Dye terminator cycle sequencing kit and analyzed with ABI Prism 377 DNA sequencer to ensure the correct constructs and to exclude any potential PCR artifacts.

**Expression of CYP1B1 Variants in Yeast.** The yeast strain INVSc1-HR *MAT α his3Δ1 leu2 trp1–289 ura3–52* (*pFL-35 human reductase*) expressing human cytochrome P450 reductase was a gift from the LINK project (a program of the University of Dundee/Biotechnology and Biology Research Council/Department of Trade and Industry/Pharmaceutical Industry) (Masimirembwa et al., 1999). Yeast cells were transfected with the pYeDP60-CYP1B1 expression plasmids described above and were subsequently inoculated into selective medium and grown as described in (McLellan et al., 2000), where also the method for isolation of microsomes is described.

**Analysis of Estradiol Metabolism.** Rates of CYP1B1-dependent metabolism of estradiol in yeast microsomes were analyzed as described previously (McLellan et al., 2000) employing reversed-phase high-performance liquid chromatography with a C18 column (Merck, 250 × 4 mm, 5 μm) and acetic acid/methanol/acetonitrile/water [1:1:38:60 (v/v)] as mobile phase. In brief, incubation mixtures contained microsomes corresponding to 7 pmol of the CYP1B1 variants, varying concentration (0–12.8 μM) of $^{14}$C-labeled 17β-Estradiol (PerkinElmer Life Sciences), and was buffered to pH 7.4 with 100 mM sodium phosphate in a final volume of 0.5 ml. After 2-min preincubation at 37°C, the reaction was initiated by adding NADPH to a final concentration of 0.5 mM. In all incubations, equivalent protein amounts (2 mg/incubation) were used by addition of microsomes from yeast transfected with the empty pYeDP60 plasmid. After 5 min, the incubations were terminated by addition of 2 ml of dichloromethane. The organic phase extracts were collected after centrifugation for 10 min at 3500 rpm and evaporated under a nitrogen stream. The residue was dissolved in 160 μl of mobile phase and 100 μl was injected into the high-performance liquid chromatography.

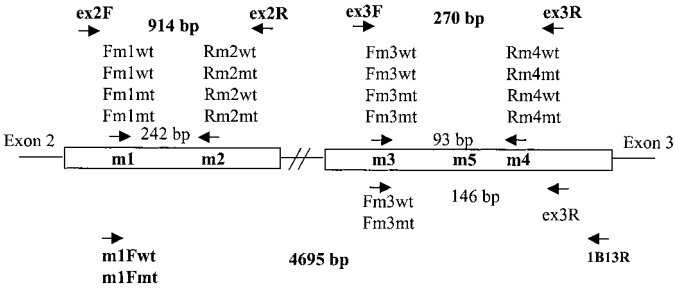


**Fig. 1.** A schematic diagram of the PCR-based strategy used for analysis of SNPs and haplotypes in the human *CYP1B1* gene. The positions of 142C→G (m1), 355G→T (m2), 4326C→G (m3), 4390A→G (m4), and 4360C→G (m5) in the coding region of *CYP1B1* gene are marked within the exon boxes. Horizontal arrows denote positions of primers used in the haplotype analysis namely, m1 versus m2, m3 versus m4, m3 versus m5, and m1 versus m3. Primers used in the first PCR (PCR 1) are indicated in bold.

## Results

**Analysis of *CYP1B1* Polymorphisms in the Ethiopian Population.** To detect new polymorphic sites in the *CYP1B1* gene, we sequenced the whole open reading frame and exon-intron boundaries of *CYP1B1* using genomic DNA from five different subjects previously genotyped for the *CYP1B1* missense mutations m1-m4. Sequencing was carried out using genomic DNA from 1) subject E1, homozygous wt for all SNPs; 2) subject E15, homozygous for both m1 and m2; 3) subject E6, homozygous for m3; 4) subject E65, homozygous for m1, m2, and m3; and 5) subject ES11, heterozygous for m1, m2, m3, and m4. The results confirmed the presence of the SNPs determined by the allele-specific PCR analysis and also detected three new SNPs in subjects E65 and ES11. Two silent mutations, 729G→C and 4110T→C, were identified in exon 2 and exon 3, respectively. Furthermore, a missense mutation 4360C→G (m5), which causes an Ala443Gly substitution, was detected in exon three in both E65 and ES11.

**Genotyping for m1, m2, m3, m4, and m5.** The five missense mutations of *CYP1B1*, m1, m2, m3, m4, and m5, were analyzed in genomic DNA from 150 healthy unrelated Ethiopian persons using allele-specific PCR methods as detailed under *Materials and Methods*. The frequencies of the SNPs were 51, 50, 53, 2, and 7%, respectively. None of the subjects was homozygous for m4 or m5.

**SNP Mapping and Determination of the *CYP1B1* Haplotypes.** We considered it important to determine the haplotypes of the various *CYP1B1* genes in the population because of the high frequency of the missense mutations and incomplete knowledge of their linkage disequilibrium. All subjects who were heterozygous for two or more SNP sites were further analyzed by three different PCR methods. Linkage of m1 in exon 2 with m3, m4, or m5 in exon 3 was achieved by separation of the two chromosomes by allele-specific long PCR using m1 wt or mutant specific forward primers and a common reverse primer in the 3′-end of exon 3 as outlined in Fig. 1. The m1 separated alleles were further genotyped for the m3, m4, or m5 using allele-specific PCR.

To study the linkage of m1 and m2 in exon 2 and m3 and m4 in exon3, we first amplified a region covering the two polymorphic sites. The PCR product was used as a template to amplify a region between the two SNP sites by simultaneous allele-specific PCR using SNP specific primers in both forward and reverse directions (Fig. 1). In heterozygous subjects, PCR amplification between the two SNP sites with all four possible combinations of primers should result in only two sets of primers that give a PCR product (Fig. 2). Based on the result, it is possible to link all SNPs together and the haplotypes can be identified.

Genotyping of the 150 subjects revealed that 43 subjects were heterozygous for m1, m2, and m3. Haplotype analyses of m1 in relation to m2 indicated that m1 was in linkage disequilibrium with m2 in all these subjects. Alleles sepa-

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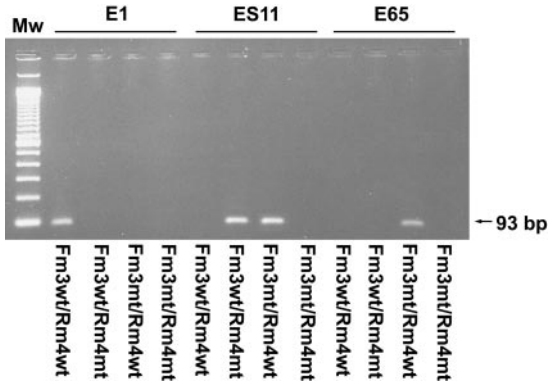


**Fig. 2.** Analysis of m3 and m4 linkage disequilibrium. Genomic DNA from subjects E1 (homozygous wt for both m3 and m4) and E65 (homozygous for the m3 SNP) were used as controls. ES11 was heterozygous for both SNPs. Part of exon 3 covering m3 and m4 was amplified with PCR (see Fig. 1) and used as a template in the second simultaneous allele-specific PCR for both m3 and m4. Combinations of primers used in PCR 2 are listed under each corresponding lane. In subject E1, PCR amplification was only obtained when both the wt-specific primers were used (lane 1) and in subject E65 PCR amplification was obtained only with the m3 mutant-specific and the m4 wt-specific primers (lane 3). Amplification of the region between m3 and m4 with all four possible combinations of primer give a PCR product in only two sets of primers in heterozygous subjects. Thus in subject ES11, PCR amplification was obtained only in lanes 2 and 3 using the m3wt/m4 mt and m3 mt/m4wt primer combinations, respectively, indicating that m3 and m4 are on separate alleles. 100 bp DNA Ladder (Invitrogen, Carlsbad, CA) was used as a marker.

rated with respect to m1 were further regenotyped for m3, which revealed that m1 and m3 were not in linkage disequilibrium in these 43 subjects. We could therefore conclude that these subjects carried m1+m2 (*CYP1B1*2*; see Table 3 for a description of the haplotypes found) on one allele and m3 (*CYP1B1*3*) on the other. In addition, two subjects, E27 and E74, were heterozygous for m1, m2, and m4. m1 was here linked with m2 in one allele (*CYP1B1*2*) and m4 was present alone on the other (*CYP1B1*4*), and the subjects were thus of a *CYP1B1*2/*4* haplotype. Three additional subjects were heterozygous for both m3 and m4, and haplotype analysis of m3 in relation to m4 indicated that these two

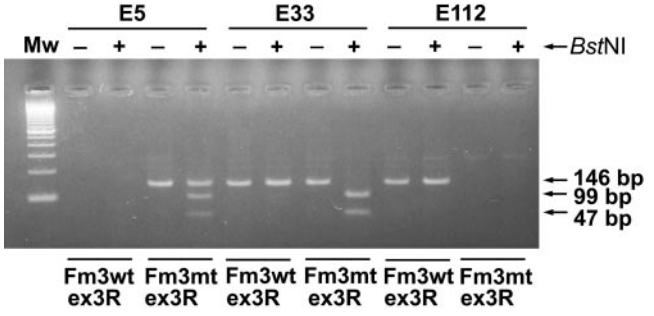


**Fig. 3.** PCR-restriction fragment length polymorphism-based analysis of linkage between m3 and m5. The two alleles were separated with respect to the presence of m3 using genomic DNA from three representative subjects. Subject E5 was homozygous mutated for m3 and heterozygous for m5, E33 was heterozygous for both m3 and m5. E112, wt for both m3 and m5, was used as control. The two forward primers, Fm3wt or Fm3 mt, and a common reverse primer, ex3R (indicated under each lane), were used to separate the two alleles with respect to m3 genotype resulting in a 146-bp PCR fragment. This fragment was subsequently subjected to digestion with *Bst*NI as the 4360C→G mutation (m5) introduces a *Bst*NI restriction site. Complete digestion is only seen in PCR fragments obtained with the m3 mt primer (Fm3 mt), indicating that m5 is genetically linked with m3. DNA fragment sizes are indicated with arrows on the right side. A 100-bp DNA Ladder (Invitrogen) was used as a marker.

SNPs were not linked. The subjects were thus assigned as *CYP1B1*3/*4* (see Fig. 2). Among all the 150 subjects genotyped, six subjects were heterozygous for m4 and this mutation was not linked to any other SNP. In all subjects except two, m1 was linked with m2. In the two subjects having m1 but not m2, linkage disequilibrium was instead found on the same allele with m3, yielding a new allele, *CYP1B1*5*. The other allele in these two subjects carrying *CYP1B1*5* was in one case *CYP1B1*2* and in the other *CYP1B1*3*.

The genotyping revealed that 21 subjects were heterozygous for m5. Of those, nine were heterozygous for m1 and m2 and homozygous for m3 (group A), seven were homozygous for m1 and m2 and heterozygous for m3 (group B), and three were heterozygous for m1, m2, and m3 (group C). Amplification of exon 3 followed by allele-specific amplification with m3 primers and *Bst*NI-RFLP for assessment of m5 was used to confirm the linkage between m3 and m5 as detailed under *Materials and Methods*. The PCR products amplified with the m3 mutant specific primer, Fm3 mt, were completely digested, whereas PCR products amplified with the wt-specific primer Fm3wt were not (Fig. 3). This confirmed that the m5 SNP is in complete linkage disequilibrium with m3. Analysis of linkage between m5 and m1 plus m2 in the 21 subjects carrying m5 was carried out by long PCR amplification using m1 wt or m1 mutant primers and the 1B1 reverse primer as detailed under *Materials and Methods*, followed by genotyping of the long PCR products with m5 specific primers. This revealed that m5 is also always linked to both m1 and m2. Thus, the linkage of m1, m2, and m3 with m5 allows the identification of the new allele *CYP1B1*7* with m1, m2, m3, and m5. The subjects in groups A, B, and C were therefore assigned the genotypes *CYP1B1*3/*7*, *CYP1B1*2/*7,* and *CYP1B1*1/7,* respectively. Furthermore, in the group of 21 subjects heterozygous for m5, one subject (E65), who was homozygous for m1, m2, and m3 and heterozygous for m5 was thus assigned as *CYP1B1*6/*7*. Subject ES11 was the only subject heterozygous for all five SNPs, m1, m2, m3, m4, and m5. Haplotype analysis showed that m1, m2, m3, and m5 were linked to each other on one allele and m4 was present on the other. The subject was therefore haplotyped as *CYP1B1*4/*7*.

To verify the efficiency of the haplotyping method, a *CYP1B1* fragment containing the whole coding region and intron 2 was amplified using non allele-specific long PCR from seven subjects previously assigned as having *1/*1, *2/*2, *2/*3, *3/*3, *6/*6, *3/*5, *4/*7. The PCR products were cloned into pBluescript. One colony from each group was picked up and allowed to grow. Plasmid DNA was purified and the entire coding region was sequenced. The plasmid inserts were found to contain *1, *2, *3, *3,*6, *5, and *7, respectively. The sequencing results agreed entirely with the haplotype assigned using the allele-specific long PCR method.

In summary, the results revealed the presence of seven different *CYP1B1* haplotypes as summarized in Table 3 where the polymorphisms were in combinations m1+m2 (*2), m3 alone (*3), m4 alone (*4), m1+m3 (*5), m1+m2+m3 (*6), and m1+m2+m3+m5 (*7). The three last three variants are newly identified in this study and named according to the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/) and incorporated into the Web page.

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TABLE 3

Description of *CYP1B1* alleles found in Ethiopian population ($n$ = 150) and their allele frequency determined by allele-specific PCR and restriction fragment length polymorphism

| Allele | Protein | Nucleotide Changes | Amino Acid Substitution(s) | Trivial Name | Allele frequency |
|---|---|---|---|---|---|
| | | | | | % |
| *CYP1B1*1* | CYP1B1.1 | None | | wt | 8.3 |
| *CYP1B1*2* | CYP1B1.2 | 142C>G, 355G>T | Arg48Gly, Ala119Ser | m1 + m2 | 36.7 |
| *CYP1B1*3* | CYP1B1.3 | 4326C>G | Leu432Val | m3 | 39.0 |
| *CYP1B1*4* | CYP1B1.4 | 4390A>G | Asn453Ser | m4 | 2.0 |
| *CYP1B1*5* | CYP1B1.5 | 142C>G, 4326C>G | Arg48Gly, Leu432Val | m1 + m3 | 0.7 |
| *CYP1B1*6* | CYP1B1.6 | 142C>G, 355G>T, 4326C>G | Arg48Gly, Ala119Ser, Leu432Val | m1 + m2 + m3 | 6.3 |
| *CYP1B1*7* | CYP1B1.7 | 142C>G, 355G>T, 4326C>G; 4360C>G | Arg48Gly, Ala119Ser, Leu432Val, Ala443Gly | m1 + m2 + m3 + m5 | 7.0 |

**CYP1B1 Haplotypes in Ethiopians.** With five missense mutations in the *CYP1B1* gene, $2^5 = 32$ possible different *CYP1B1* haplotypes could theoretically be present, but only seven were found. These (*CYP1B1*1, *2, *3, *4, *5, *6*, and **7*) were present at allele frequencies of 8, 37, 39, 2, 0.7, 6, and 7%, respectively, in the Ethiopian population examined (Table 3). Eighteen different combinations of these haplotypes were present among the subjects examined, of which the *CYP1B1*2/*3* combination was found to be the most frequent one (Table 4). One subject was found to be homozygous for *CYP1B1*6,* whereas no subject was homozygous for *CYP1B1*4, CYP1B1*5,* or *CYP1B1*7.* In fact, only one subject was homozygous for the *CYP1B1*1* allele. The frequency distribution of the observed haplotype combination did not significantly deviate from the ones expected by the Hardy-Weinberg law using the $\chi^2$ test by performing a 2 × 2 cross table for each genotype group against the rest using the computer program Statistica (version 5.5; StatSoft, Tulsa, OK). Fisher's exact test was used for genotype groups where number of expected frequencies was less than 5.

**Evaluation of Catalytic Properties of the Variant Forms of CYP1B1.** The human CYP1B1 cDNAs corresponding to the different alleles were cloned into the pYeDP60 expression vector and expressed in *Saccharomyces cerevisiae* strain INVSc1-HR. This strain is genetically modified to over express the human P450 reductase gene (see above). The kinetic properties of the six most common variants CYP1B1.1, CYP1B1.2, CYP1B1.3, CYP1B1.4, CYP1B1.6, and CYP1B1.7, as well as constructs with the m3+m4 and m3+m5 in combination, were analyzed using 17$\beta$-estradiol as a substrate and microsomes from yeast transfected with the respective cDNA variant. All CYP1B1 variants expressed well in yeast and folded properly to give a typical P450 spectrum in the CO-reduced form with a peak at 450 nm. The expression levels were similar, although not identical for all forms (Table 5). These results indicate that neither of the missense mutations alone or in combination influences the gross structure of the enzyme or the folding efficiency.

Incubations with estradiol revealed that CYP1B1.2, CYP1B1.3, and CYP1B1.4 exhibited similar kinetic properties as CYP1B1.1. In contrast, both CYP1B1.6 and CYP1B1.7 had significantly reduced $V_{max}$ and increased apparent $K_m$ for both the 2- and 4-hydroxylation of estradiol (Table 5). The magnitude of $K_m$ and $V_{max}$ alterations was 2- to 5-fold, which were statistically significant in all cases, except for the $V_{max}$ of the 2-hydroxylation. Accordingly, the intrinsic clearance ($V_{max}/K_m$) was decreased in CYP1B1.6 and CYP1B1.7 to only about 20% of the corresponding value exhibited by CYP1B1.1 ($p < 0.001$). No significant alterations in the kinetic properties were observed in the two constructs in which the

TABLE 4

Frequency of *CYP1B1* genotypes in Ethiopian population listed in decreasing order of frequency

Eighteen different combinations of *CYP1B1* alleles were found among 150 Ethiopians. The observed genotype distribution pattern was consistent with the expected frequencies according to Hardy-Weinberg equilibrium.

| Genotype | Trivial Name | No. of subjects | |
|---|---|---|---|
| | | Observed | Predicted (Hardy-Weinberg) |
| *CYP1B1*2/*3* | m1 + m2/m3 | 44 (29.3%) | 43 |
| *CYP1B1*3/*3* | m3/m3 | 21 (14.0%) | 23 |
| *CYP1B1*2/*2* | m1 + m2/m1 + m2 | 19 (12.7%) | 20 |
| *CYP1B1*1/*2* | wt/m1 + m2 | 10 (6.7%) | 9.0 |
| *CYP1B1*1/*3* | wt/m3 | 10 (6.7%) | 10 |
| *CYP1B1*3/*7* | m3/m1 + m2 + m3 + m5 | 9 (6.0%) | 8.0 |
| *CYP1B1*2/*6* | m1 + m2/m1 + m2 + m3 | 8 (5.3%) | 7.0 |
| *CYP1B1*3/*6* | m3/m1 + m2 + m3 | 8 (5.3%) | 7.4 |
| *CYP1B1*2/*7* | m1 + m2/m1 + m2 + m3 + m5 | 7 (4.7%) | 7.7 |
| *CYP1B1*1/*7* | wt/m1 + m2 + m3 + m5 | 3 (2.0%) | 1.7 |
| *CYP1B1*3/*4* | m3/m4 | 3 (2.0%) | 2.3 |
| *CYP1B1*2/*4* | m1 + m2/m4 | 2 (1.3%) | 2.2 |
| *CYP1B1*3/*5* | m3/m1 + m3 | 1 (0.7%) | 0.7 |
| *CYP1B1*6/*7* | m1 + m2 + m3/m1 + m2 + m3 + m5 | 1 (0.7%) | 1.3 |
| *CYP1B1*6/*6* | m1 + m2 + m3/m1 + m2 + m3 | 1 (0.7%) | 0.6 |
| *CYP1B1*4/*7* | m4/m1 + m2 + m3 + m5 | 1 (0.7%) | 0.4 |
| *CYP1B1*2/*5* | m1 + m2/m1 + m3 | 1 (0.7%) | 0.6 |
| *CYP1B1*1/*1* | wt/wt | 1 (0.7%) | 1.0 |

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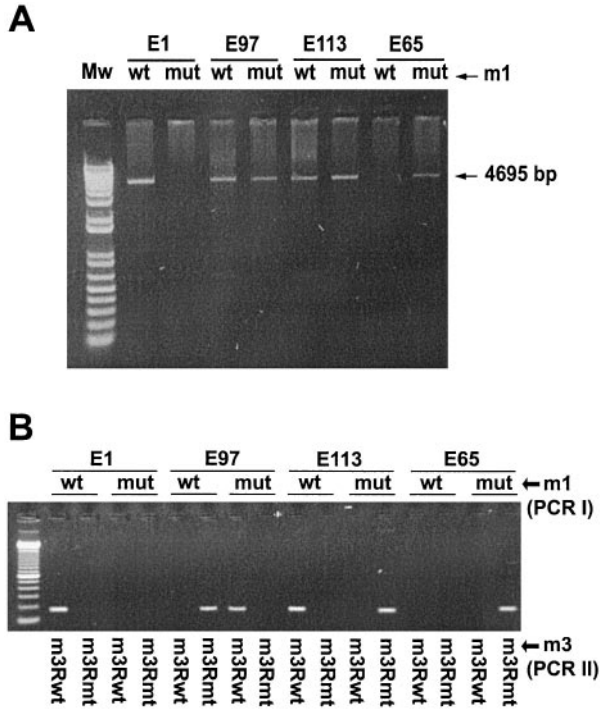


**Fig. 4.** Linkage analysis of m1 and m3. A, alleles were separated with respect to the presence of m1 by allele-specific long PCR with two forward primers, m1Fwt or m1Fmt, and a common reverse primer, 1B13R. The 1-kilobase-plus DNA ladder (Invitrogen) was used as a marker. Subjects E1, homozygous for m1wt, and E65, homozygous for m1 and m3 mutated, were used as control. E97 and E113 were heterozygous for both m1 and m3. B, analysis of PCR fragments from A, for the presence of m3 (PCR 2). Fragments from PCR 1 (A) were subjected to PCR amplification using a common exon 3 forward primer (ex3F) and reverse m3Rwt/m3Rmt primers (indicated under each lane). As expected, E1 was only amplified with the m3Rwt primer and E65 with the m3Rmt primers. E97, positive for both m1wt and m1 mt PCR fragments in PCR 1, amplified in the second PCR with both m3Rmt and m3Rwt primers, respectively, indicating the absence of linkage between m1 and m3. Individual E113, heterozygous for m1, fragment obtained with m1wt in PCR 1 was amplified with the m3Rwt in PCR 2 and the fragment obtained with m1 mt primer in PCR 1 was positive with m3Rmt in PCR 2, indicating the presence of m1 and m3 on the same allele and the other allele being wt for both SNPs.

Leu432Val (m3) amino acid change had been combined with either Asn453Ser (m4) or Ala443Gly (m5) (Table 5). In conclusion, these results emphasize the necessity to evaluate the kinetic properties of the different complete haplotypes rather than to study the influence of each specific SNP.

## Discussion

Based on the assumption that allele-specific PCR analysis for the known SNPs in the human *CYP1B1* gene might not completely reveal the true haplotypes, we sequenced the complete open reading frame of the *CYP1B1* genes from five different subjects and identified three novel SNPs, one causing an amino acid change. Methods for haplotype analysis of the four previously known missense mutations and for the newly identified missense mutation (m5) were developed and genomic DNA from 150 unrelated healthy Ethiopians were analyzed, revealing the presence of seven different *CYP1B1* haplotypes in the population. In total, we detected 18 different combinations of haplotypes in the population (Table 4), illustrating the complexity by which the various SNPs are distributed and the manner by which they would influence the phenotype with respect to CYP1B1 enzyme function. Only two of the haplotypes, however, having a combination of three or four different amino acid substitutions, respectively, exhibited altered kinetic properties for estradiol metabolism compared with the wild-type enzyme. The amino acid changes in common of the enzyme variants with altered properties were Arg48Gly, Ala119Ser, and Leu432Val.

Previous work by Stoilov and collaborators has identified 17 different inactivating mutations in the human *CYP1B1* gene, cosegregating with primary congenital glaucoma (Stoilov et al., 1998). In addition, six SNPs in the *CYP1B1* gene that are not associated with the disease phenotype have been described previously (Stoilov et al., 1998; Bejjani et al., 2000). Thus, together with the three novel SNPs identified in this study, there are at present at least nine polymorphic sites in the *CYP1B1* gene, of which five cause amino acid substitutions.

The frequency of m1, m2, and m3 among Ethiopians, the first African population to be studied with respect to the *CYP1B1* genotype, was higher than that described for whites (Stoilov et al., 1997) and Japanese (Inoue et al., 2000). The frequency of m3 in the Ethiopian population (54%) was somewhat lower than among African Americans (75%) but higher than among whites (43%) and Chinese (17%) (Tang et al.,

TABLE 5

Kinetic analysis of the 4- and 2-hydroxylation of 17$\beta$-estradiol by human CYP1B1 variants expressed in *S. cerevisiae*

Kinetic parameters are presented as means ± S.D. from three independent analyses using two separate microsomal preparations for each enzyme variant.

| CYP1B1 variant | Amino acid substitutions | Expression level | $K_m$ | | $V_{max}$ | | $V_{max}/K_m$ ratio | |
|---|---|---|---|---|---|---|---|---|
| | | | 4-Hydroxy | 2-Hydroxy | 4-Hydroxy | 2-Hydroxy | 4-Hydroxy | 2-Hydroxy |
| | | *pmol/mg* | *μM* | | *pmol/pmol P450/min* | | | |
| CYP1B1.1 | None | 14.6 | 5.3 ± 0.3 | 8.9 ± 1.1 | 3.8 ± 0.1 | 0.5 ± 0.10 | 0.71 | 0.05 |
| CYP1B1.2 | Arg48Gly, Ala119Ser | 3.6 | 6.4 ± 1.9 | 7.7 ± 0.1 | 4.7 ± 0.2 | 0.5 ± 0.10 | 0.73 | 0.06 |
| CYP1B1.3 | Leu432Val; | 7.1 | 6.4 ± 0.2 | 8.7 ± 2.4 | 3.3 ± 0.2 | 0.65 ± 0.10 | 0.51 | 0.07 |
| CYP1B1.4 | Asn453Ser | 5.2 | 7.0 ± 1.0 | 9.4 ± 1.8 | 3.6 ± 0.6 | 0.55 ± 0.06 | 0.51 | 0.06 |
| CYP1B1.6 | Arg48Gly, Ala119Ser, Leu432Val | 16.1 | 12.1 ± 1.0[b] | 16.6 ± 0.1[a] | 2.1 ± 0.1[b] | 0.3 ± 0.05 | 0.17[c] | 0.01[a] |
| CYP1B1.7 | Arg48Gly, Ala119Ser, Leu432Val, Ala443Gly | 7.4 | 13.4 ± 1.3[b] | 39.1 ± 2.4[b] | 1.7 ± 0.2[b] | 0.7 ± 0.08 | 0.12[c] | 0.01[b] |
| L432V + N453S | Leu432Val, Asn453Ser | 11.5 | 7.9 ± 0.6 | 7.5 ± 0.5 | 2.6 ± 0.1 | 0.3 ± 0.05 | 0.32 | 0.04 |
| L432V + A443G | Leu432Val, Ala443Gly | 10.0 | 8.9 ± 0.6 | 9.7 ± 1.7 | 3.1 ± 0.1 | 0.5 ± 0.10 | 0.34 | 0.05 |

[a] Significant difference ($p < 0.05$) compared with CYP1B1.1 using unpaired $t$ test.
[b] Significant difference ($p < 0.01$) compared with CYP1B1.1 using unpaired $t$ test.
[c] Significant difference ($p < 0.001$) compared with CYP1B1.1 using unpaired $t$ test.

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2000). The m4 polymorphism, was rare in the Ethiopian population examined and has previously been shown to be more frequent in whites compared with African-Americans (Bailey et al., 1998), and not at all detected in Japanese population (Inoue et al., 2000). The variant having m4 alone (Asn453Ser) has been shown to be present in MCF-7 cells (Spink et al., 2000). These findings indicate the existence of pronounced interethnic variations in the distribution of different *CYP1B1* alleles.

We considered it important to study the functional properties of the haplotypes of CYP1B1 found in the population. For this purpose, we employed the heterologous yeast system with a strain over expressing human cytochrome P450 reductase. All CYP1B1 enzyme variants displayed typical P450 spectra indicating no influence on major structural aspects of the enzyme. The kinetics was, however, significantly different in enzymes having a combination of m1, m2, and m3 (i.e., for CYP1B1.6 and CYP1B1.7) in which the $V_{max}$ was lower and $K_m$ was higher for estradiol hydroxylation (Table 5). The influence of the new missense mutation m5 (Ala443Gly) identified in the present study was small, but the affinity for estradiol was further reduced in CYP1B1.7 that had this SNP compared with CYP1B1.6. The Ala443 amino acid is conserved both in the mouse, rat and human CYP1B1 sequences. Because glycine residues are often involved in protein bending, it is reasonable to speculate that this amino acid substitution might be of some structural importance.

These results indicate that the true haplotype is important in determining the overall function of the specific form of CYP1B1, indicating that combinations of SNPs would influence the structure of the active site after protein folding. Such an example has previously been registered (e.g., with the CYP2D6.17 variant having the amino acid substitutions Thr107Ile, Arg296Cys, and Ser486Thr). Incorporation of these polymorphisms separately into wt CYP2D6 cDNA did not influence the catalytic function when the enzyme was expressed in yeast and in mammalian COS-1 cells. However, constructs having both the Thr107Ile and Arg296Cys substitutions displayed a reduced affinity for the CYP2D6 substrates bufuralol and codeine compared with the wt enzyme (Oscarson et al., 1997). These findings are in agreement with phenotype analysis in vivo, where the activity of CYP2D6.17 has been found to be severely impaired when debrisoquine was given as a probe drug (Masimirembwa and Hasler, 1997). It can be speculated that the folding of the enzyme is affected by the combination of substitutions resulting in enzyme products with a different active site conformation. A similar mechanism for the specific effects seen for CYP1B1.6 and CYP1B1.7 can therefore be hypothesized. Thus, altered active site structure is only achieved when the substitutions Ala48Gly, Ala119Ser, and Leu432Val exist together as in CYP1B1.6 and CYP1B1.7 (see Table 4).

It should be noted that the folding is partially dependent on the expression system and that a similar relationship might not be evident when the constructs are expressed in, for example, bacteria. Thus, variable $K_m$ values for estradiol metabolism catalyzed by different CYP1B1 variants has been achieved in different expression systems, but variations have also been registered between laboratories (Shimada et al., 1999; Hanna et al., 2000; Li et al., 2000; McLellan et al., 2000; Spink et al., 2000). Although all these discrepancies cannot easily be explained, it is considered important to have similar conditions and expression systems when comparing allelic variants with different missense mutations. Moreover, a similar haplotype activity relationship as observed in the yeast expression system may not hold in mammalian cells or in humans in vivo. For instance, higher levels of testosterone hydroxylation by CYP1B1 are observed when expressed in human lymphoblastoid cells (Crespi et al., 1997) compared with *S. cerevisiae* (Shimada et al., 1997). Furthermore, as seen with CYP2D6.17 (Oscarson et al., 1997), the haplotypes would be expected to influence the efficiency of metabolism in a substrate-specific manner. Use of in vitro metabolic conditions to assess the activity profile of the various CYP1B1 variants is justified because, unlike several CYP enzymes, such as CYP2D6 and CYP2C19, currently there is no CYP1B1-specific probe drug available for use in vivo. Further studies, if possible employing CYP1B1-specific probes, can perhaps give an answer to the in vivo relevance of these alleles. In addition, it is of importance to study the effect of the CYP1B1.6 and CYP1B1.7 on the metabolism of other CYP1B1 substrates such as benzo[*a*]pyrene and 7,12 dimetylbenza[*a*]anthracine, known procarcinogens and substrates of CYP1B1 in vitro.

CYP1B1 is known to metabolize a variety of putative human carcinogens as well as estrogens In addition, CYP1B1 shows elevated expression in a wide range of human tumors including mammary and ovarian tumors. Thus, CYP1B1 might have a potential role in tumor development and progression. Polymorphisms in this enzyme may thus influence the metabolism of environmental estrogens and hormone-like substances and affect the interindividual risk of developing cancer. Thus, it would be of potential importance to investigate for any association of the *CYP1B1* haplotypes here identified yielding enzyme products with altered function and incidence of, for example, breast or ovarian cancer.

In conclusion, our study shows the extreme complexity by which five different SNPs in the human *CYP1B1* gene are distributed into a human population, resulting in seven different haplotypes and CYP1B1 enzyme variants, of which two exhibit altered kinetic properties for estradiol hydroxylation. Consequently, the *CYP1B1* genetic polymorphism may contribute to interindividual and interethnic variation in susceptibility toward the development of cancer upon exposure of procarcinogens or estrogen over a long period of time, but a true investigation regarding the genetic influence of the polymorphic CYP1B1 on the susceptibility of different forms of cancer requires determination of the *CYP1B1* haplotypes.

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**Address correspondence to:** Dr. Eleni Aklillu, Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, SE-171 77 Stockholm, Sweden.

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