Functional polymorphism in human CYP4F2 decreases 20-HETE production

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Stec DE, Roman RJ, Flasch A, Rieder MJ. Functional polymorphism in human Cyp4F2 decreases 20-HETE production. Physiol Genomics 30: 74–81, 2007. First published March 6, 2007; doi:10.1152/physiolgenomics.00003.2007.—20-Hydroxyeicosatetraenoic acid (20-HETE) plays an important role in the regulation of renal tubular and vascular function and a deficiency in the renal formation of 20-HETE has been linked to the development of hypertension. The cytochrome P450 4F2 (CYP4F2) gene encodes for the major CYP enzyme responsible for the synthesis of 20-HETE in the human kidney. We screened two human sampling panels (African and European Americans: n = 24 and 23 individuals, respectively) using PCR and DNA resequencing to identify informative SNPs in the coding region of the CYP4F2 gene. Two nonsynonymous SNPs that lead to amino acid changes at position 12 (W12G) and 433 (V433M), were identified. Both of these variants were found to be frequent in both African and European American sampling panels (9–21% minor allele frequency), and the W12G polymorphism exhibited extensive linkage disequilibrium with surrounding SNPs. To determine the functional significance of these mutations on the ability of the CYP4F2 enzyme to metabolize arachidonic acid and leukotriene B4 (LTB4), recombinant baculoviruses containing four different human CYP4F2 variants (i.e., W12/V433, W12/M433, G12/V433, G12/M433) were generated and the proteins were expressed in S9 insect cells. The presence of the M433 allele, W12/M433, or G12/M433 decreased 20-HETE production to 56–66% of control. In contrast these variants had no effect on the ω-hydroxylation of LTB4. These findings are the first to identify a functional variant in the human CYP4F2 gene that alters the production of 20-HETE.

METHODS

Sampling panel screening and mapping of nonsynonymous CYP4F2 polymorphisms. Directed PCR and DNA resequencing of the genomic region encompassing CYP4F2 (all intronic and exonic sequence including ~2 kb upstream and 1.1 kb downstream) was performed in an African American (n = 24 individuals) and European American (n = 23 individuals) sampling panels to identify single nucleotide polymorphisms (SNPs) as part of the SeattleSNPs Program for Genomic Applications using previously described samples and methods (3). The complete data set from this work is available at http://pga.gs.washington.edu/data/cyp4f2/ along with detailed information for all SNP data. Due to the close homology between CYP4F2 and CYP4F3, all intronic and exonic sequence was covered in the mapping of nonsynonymous polymorphisms in this study. This was done in order to identify functionally relevant variants in regions of high sequence similarity within the CYP4F2 gene.

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20-HYDROXYEICOSATETRAENOIC ACID (20-HETE) is a metabolite of arachidonic acid (AA) produced in the kidney by cytochrome P450 enzymes. 20-HETE has potent actions on renal tubular and vascular function including: vasoconstriction secondary to inhibition of large conductance Ca2+ channels in vascular smooth muscle cells and inhibition of Cl− transport in the thick ascending loop of Henle (6, 10, 32). 20-HETE has also been implicated as a second messenger mediating the inhibitory effects of dopamine, PTH, and angiotensin II on Na+K+-ATPase activity and sodium transport in the proximal tubule (20, 23). Although some studies have suggested that increased levels of 20-HETE in the renal vasculature may underlie the development of hypertension (29), most of the available evidence in rodent models suggests that a deficiency in the renal production of 20-HETE is associated with the development of several salt-sensitive forms of hypertension. For example, the renal production of 20-HETE is reduced in Dahl salt-sensitive (DS) rats and in mice with DOCA-salt hypertension (9, 16). Induction of the renal formation of 20-HETE lowers blood pressure in the DS rat, obesity-induced and angiotensin II-dependent hypertension and the stroke-prone spontaneously hypertensive rat (22, 24, 28, 30). Chronic blockade of the formation of 20-HETE has also been shown to induce the development of salt-sensitive hypertension in normally salt-resistant strains of rats (8, 26). While the role for alterations in renal 20-HETE in the development of salt-sensitive hypertension has been well established in rodent models, the role of renal 20-HETE in the development of hypertension in humans has yet to be definitively examined. The urinary excretion of 20-HETE has been reported to be reduced in salt-sensitive patients challenged with an infusion of saline or furosemide (13, 14). There are also recent studies linking a C-to-T mutation in the CYP4A11 gene that reduces the production of 20-HETE with elevated blood pressure in three large human populations (7, 18, 19).

In humans, there are several CYP ω-hydroxylases that have the potential to convert AA to 20-HETE. CYP 4F2, 4F3b, 4F11, 4F12, and 4A11 isoforms are all expressed in the human kidney, and each has the capability of converting AA to 20-HETE. However, immunoprecipitation studies performed with human kidney microsomes suggest that the 4F2 isoform accounts for up to 70% of the 20-HETE production in human renal microsomes (15). The CYP 4F2 isoform is also expressed in the liver, lung, and white blood cells and is the main enzyme responsible for the ω-hydroxylation of leukotriene B4 (LTB4) although several other CYP 4F isoforms can ω-hydroxylate LTB4 as well. This metabolite undergoes further metabolism to 20-carboxy-LTB4, which can undergo B-oxidation from its ω-side and along with traditional β-oxidation from the C-1 carbon, which leads to the inactivation of this proinflammatory agent (11). The purpose of the present study was determine whether variants exist in the coding region of the human CYP 4F2 gene and to determine the effect of these variants on the metabolism of AA and LTB4.

Not all SNPs identified were successfully amplified using PCR. Two nonsynonymous SNPs that lead to amino acid changes at position 12 (W12G) and 433 (V433M) were identified. Both of these variants were found to be frequent in both African and European American sampling panels (9–21% minor allele frequency), and the W12G polymorphism exhibited extensive linkage disequilibrium with surrounding SNPs. To determine the functional significance of these mutations on the ability of the CYP4F2 enzyme to metabolize arachidonic acid and leukotriene B4 (LTB4), recombinant baculoviruses containing four different human CYP4F2 variants (i.e., W12/V433, W12/M433, G12/V433, G12/M433) were generated and the proteins were expressed in S9 insect cells. The presence of the M433 allele, W12/M433, or G12/M433 decreased 20-HETE production to 56–66% of control. In contrast these variants had no effect on the ω-hydroxylation of LTB4. These findings are the first to identify a functional variant in the human CYP4F2 gene that alters the production of 20-HETE.

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FUNCTIONAL HUMAN 4F2 VARIANTS

and CYP4F3 (92% identity overall), extensive validation of all PCR amplification primers and SNP data was performed to assure specificity of this locus. From these data, two nonsynonymous coding SNPs at reference sequence position 2631 (cDNA position 84-T/G: dbSNP rs3093105 and 20597 (cDNA position 1347-G/A: rs2108622) relative in the genomic sequence of CYP4F2 (GenBank accession no. AF467894 and mRNA RefSeq accession no. NM_001082) (Table 1) were selected for further analysis. These SNPs lead to amino acid changes at position 12 (W12G) and position 433 (V433M), respectively, in the CYP4F2 protein sequence (relative to GenBank accession no. AAL67578). Both of these variants were found to be common in both African and European American sampling panels. Polymorphism allele frequencies were 21 and 11% for the rs3093105-T minor allele and 9 and 17% for the rs2108622-A minor allele in the African American and European American panels, respectively. Both polymorphisms were in Hardy-Weinberg equilibrium in the African American and European American panels. All SNPs identified by PCR were confirmed by sequencing as above. Recombinant baculoviruses containing the individual variants were ligated into the pCR4Zero Topo vector (Invitrogen) and sequenced as above to confirm identity. The full-length 4F2 cDNA, was then excised, gel purified, and ligated to the vector pCRAZero using a topo-isomerase based ligation kit (Invitrogen). The ligation reactions were then transformed into bacteria and plated on agar plates containing ampicillin. Plates were grown overnight at 37°C. The next day, 30 individual colonies were selected and grown in 5 ml of Luria broth supplemented with ampicillin. Plasmid DNA from all of the selected bacterial colonies (pCRAZero Topo) was used to verify the CYP4F2 cDNA across the full length of the insert. DNA sequencing was performed using flanking and internal oligos as priming sites for fluorescence based sequencing (BigDye Terminator; Applied Biosystems, Foster City, CA) under standard conditions and run on an ABI 3700 DNA analyzer. Four clones having the full-length cDNA sequence and each combination of the two nonsynonymous alleles at cDNA positions 84 and 1347 were identified [i.e., 84-T and 1347-G (t6), 84-G and 1347-G (t16), 84-T and 1347-A (t18), 84-G and 1347-A (t24)]. All other alleles within these sequences were identical, coded for synonymous amino acids, or were present in the untranslated regions.

Cloning of the human 4F2 variants. The human 4F2 variants were cloned from a mixed pool of human full-length cDNAs derived from RNA isolated from 21 different organs including the kidney and liver (Panomics, Redwood City, CA). PCR was performed using Phusion polymerase and 1 μL of the human cDNA library using the following primers: 4F2++, 5’-AGCAGACAGAGAGGAGA-TTCTAGAGATGATTTAATGT. The PCR products were separated on 1% agarose gels and viewed under UV light after ethidium bromide staining. A band at ~2,400 bp, corresponding to the full-length 4F2 cDNA, was then excised, gel purified, and ligated to the vector pCRAZero using a topo-isomerase based ligation kit (Invitrogen). The ligation reactions were then transformed into bacteria and plated on agar plates containing ampicillin. Plates were grown overnight at 37°C. The next day, 30 individual colonies were selected and grown in 5 ml of Luria broth supplemented with ampicillin. Plasmid DNA from all of the selected bacterial colonies (pCRAZero Topo) was used to verify the CYP4F2 cDNA across the full length of the insert. DNA sequencing was performed using flanking and internal oligos as priming sites for fluorescence based sequencing (BigDye Terminator; Applied Biosystems, Foster City, CA) under standard conditions and run on an ABI 3700 DNA analyzer. Four clones having the full-length cDNA sequence and each combination of the two nonsynonymous alleles at cDNA positions 84 and 1347 were identified [i.e., 84-T and 1347-G (t6), 84-G and 1347-G (t16), 84-T and 1347-A (t18), 84-G and 1347-A (t24)]. All other alleles within these sequences were identical, coded for synonymous amino acids, or were present in the untranslated regions.

Generation of baculovirus. Full-length 4F2 cDNA variants were reamplified with Phusion platinum polymerase with BamHI and XhoI sites on the forward and reverse primers, respectively. PCR products of the individual variants were ligated into the pCRAZero Topo vector (Invitrogen) and sequenced as above to confirm identity. The full-length 4F2 cDNAs were then cloned into the BamHI/XhoI sites of the baculovirus expression vector pAcUw51-CYPOR (kindly provided by Drs. Cosette Serabjit-Singh and Darryl C. Zeldin). The vector pAcUw51-CYPOR contains two independent promoters that drive the expression of P450 reductase as well as the Cyp4F2 variant (12). The identity of each of the variants in the pAcUw51-CYPOR vector was also confirmed by sequencing as above. Recombinant baculoviruses were obtained by cotransfection with BaculoGold DNA (0.5 μg BaculoGold DNA: 5 μg recombinant pAcUw51-CYPOR vector con

Table 1. Description of functional nonsynonymous polymorphisms in CYP4F2 and correlated tagSNPs

<table>
<thead>
<tr>
<th>dbSNP Reference ID</th>
<th>Genomic Position (chr. 19)*</th>
<th>Reference Sequence Position†</th>
<th>cDNA Position‡</th>
<th>DNA Sequence Context</th>
<th>African American MAF§</th>
<th>African American tagSNPs¶</th>
<th>European American MAF∥</th>
<th>European American tagSNPs∥</th>
<th>Amino Acid Sequence Position†</th>
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<td>2631</td>
<td>84</td>
<td>GCTGGGCTTC[T/G]GGCCAGTGGC</td>
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<td>rs3093112, rs3093114, rs2074902, rs3093092, rs3093098, rs3093173, rs2006193, rs2365178, rs30393100, rs30393115, rs2365178, rs30393092, rs3093100, rs30393096, rs3093106, rs3093106, rs3093101, rs3093102, rs30393112, rs30393112, rs30393114, rs3093115, rs3093116, rs3093120, rs3093120, rs3093121, rs3093121, rs3093122, rs3093122, rs3093123, rs3093123, rs3093124, rs3093124, rs3093125, rs3093125</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>rs2108622</td>
<td>15851431</td>
<td>20597</td>
<td>1347</td>
<td>CAACCCAGCT[G/A]TGTGGCCGG</td>
<td>0.09</td>
<td>NA</td>
<td>0.17</td>
<td>NA</td>
<td>V433M</td>
</tr>
</tbody>
</table>

*Build hg_16, May 2004, NCBI build 35. †All positions are relative to GenBank accession numbers AF467894 (Reference), NM_001082 (cDNA), and AAL67578 (protein). §MAF-minor allele frequency, $r^2$ = 1.0 (perfect correlation).
were grown in media supplemented with hemin (conjugated to bovine
infection (MOI) of 5–10 for isolation of recombinant microsomes.
picked, amplified, and used to infect Sf9 cells at a multiplicity of
baculovirus agarose as previously described (4). Clear plaques were
and incubated with Sf9 cells (1×10^6 cells) covered in 1.25% baculovirus agarose as previously described (4). Clear plaques were
and incubated with Sf9 cells at a multiplicity of
MOI of 5–10 for isolation of recombinant microsomes.

Preparation of recombinant cell microsomes. Sf9 cells were in-
fected with recombinant baculoviruses at MOI of 5–10. The Sf9 cells were grown in media supplemented with hemin (conjugated to bovine serum albumin) at a final concentration of 4 µM at the time of infection to ensure the production of spectrally active P450 enzymes. The cells were then incubated at 27°C for 3 days following infection. After this time, cells were washed in PBS and resuspended in sucrose buffer (50 mM potassium phosphate, pH 7.4, and 0.5 M sucrose). The cells were lysed by brief sonication and then centrifuged at 10,000 g at 4°C. The supernatant was then subjected to high-speed centrifugation at 100,000 g for 1 h at 4°C. The microsomal pellet was then resuspended in a 100 mM KPO4 buffer (pH 7.25) containing 1 mM EDTA, 1 mM dithiothreitol, and 30% glycerol by brief sonication and stored at −80°C. The protein concentration was measured using a Bradford-based Bio-Rad protein assay with BSA as a standard.

Immunoblots. Western blots for Cyp4F2 protein were performed on the microsomes prepared from Sf9 cells as described above. A standard curve of human CYP 4F2 super-somes (Gentest, Woburn, MA) ranging from 5 to 500 nmol was also run on each gel. Samples of 10 µg of microsomal protein were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 5 min, electrophoresed on 7.5% SDS-polyacrylamide gels, and blotted onto nitrocellulose membrane. Mem-

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Measurement of AA metabolism. The CYP-dependent metabolism of AA was determined by incubating 250 µg of microsomal protein prepared from the insect cells infected with baculovirus containing specific 4F2 variants in a 0.5-ml volume with a saturating concentra-
tion of [14C]AA (1 µCi, 42 µM) in an NADPH-regenerating system as previously described (28). The reactions were terminated by the acidification with formic acid, extracted twice with ethyl acetate, and dried under N2 gas. The metabolites were then resuspended in 500 µl of 100% ethanol and separated by HPLC, and the metabolites formed were monitored by a radioactive flow detector as previously described (17). The production of each metabolite was calculated and expressed as picomoles product formed per minute per milligram of protein. The production rate of each metabolite was then normalized to the amount of the CYP 4F2 protein in the individual microsomal preparations as determined by Western blot and presented as picomoles metabolite formed per minute per nanomole CYP 4F2 protein.

The CYP-dependent metabolism of LTB4 was also determined by incubating microsomes prepared from insect cells infected with baculovirus containing a specific 4F2 variant with 30 µM LTB4 (Cayman Chemicals) in a NADPH-regenerating system as previously de-
scribed (2). The reactions were terminated with four volumes of ethanol, and the formed metabolites were separated by HPLC and monitored by an on-line UV detector at 278 nm. The production of each metabolite was calculated and expressed as picomoles metabolite formed per minute per milligram of protein. The production rate of each metabolite was then normalized to the amount of 4F2 protein in the microsomal preparation as determined by Western blot and pre-

RESULTS

Functional SNP descriptions and linkage disequilibrium relationships. For each of the two nonsynonymous coding SNPs tested in this study, we determined the set of perfectly correlated SNPs discovered through directed resequencing in two diverse human sampling panels. For the W12G (rs3093104) polymorphism, 15 SNPs and 27 SNPs were found to be perfect surrogate markers (tagSNPs) in the African American and European American panels, respectively (Table 1). This set of tagSNPs extended ~1.2 kb upstream and ~13.2 kb downstream from W12G (rs3093104) in the European Americans and ~80 bp upstream and ~4 kb downstream in the African Americans. The V433M (rs2108622) polymorphism did not show perfect linkage disequilibrium with any SNP in our dataset; however, lower levels of r2 (r2 = 0.75) did result in a set of correlated surrogate SNPs present in the European American (rs3093206, rs3093209, rs3093193, rs3093199, rs3093207, rs3093211, rs3093216), but not in the African-American panel.

Expression of 4F2 variants in Sf9 insect cells. The results of the Western blot experiments are presented in Fig. 1. The four different variants of the CYP4F2 gene W12/V433 (wild-type [WT]), G12/V433, W12/M433, and G12/M433 all expressed the 4F2 protein as detected by Western blot using an anti-human 4F2 polyclonal antibody. All of the variants migrated at the predicated molecular mass of the intact 4F2 protein (60 kDa). The levels of P450 reductase in each of these preparations were well correlated with the level of CYP 4F2 protein expressed in each sample (Fig. 1).

20-HETE production by 4F2 variants. One major metabolite with a retention time of 15 min was observed upon incubation of the CYP4F2 variants with radiolabeled AA (Fig. 2). Previous studies have demonstrated that CYP 4F isoforms can generate 18- and 19-HETE in addition to 20-HETE (1). Since all of these metabolites migrate with similar HPLC retention times, additional experiments were performed to establish the identity of this metabolite. The metabolite comigrated with an
authentic $^{14}$C-20-HETE standard, and the spectrum of this metabolite as determined by LC/MS/MS was identical to that observed with a 20-HETE standard (Fig. 3). As can be seen in Fig. 3, the MS2 spectrum of the m/z 319 carboxylate anion formed by the WT variant revealed major peaks at m/z 301, 275, 273, 257, and 245, which was identical to that seen when the 319 carboxylate ion of 20-HETE was fragmented. 20-HETE production averaged $84 \pm 14$ pmol·min$^{-1}$·nmol$^{-1}$ P450, respectively when CYP4F2 variants containing the presence of the M433 allele, W12/M433, or G12/M433 were incubated with AA (Fig. 4).

LTB$_4$ metabolism by 4F2 variants. Incubation of all of the variants produced one major LTB$_4$ metabolite with a UV absorbance of 278 nm that migrated with a retention time of 6 min (Fig. 5). Since several hydroxy-LTB$_4$ metabolites, including 18- and 19-OH LTB$_4$, produced by CYP 4F isoforms can migrate with similar retention times (2), additional experiments were performed to verify the identity of this metabolite. This metabolite generated a peak at m/z of 351 that comigrated with an authentic 20-OH-LTB$_4$ standard on LC/MS (Fig. 6). As can be seen in Fig. 6, the MS/MS spectrum of m/z 351 carboxylate anion of the metabolite formed when the WT variant was incubated with LTB$_4$ revealed major peaks at m/z 333, 315, 289, 271, 261, and 195 and was identical to that generated by fragmentation of the carboxylate ion generated from an authentic LTB$_4$ standard. Substitutions at amino acids 12 (G12) and 433 (M433) had no effect on the rate of formation of 20-hydroxy-LTB$_4$, which averaged $18 \pm 5$, vs. $13 \pm 1$, vs. $16 \pm 4$, vs. $11 \pm 3$ pmol·min$^{-1}$·nmol$^{-1}$ P450 in W12/V433, G12/V433, W12/M433, and G12/M433 variants, respectively.

DISCUSSION

The present study identified a functional polymorphism in the coding region of the human CYP4F2 gene that decreases the production of 20-HETE. The M433 allele encodes for a methionine-to-valine substitution at amino acid position 433. This substitution decreased 20-HETE production by $50\%$, but it had no significant effect on the $\omega$-hydroxylation of LTB$_4$. The G12 allele that encodes for a glycine-to-tryptophan substitution at amino acid 12 did not have any significant effect on the $\omega$-hydroxylation of AA or LTB$_4$. Both of these variants were common in African and European American sampling.

![Fig. 1. Representative Western blots of 4F2 variants and P450 reductase from purified baculovirus microsomes. A: Western blot using a rabbit polyclonal anti-human Cyp4F2 antibody detected a 53-kDa protein that comigrated with a commercially available Cyp4F2 standard. Lanes 1–4: Cyp4F2 standard at 500, 100, 10, 5 nmol, respectively. Lanes 5–8: Cyp4F2 variants, W12/V433, G12/V433, W12/M433, G12/M433. B: Western blot of P450 reductase. Lanes 1–4: P450 reductase standard at 500, 100, 10, 5 nmol, respectively. Lanes 5–8: Cyp4F2 variants, W12/V433, G12/V433, W12/M433, G12/M433.](http://physiolgenomics.physiology.org/)

![Fig. 2. HPLC chromatograms of metabolites produced by incubation of Cyp4F2 variant microsomes with radiolabeled arachidonic acid. One major metabolite, 20-hydroxycis-etaetaenoic acid (20-HETE), with a retention time of 15 min was detected in all of the variants.](http://physiolgenomics.physiology.org/)
panels and exhibited allele frequencies ranging between 9 and 21%. Numerous perfectly correlated SNPs (tagSNPs) were identified that can serve as surrogate markers for the W12G polymorphism, but no perfectly correlated tagSNPs could be identified for the V433M polymorphism.

Several lines of evidence support the idea that the CYP4F2 isoform serves as the major 20-HETE producing enzyme in the human kidney. Lasker et al. (15) reported that antibodies directed against the CYP4F2 isoform produced a greater inhibition of the formation of 20-HETE in microsomes prepared from human kidneys than antibodies directed against the CYP4A11 isoform. They also demonstrate a greater sensitivity of 4F2-dependent 20-HETE production to substrate specific inhibitors that that seen using recombinant 4A11 protein (15).

Previous studies have also indicated that the renal production of 20-HETE plays a vital role in the regulation of blood pressure at least in rodent models. 20-HETE is an important regulator of renal electrolyte absorption in the kidney (6, 31), and alterations in the production of 20-HETE have been linked to the development of hypertension in several different animal models (22, 28, 30). Studies in DS rats have demonstrated linkage of reduced 20-HETE producing 4A isoforms in the kidney with hypertension (25). Furthermore, transfer of a segment of chromosome 5 containing fully functional renal 4A isoforms from normotensive Lewis rats, which restores renal 20-HETE production in the kidney, attenuates the development of hypertension in the resulting congenic strain of DS rats (21). This evidence provides a strong rationale for the hypothesis that a deficiency in the renal formation of 20-HETE may contribute to the development of salt-sensitive forms of hypertension.

In humans, decreased 20-HETE excretion has been demonstrated in salt-sensitive vs. salt-resistant hypertensive patients in response to acute volume expansion with saline and following furosemide administration (13, 14). These results suggest that alterations in renal 20-HETE production may contribute to salt-sensitive hypertension in humans as well. The mechanism underlying the decreased excretion of 20-HETE in salt-sensitive hypertensive patients is not known; however, our data demonstrating decreased 20-HETE production by the CYP4F2 M433 variant could possibly explain these observations if this variant is more prevalent in salt-sensitive hypertensive subjects. There is precedent for this in that a variant that exhibits reduced enzyme activity has been identified in the CYP4A11 isoform. This variant has been associated with hypertension in three large human population studies (7, 18, 19). It is possible that functional polymorphisms in the CYP4A11 and CYP4F2 genes can lead to additive or synergistic effects to reduce 20-HETE production in carriers of the low activity alleles of these genes.

LTB4 is a potent proinflammatory mediator synthesized by 5-lipoxygenase and leukotriene A4 hydrolase. LTB4 is increased by inflammatory mediators including endotoxin, complement fragments, tumor necrosis factor, and interleukins (5). Two cell surface G protein-coupled receptors for LTB4 (BLT1
and BLT2) have been isolated and extensively characterized in the last few years. The two receptors differ in their pattern of expression with BLT1 being expressed primarily in leukocytes, whereas BLT2 is expressed more ubiquitously (27). CYP4F2 has previously been reported to mediate the /H9275-H9275-hydroxylation to 20-OH LTB4 in microsomal protein preparations derived from human liver (11). 20-OH LTB4 then can undergo further conversion to the 20-carboxy-LTB4, which can be further broken down by β-oxidation on both the C-1 and w-termini of the molecule. We further demonstrated that the metabolism of LTB4 to the hydroxyl metabolite is not affected by any of the CYP4F2 variants studied.

Fig. 5. HPLC chromatograms of metabolites produced by incubation of Cyp4F2 variant microsomes with leukotriene B4 (LTB4). One major metabolite, 20-OH LTB4 with a retention time of 6 min, was detected in all of the variants.

Fig. 6. Confirmation of 20-OH LTB4 as metabolite produced by incubation of Cyp4F2 variant microsomes with LTB4. A: HPLC chromatogram demonstrating elution of 20-OH LTB4 standard with metabolite derived from Cyp4F2 variant microsomes. B: LC/MS/MS analysis of metabolite demonstrating similar retention time of the primary ion m/z 351 and identical MS/MS spectra generated from a 20-hydroxy-LTB4 standard and the product formed by incubation of the variants with LTB4.
The analysis of linkage disequilibrium between the two functional polymorphisms tested in this study and other SNPs discovered by comprehensive resequencing provides information on other surrogate markers (tagSNPs) and linked polymorphisms. At the most stringent r^2 levels (r^2 = 1.0), numerous tagSNPs, in both panels, could be used as a representative SNP for the W12G variant (Table 1). This information is important when selecting highly informative SNPs to be assayed in genetic association studies with a quantitative trait of interest (e.g., renal injury) or for interpreting results to identify “functional” polymorphisms (i.e., a nonfunctional, surrogate SNP could have been selected without knowledge of a linked functional SNP). Linkage disequilibrium also describes the set of SNPs that co-occur on the same haplotype background and could give rise to interactions or synergistic effects between functional polymorphisms (e.g., SNPs affecting transcription of a specific nonsynonymous allele). It is important to note that none of the polymorphisms show perfect linkage disequilibrium with the V433M SNPs affecting 20-HETE production. Therefore, genotyping this SNP directly would be required in any association study to achieve maximal statistical power, especially in the African American population that does not have any surrogate tagSNPs even at lower levels of linkage disequilibrium. However, several SNPs show high level linkage disequilibrium (r^2 = 0.75) with this polymorphism in the European population. Understanding the complete genetic structure of CYP4F2 for these functional SNPs allows accurate and rational selection of appropriate polymorphisms for association studies and could assist in the design and interpretation of functional assays (e.g., regulation of mRNA expression). Even though these polymorphic alleles occur at an appreciable frequency in the general sampling panels studied (9–21%), the genotype of individuals homozygous for each of the alleles would be rare. For the 20-HETE lowering polymorphism (433M), only 4.4 and 1.1% of African and European Americans would be homozygous for this less functional allele. The genotypic effect of these polymorphisms for influencing hypertension or the development of renal disease is not clear, but a larger population effect may be observed if heterozygotes also demonstrate a measurable phenotype (i.e., additive or dominant effects). Studies in which these CYP4F2 variants can be linked to levels of hypertension or kidney disease measures are needed to fully resolve this issue.

In conclusion, we have identified a functional variant in the human CYP4F2 gene (M433) that results in a decrease in 20-HETE production from AA but does not affect the metabolism of LTB4 to 20-hydroxy-LTB4. The other variant, G12, had no effect on either the metabolism of AA or LTB4. Both of these variants are found in high frequencies in African American- and European American-derived sampling panels. Whether or not these functional variants of the CYP4F2 gene can be related to the development of hypertension or other phenotypes associated with reduced renal excretion of 20-HETE will need to be determined in large, population-based studies. However, the present identification of a functional SNP in the coding region of this enzyme that reduces enzyme serves as a key first step in this type of translational studies.

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