

Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsomal P450-catalyzed drug metabolism

Steven N. Hart^a, Shuang Wang^b, Kaori Nakamoto^a, Christopher Wesselman^a, Ye Li^a and Xiao-bo Zhong^a

Objectives Cytochrome P450 oxidoreductase (POR) is the only flavoprotein that donates electrons to all microsomal P450 enzymes, which catalyze the biosynthesis of steroids, fatty acids, and bile acids, as well as metabolism of more than 80% of prescription drugs. Although mutations in POR have been identified in several disease states with disordered steroidogenesis, effects of polymorphisms on drug metabolism in the general population are unclear. In this report, we performed a comprehensive study to correlate POR polymorphisms with POR gene expression, POR activity, and P450-catalyzed drug metabolism.

Methods A set of human liver samples ($n=99$) were used in this study. POR polymorphisms were identified by sequencing the exons and surrounding introns of the POR gene and mRNA levels were quantified by branched DNA technology. POR activity was quantified by measuring cytochrome *c* reduction in liver microsomes and activities of 10 drug-metabolizing P450 enzymes were quantified by high performance liquid chromatography methods with drugs known to be specific for each enzyme.

Results Of the 34 polymorphisms identified in this cohort, four polymorphisms changed an amino acid: K49N, L420M, A503V, and L577P. L577P likely resulted in an α helix change, possible disruption of the nicotinamide adenine

dinucleotide phosphate interaction, and decreased POR activity ($P=0.003$) and several drug-metabolizing P450 activities. We also found an intronic polymorphisms rs41301427, which was associated with altered POR, but not P450 activities.

Conclusion Polymorphisms in the POR gene can affect POR and P450-catalyzed drug oxidation. These results suggest that POR has the potential to serve as a predictive biomarker for pharmacogenomic testing. *Pharmacogenetics and Genomics* 18:11–24 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Microsomal P450 enzymes are heme-containing proteins that catalyze biosynthesis of steroids, fatty acids, and bile acids [1], as well as metabolism of more than 80% of prescription drugs [2]. In the last few decades, pharmacogenomic studies have revealed that genetic polymorphisms can affect P450-catalyzed drug metabolism in different ways. First, a mutation in a P450 enzyme can affect the metabolic rates for drugs oxidized by that P450 enzyme. For example, mutations in CYP2C9 can decrease warfarin metabolism, leading to hemorrhagic complications [3,4]. Second, because several P450 enzymes share the same mechanisms for activation, suppression, and regulation, then genetic polymorphisms in coactivators, cosuppressors, or regulators may affect metabolic rates of drugs catalyzed by these P450 enzymes and influence a larger set of drugs. Genetic polymorphisms in the nuclear receptor pregnane X receptor [5] and the membrane

transporter multidrug resistance 1 [6] are two such examples. Third, all microsomal P450 enzymes require cofactors for their functions. Genetic polymorphisms in the cofactor genes may influence metabolic rates of all P450-catalyzed drugs. Cytochrome P450 oxidoreductase (POR) is one such cofactor.

POR is the only flavoprotein that donates electrons to microsomal P450 enzymes [7]. Oxidation of drugs by the P450s requires two sequential one-electron donations, but the source of these electrons comes from nicotinamide adenine dinucleotide phosphate (NADPH), which gives up a pair of electrons. POR compensates for this discrepancy by stabilizing the one-electron reduced form of the flavin cofactors of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Electrons pass from NADPH through FAD to FMN in the POR protein. Following a conformational change, the FMN-binding

domain of the POR interacts with the oxidation/reduction-partner binding site of the P450 enzymes so that electrons reach the P450 heme iron (Fe^{3+}) to achieve catalysis of drug oxidation.

It is reasonable to assume that disruption of electron flow in the POR protein would have destructive effects on oxidation of drugs by all microsomal P450 enzymes. This assumption has been supported by studies in animal models. POR knockout mice are embryonically lethal, giving rise to multiple developmental defects [8,9]. Mice with liver-specific deletion of POR are reproductively and morphologically normal, but they show a profound decrease of capabilities in the metabolism of steroids and drugs [10,11].

The gene encoding human POR is quite genetically polymorphic. Located on chromosome 7q11.2 [12], the POR gene (Gene ID 5447 in the National Center for Biotechnology Information database, NCBI) is a 71,753-bp gene (NT 007933) containing 16 exons that transcribe a 2509-bp mRNA (NCBI NM_000941.2) and encodes an 82-kDa membrane-bound protein with 680 amino acids (NCBI NP_000932.3). Currently, the NCBI single nucleotide polymorphism database (dbSNP) has reported ~320 SNPs in the 72-kb genomic region (4.4 SNP per 1 kb, higher than 0.8 SNP per 1 kb, an estimate of the average density of SNPs in human genome [13]). Fifteen of these SNPs are located in the exonic regions, in which eight are synonymous and seven are nonsynonymous. Five of the SNPs, rs10262966 (G5G), rs1135612 (P129P), rs2228104 (A485A), rs1057868 (A503V), and rs1057870 (S572S), have minor allele frequencies of more than 10% in at least one examined ethnic population [14].

Genetic polymorphisms in the POR gene have recently been associated with an autosomal recessive genetic disease, P450 oxidoreductase deficiency [15–20]. Its clinical phenotypes include ambiguous genitalia, congenital adrenal hyperplasia, Antley–Bixler syndrome, and polycystic ovary syndrome. These phenotypes typically link to abnormal steroid profiles with accumulation of steroid metabolites. Molecular genetic analyses first focused on steroid 17 α -hydroxylase (CYP17) and steroid 21-hydroxylase (CYP21), which are P450 enzymes involved in steroid metabolism. Deficiencies of CYP17 and CYP21 were observed in patients with glucocorticoid deficiency, skeletal dysplasia, and Antley–Bixler syndrome, but no mutations in the CYP17 and CYP21 genes could be identified [21,22]. These findings suggested a defect in a cofactor that interacts with these P450 enzymes. Flück *et al.* [15] first reported five missense POR mutations (A287P, R457H, V492E, C569Y, and V608F) and a splicing mutation in an initial study with four patients who had disordered steroidogenesis and Antley–Bixler syndrome. Later Arlt *et al.* [16] identified another POR missense mutation (Y181D) in three

patients who had congenital adrenal hyperplasia, and also reported three POR mutations (A287P, R457H, and C569Y) originally described by Flück *et al.* [15]. Furthermore, in a study with a larger patient sample size (32 individuals), Huang *et al.* [17] identified additional missense and frameshift mutations (A115V, T142A, Q153R, P228L, M263V, R316W, G413S, Y459H, A503V, G504R, G539R, L565P, R616X, V631I, and F646del) in the POR gene and recognized a distinct new disease: POR deficiency. In that study, 15 of 19 patients having abnormal genitalia and disordered steroidogenesis were homozygous or heterozygous for POR mutations that eliminated or dramatically decreased POR activity. The R457H mutation was found at very high allele frequency (65%) in a study with 10 Japanese patients from eight families with Antley–Bixler syndrome, abnormal genitalia, and impaired steroidogenesis [18]. Four other mutations were also identified in these patients: a missense mutation (Y578C), a silent transition (G5G), a 1-bp insertion (I444fsX449), and a 24-bp deletion (L612_W620delinsR). The mutations of Y181D, A287P, R457H, V492E, and V608F also significantly increased cytotoxicity in cultured Chinese hamster ovary cells induced by paraquat, a widely used herbicide [23], and mitomycin C, a highly active anticancer prodrug [24].

POR deficiency is a very rare genetic disease. Mutations causing the POR deficiency may not be common in the general population. It is, however, unclear whether genetic polymorphisms in the POR gene affect P450-catalyzed drug metabolism. Recently, we identified novel SNPs in the POR gene in individuals without POR deficiency [25]. In this report, we performed a comprehensive study to establish correlations of genetic polymorphisms in the POR gene with POR gene expression, POR activity, and POR-assisted P450 activities using a set of human liver tissue samples. Our data indicate that genetic polymorphisms in the POR gene indeed influence P450-catalyzed drug metabolism.

Materials and methods

Human livers

Human liver tissue samples ($n = 99$) were purchased from XenoTech LLC (Lenexa, Kansas, USA) in the form of three 5 ml lysates in DNA, RNA, and microsome isolation buffers. The samples were acquired by XenoTech through the Midwest Transplant Network (Westwood, Kansas, USA), the National Disease Research Interchange (Philadelphia, Pennsylvania, USA), and the Anatomical Gift Foundation (Woodbine, Georgia, USA). Livers were initially harvested for transplantation purposes, but were not used for various reasons and subsequently were donated for research. The livers were cooled immediately after procurement with a cold perfusion solution and frozen within 1–36 hrs. All liver samples were tested for and declared free of infectious agents, including human immunodeficiency virus (HIV),

hepatitis B (HBV), and hepatitis C (HCV). Demographic information such as sex, age, ethnicity, and confounding factors are listed in Table 1.

Cytochrome P450 oxidoreductase and P450 activities

P450 enzyme profiles of this liver cohort were characterized by Xenotech LLC. Liver microsomes were prepared by using differential ultracentrifugation [26]. The rate of cytochrome *c* reduction by liver microsomes was determined spectrophotometrically based on a previously described method [27] with some modifications [28]. The reaction was conducted in a 1 ml solution with 50 $\mu\text{mol/l}$ cytochrome *c*, 100 $\mu\text{mol/l}$ NADPH, and $\sim 50 \mu\text{g}$ liver microsomal protein at room temperature for 10 min. The rate of cytochrome *c* reduction was determined from the rate of increase in absorbance at 550 nm by reduced form of cytochrome *c* with a DW2C dual beam spectrophotometer (SLM-Aminco, Urbana, Illinois, USA). P450 enzyme activities were determined by measuring the rates of the following reactions with a spectrofluorometer or high performance liquid chromatography according to previously described procedures: 7-ethoxyresorufin *O*-dealkylation (CYP1A2) [28], coumarin 7-hydroxylation (CYP2A6) [28], *S*-mephenytoin *N*-demethylation (CYP2B6) [28,29], paclitaxel hydroxylation (CYP2C8) [30], diclofenac 4'-hydroxylation (CYP2C9) [30], *S*-mephenytoin 4'-hydroxylation (CYP2C19) [28], dextromethorphan *O*-demethylation (CYP2D6) [28], chlorzoxazone 6-hydroxylation (CYP2E1) [28], testosterone 6 β -hydroxylation (CYP3A4/5) [28], and lauric acid 12-hydroxylation (CYP4A9/11) [28]. With the substrate concentrations and amount of liver microsomal protein used in each reaction (Table 2), the probe drugs are considered to be specific for each P450 enzyme.

Sequencing the cytochrome P450 oxidoreductase gene

Genomic DNA was isolated from liver tissue using the ChargeSwitch gDNA Mini Tissue Kit from Invitrogen (Carlsbad, California, USA), following the manufacturer's protocol. Exonic regions of POR were amplified by PCR from the genomic DNA using forward and reverse primers designed by DS Gene Software (Accelrys, Cambridge, UK). Primer sequences and PCR product sizes have

been previously reported by our lab [25]. Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA) and the subsequent PCR reactions were performed using Go Taq DNA Polymerase (Promega, Madison, Wisconsin, USA), with cycling conditions of 95°C for 3 min, 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s, followed by 72°C for 5 min. PCR products were purified with the Pre-sequencing Kit provided by USB (Cleveland, Ohio, USA). DNA sequencing reactions were carried out using BigDye Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) with the forward primers. For dye terminator removal, PERFORMA DTR Gel Filtration cartridges (Edge BioSystems, Gaithersburg, Maryland, USA) were used, and sequences were analyzed with a 3130 DNA Analyzer (Applied Biosystems).

Cytochrome P450 oxidoreductase gene expression

Total RNA was isolated from each liver tissue with TRIZOL reagent (Invitrogen) following the manufacturer's protocols. Human POR mRNA levels were assessed by branched DNA technique [31,32] with a Quantigene expression Kit (Bayer, Walpole, Massachusetts, USA) as described in the manufacturer's protocol. The specific oligonucleotide probe sets (capture extenders, label extenders, and blockers) were designed to have a melting temperature around 65°C using the "Probe Designer" software, version 1.0 (Bayer, Emeryville, California, USA). Sequences of the probes are listed in supplemental Table 1. A luminescent readout was measured with a Quantiplex 320 Luminometer (Chiron Corp., Emeryville, California, USA).

Prediction of protein structure changes

Amino acid sequences from POR homologs of human (NCBI NP_000932.3), rat (NP_113764.1), frog (*Xenopus*, AAH59318.1), fruit fly (*Drosophila*, NP_477158.1), and yeast (NP_596046.1) were aligned and displayed by ClustalW [33]. Molecular modeling of the identified nonsynonymous mutations were performed with ESy-Pred3D [34] using the crystalline rat *Por* structure (PDB:1AMO) chain 'A' as a template. The rat *Por* protein shares 92.1% identity and 96.3% similarity with the human POR in alignment by the Needle EMBOSS pairwise tool [35]. Models were visualized and displayed using PyMOL [36]. Protein secondary structure and membrane topology for the POR mutations were predicted by the Quick2D (the Max-Planck Institute for Development Biology, Tübingen, Germany) analysis tool with PSIPRED [37] and MEMSAT2 [38].

Statistical analysis

We used multiple linear regressions to assess the effect of POR SNPs on POR enzyme activity. Age, sex, ethnicity, reason for death, smoking history, drinking history, and cytomegalovirus (CMV) infection were included in the model to adjust for potential confounding effects.

Table 1 Demographic information of confounding factors in the human liver cohort

Confounding factors	Distributions (n)
Sex	Male (59) and female (40)
Ethnicity	Caucasian (77), African-American (9), Hispanic (10), and Asian (3)
Age	Year 0–1, (4); year 1–18, (7); year 18–45, (27); year 45–60, (39); and year older than 60, (22)
Smoking	Nonsmoker (61) and smoker (38)
Alcohol drinking	Nondrinker (47), drinker (51), and unknown (1)
Reason for death	Anoxia (18), aortic aneurysm (1), cerebrovascular aneurysm (61), head trauma (14), myocardial infarction (3), and motor vehicle accident (2)
CMV infection	Negative (67), positive (30), and not determined (2)

CMV, cytomegalovirus.

The same linear regression was also applied to assess the association between POR gene expression and POR enzyme activity.

Results

Interindividual variation of cytochrome P450 oxidoreductase enzyme activity

Interindividual variation of the POR enzyme activity was observed in this liver cohort. Although cytochrome *c* has been shown not to be the natural substrate of POR as initially thought [39,40], cytochrome *c* reduction is still used by many researchers in the field to quantify POR activity [15,17,22]. Cytochrome *c* reduction was also used to quantify the POR activity in this study. Figure 1 shows the histogram of POR activity with a mean of 177 nmol/mg protein/min and a standard deviation (SD) of 53. Seventy-five individuals had POR activity within one SD around the mean, which was considered as normal POR

activity. Ten individuals had POR activity between -1 SD and -2 SD, which was considered low POR activity. One individual had extremely low POR activity (< -2 SD). In contrast, 10 individuals had high POR activity (between $+1$ SD and $+2$ SD) and three had extremely high POR activity ($> +2$ SD). Between $+2$ SD and -2 SD, there is about a four-fold difference.

Correlation of enzyme activities between cytochrome P450 oxidoreductase and P450 enzymes

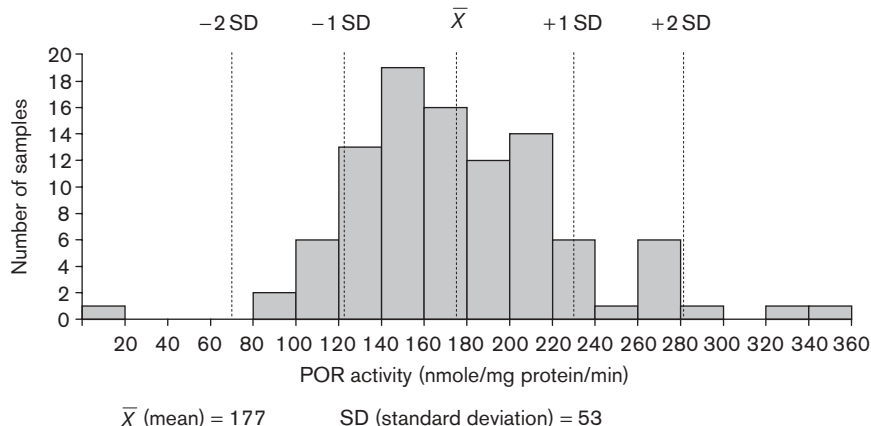
Significant associations between the activity of POR and activities of most drug-metabolizing P450 enzymes were observed in this study. Activities of 10 P450 enzymes were quantified in the 99 liver microsomes using probe drugs known to be specific for each enzyme. Table 2 lists descriptive statistics for each P450 enzyme activity. Pearson's correlations between POR activity and a P450 enzyme activity together with the *P* value (testing

Table 2 The POR and P450 enzyme activities in the human liver cohort

Enzyme and assay	Substrate concentration ^a (μmol/l)	Liver microsomal protein (μg) used in each reaction	Enzyme activity (pmol/mg protein/min)			
			Highest	Lowest	Mean	SD
POR: cytochrome <i>c</i> reduction	50	50	343000	505	177382	53185
CYP1A2: 7-ethoxyresorufin <i>O</i> -dealkylation	10	100	258	NPD	51	45
CYP2A6: Coumarin 7-hydroxylation	50	100	7310	5	984	1167
CYP2B6: S-mephenytoin <i>N</i> -demethylation	400	400	1280	10	107	160
CYP2C8: paclitaxel 6 α -hydroxylation	10	50	2040	NPD	341	340
CYP2C9: diclofenac 4'-hydroxylation	100	50	5870	127	1810	867
CYP2C19: S-mephenytoin 4'-hydroxylation	400	400	895	NPD	101	141
CYP2D6: dextromethorphan <i>O</i> -demethylation	80	500	1160	18	308	192
CYP2E1: chlorzoxazone 6-hydroxylation	500	200	11500	201	2109	1505
CYP3A4/5: testosterone 6 β -hydroxylation	250	200	20300	NPD	3421	3670
CYP4A9/11: lauric acid 12-hydroxylation	100	400	4350	NPD	1699	818

^aThe substrate concentration is near the 10 km for the reaction and has been shown to be appropriate for metabolite formation. NPD, no catalyzed product detected; POR, cytochrome P450 oxidoreductase; SD, standard deviation.

Fig. 1



Distribution of cytochrome P450 oxidoreductase (POR) activity quantified by measuring cytochrome *c* reduction in the liver cohort.

Table 3 Pearson's correlation between POR activity and P450 activity

POR vs. P450 enzyme	Pearson's correlation	<i>P</i> value
CYP1A2	0.102	0.316
CYP2A6***	0.396	<0.001
CYP2B6***	0.354	<0.001
CYP2C8***	0.437	<0.001
CYP2C9***	0.482	<0.001
CYP2C19	0.075	0.460
CYP2D6*	0.204	0.043
CYP2E1***	0.414	<0.001
CYP3A4/5***	0.406	<0.001
CYP4A9/11***	0.500	<0.001

POR, cytochrome P450 oxidoreductase.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

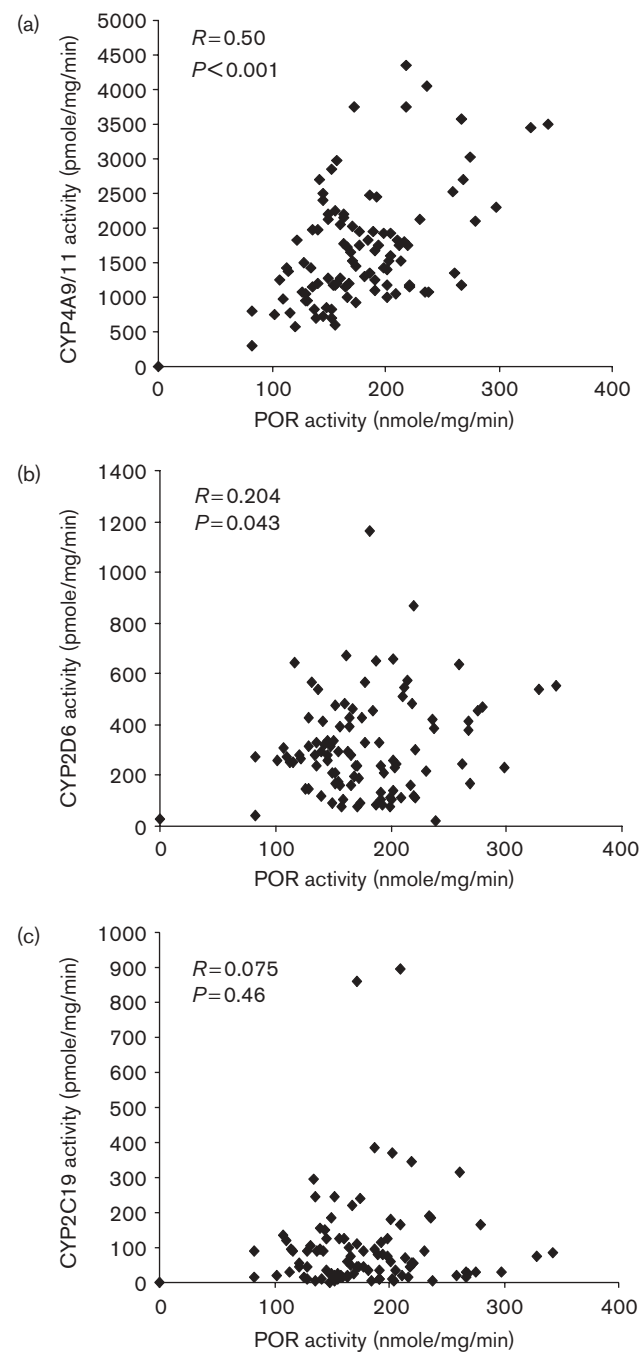
correlation equals 0) are summarized in Table 3. Seven of the P450 enzyme activities (CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2E1, CYP3A4/5, and CYP4A9/11) correlated with POR activity at significant levels of $P < 0.001$. CYP2D6 correlated with POR activity at a significant level of $P < 0.05$. Only CYP1A2 and CYP2C19 did not significantly correlate with POR ($P > 0.05$). As examples, Fig. 2 shows the scatter plots of enzyme activity of POR vs. CYP4A9/11 ($P < 0.001$), CYP2D6 ($P = 0.043$), and CYP2C19 ($P = 0.460$).

Identification of genetic polymorphisms in the cytochrome P450 oxidoreductase gene

Genetic polymorphisms were identified in the exons and surrounding introns (~7.7 kb genomic sequences) in the POR gene by sequencing PCR products amplified from genomic DNAs isolated from the 99 human liver samples. Thirty-four SNPs were identified in these areas. Of these, 20 were in the introns, five in the 3'-UTR, and nine in the exons (Table 4). Nine of the 34 SNPs were novel polymorphisms recently reported for the first time [25]. Of the nine exonic polymorphisms, five were synonymous polymorphisms (G5G, T29T, P129P, S485S, and S572S) and four were nonsynonymous polymorphisms resulting in amino acid changes at K49N, L420M, A503V, and L577P that had minor allele frequencies of 0.005, 0.045, 0.219, and 0.020, respectively. As expected, we did not observe any of the missense or frameshift mutations (T142A, Q153R, Y181D, M263V, A287P, R457H, Y459H, V492E, G539R, L565P, C569Y, Y578C, V608F, R616X, F646del, I444fsX449, and L612W620delinsR) that have been associated with POR deficiency [15–18]. All SNPs but rs2286816 in intron 6 had a Hardy-Weinberg P value greater than 0.001.

Prediction of functional influence of the cytochrome P450 oxidoreductase mutations

Four nonsynonymous SNPs, which result in the amino acid changes at K49N, L420M, A503V, and L577P, were identified in this liver cohort. K49N resides in the amino-terminal tail, L420M in the connecting domain, A503V in the FAD-binding domain, and L577P in the NADPH-

Fig. 2

Scatter plots of cytochrome P450 oxidoreductase (POR) activity vs. P450 activity of CYP4A9/11 (a), CYP2D6 (b), and CYP2C19 (c). Pearson's correlations (R) and corresponding P values are listed in the upper left portion of each plot.

binding domain. A503V has been reported in the NCBI dbSNP and has moderate influence on POR activity (69% of wild type) [17]. We first reported K49N, L420M, and L577P [25]. To predict the potential influence of K49N, L420M, and L577P on POR functions, we

Table 4 Genetic polymorphisms in the POR gene identified in the liver cohort

SNP ID	rs10262966	rs412952381	SNP1 ^a	rs1135612	rs2286819	rs2286820	SNP2 ^a	SNP3 ^a	rs41299517	rs3815455	rs13223707	rs13240147	rs41301394	rs4732514	rs6971082	rs4732515	rs4732516
Location	Exon 2	Exon 2	Exon 2	Exon 5	Intron 6	Intron 7	Intron 7	Intron 7	Intron 7	Intron 8	Intron 8	Intron 8	Intron 8	Intron 10	Intron 10	Intron 10	Intron 10
Amino acid change	G5G	T29T	K49N	P129P													
Major allele	A	G	G	A	A	G	G	C	A	C	G	A	C	T	C	C	G
Minor allele	G	A	C	G	G	A	A	T	G	T	C	G	T	C	T	T	C
Samples with genotype	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	93	96
Allele frequency (%)																	
Major allele	95.5	99	99.5	78.8	91.9	99	99.5	99	97	77.3	96.5	94.4	81.8	82.8	99	93.5	95.3
Minor allele	4.5	1	0.5	21.2	8.1	1	0.5	1	3	22.7	3.5	5.6	18.2	17.2	1	6.5	4.7
Genotype																	
Homo major allele (n)	92	97	98	63	88	97	98	97	93	57	94	91	66	73	97	84	89
Homo minor allele (n)	2	0	0	6	5	0	0	0	0	3	0	3	3	6	0	3	2
Heterozygous (n)	5	2	1	30	6	2	1	2	6	39	5	5	30	20	2	6	5
Observed hetero (%)	5.1	2	1	30.3	6.1	2	1	2	6.1	39.4	5.1	5.1	30.3	20.2	2	6.5	5.2
Predicted hetero (%)	8.7	2	1	33.4	13.1	2	1	2	5.9	35.1	6.8	10.5	29.8	28.4	2	11.4	8.8
HW <i>P</i> value	0.02	1.00	1.00	0.48	0.0009*	1.00	1.00	1.00	1.00	0.38	0.21	0.002	1.00	0.02	1.00	0.004	0.02
SNP ID	rs2286822	rs2286823	SNP4 ^a	SNP5 ^a	SNP6 ^a	rs41301427	rs2302431	rs2302432	rs2228104	rs1057868	rs1057870	SNP7 ^a	rs41302345	SNP8 ^a	SNP9 ^a	rs41302348	rs17685
Location	Intron 11	Intron 11	Exon 12	Intron 12	Intron 12	Intron 12	Intron 12	Intron 12	Intron 12	Exon 13	Exon 13	Exon 14	Exon 14	3'-UTR	3'-UTR	3'-UTR	3'-UTR
Amino acid change			L420M							S485S	A503V	S572S	L577P				
Major allele	C	A	C	T	T	G	C	G	G	C	C	G	T	T	G	G	C
Minor allele	T	G	A	A	C	A	T	T	T	T	T	A	C	C	A	A	G
Samples with genotype	99	99	99	99	99	99	98	98	98	99	99	97	99	99	99	99	99
Allele frequency (%)																	
Major allele	64.1	62.6	95.5	99.5	99	90.4	93.4	96.4	92.3	78.1	69.1	98	99.5	99.5	99.5	99	80.3
Minor allele	35.9	37.4	4.5	0.5	1	9.6	6.6	3.6	7.7	21.9	30.9	2	0.5	0.5	0.5	1	19.7
Genotype																	
Homo major allele (n)	47	45	90	98	97	82	88	91	86	63	63	95	98	98	98	97	63
Homo minor allele (n)	19	21	1	0	0	2	3	0	3	6	0	0	0	0	0	0	3
Heterozygous (n)	33	33	8	1	2	15	7	7	9	29	34	4	1	1	1	2	33
Observed hetero (%)	33.3	32.3	9.1	1	2	15.2	7.1	7.1	9.2	29.6	35.1	4	1	1	1	2	33.3
Predicted hetero (%)	46	46.8	8.7	1	2	17.4	12.4	6.9	14.1	34.3	42.7	4	1	1	1	2	31.6
HW <i>P</i> value	0.01	0.004	1.00	1.00	1.00	0.42	0.01	1.00	0.02	0.27	0.11	1.00	1.00	1.00	1.00	1.00	0.90

^aNovel SNPs identified in this study. HW *P* value: Hardy–Weinberg *P* value.

**P* value is significant when $P < 0.001$.

POR, cytochrome P450 oxidoreductase; SNP, single nucleotide polymorphism.

performed a series of modeling analyses to establish conservation of residues; influence on secondary structures and 3-dimensional (3D) configurations, and interactions with cofactors.

The amino acids are conserved to varying degrees

Human POR protein was aligned with homologues from the rat, frog, fruit fly, and yeast (Fig. 3). The rationale is that if an amino acid is highly conserved, then a change would likely have a great impact on POR function as there would have been an evolutionary force to retain the amino acid. The K49 amino acid is located in a FRKKKEE motif that is conserved in the human, rat, and frog, but not in the fruit fly or yeast. The L420 is also conserved among the human, rat, and frog in a LYLSWVVE motif, but almost no conservation is seen in the fruit fly or yeast. Unlike the previous two amino acids, the L577 is highly conserved in a DYLYR motif. In the fruit fly, the leucine is replaced by an isoleucine, an isomeric form of leucine. As this residue is so highly conserved, it is predicted that a change at L577 may influence POR function.

The amino acid changes affect α helix or β sheet formation

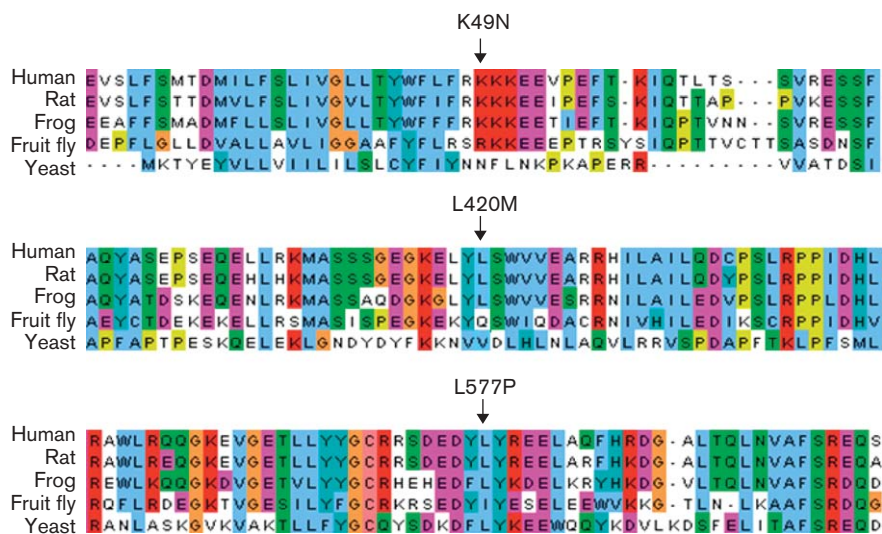
Given the sequence conservation throughout evolution, we sought out predictive modeling to (i) identify secondary structures in areas where amino acid changes occur, and (ii) predict the outcome of the changed amino acids on protein secondary structure. PSIPRED [37] was used to predict helix or β sheet structure and MEMSAT2 [38] was applied to identify transmembrane domains.

First, the full-length POR amino acid sequence with 100% major alleles was uploaded to the Quick2D analysis tool. The major K49 allele was predicted to occur in a random coil directly adjacent to an α helical region from S16 to F47 and a transmembrane domain spanning from M31 to F47 (Fig. 4a, top row). The L420 major allele was predicted to occur in the middle of an α helix that forms between K416 and E425 (Fig. 4b, top row). The L577 major allele was found in an α helix beginning at Y576 and ending at R587 (Fig. 4c, top row). When the major alleles of K49, L420, and L577 were replaced by the minor alleles of N49, M420, and P577, the Quick2D analysis revealed some disparities between major and minor allele simulations in the prediction (Fig. 4a–c, bottom rows). The L420M simulation did not predict any functional changes, but the K49N made F26 involving an α helix from E17 to F47 and V9-D10 forming a β -sheet. The L577P replacement prevented the Y576 residue from participating in the α helix.

The amino acid changes affect interaction with cofactors

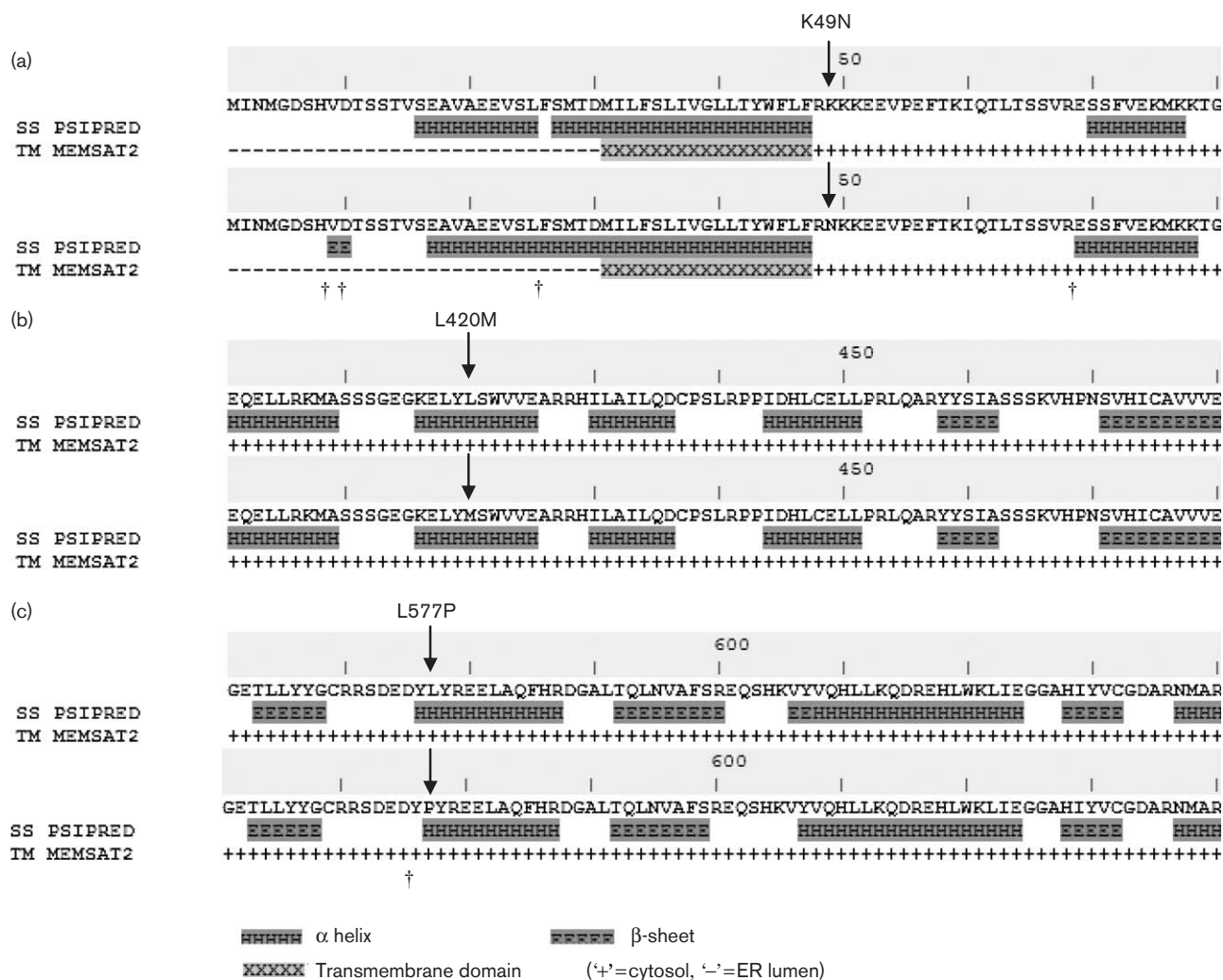
A 3D structure of the human POR protein was predicted with the ESyPred3D modeling server and visualized with PyMOL, using the fully crystallized rat *Por* structure (PDB: 1AMO chain 'A') as a template [41]. Crystallography methods used to generate the rat *Por* structure involve trypsinolysis, which truncates the protein at I53 (human V54) [41]. Thus, our homology model begins at V67 and the K49N polymorphism could not be modeled and evaluated for its impact on structure and function.

Fig. 3



Alignment fragments of amino acid sequences of cytochrome P450 oxidoreductase from five species. Representative amino acid sequences are human (NCBI NP_000932.3), rat (NP_113764.1), frog (AAH59318.1), fruit fly (NP_477158.1), and yeast (NP_596046.1). Alignment was made by ClustalW and shown in default ClustalX color scheme. NCBI, National Center for Biotechnology Information.

Fig. 4



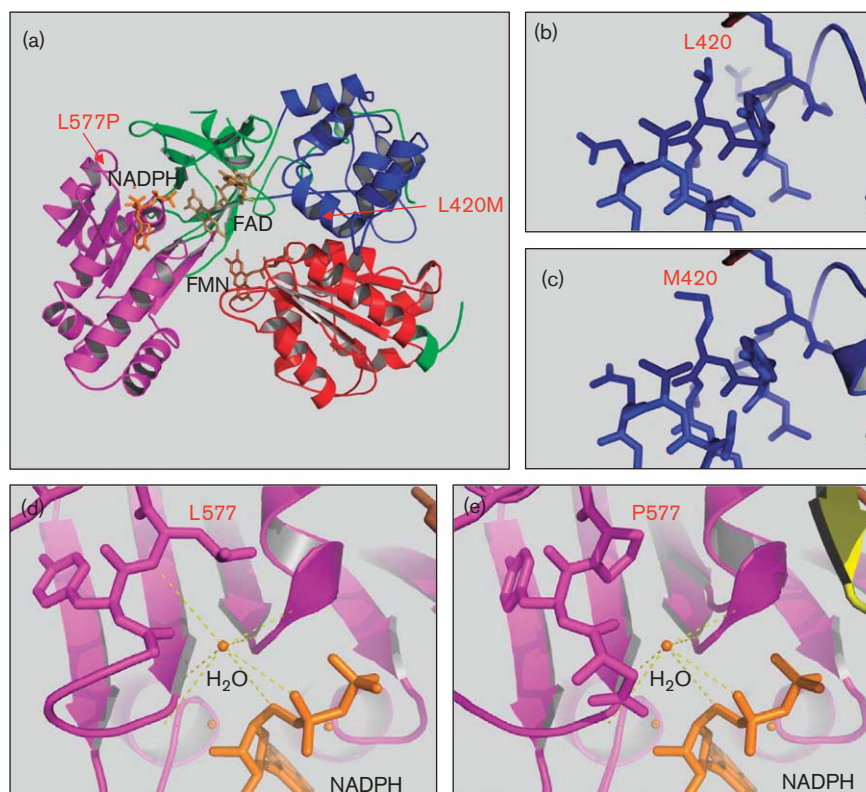
Predicted secondary structures and membrane topology for K49N (a), L420M (b), and L577P (c). † indicates difference between minor alleles and major alleles. Protein α helix and β sheet were predicted by SS PSIPRED and transmembrane helices were predicted by TM MEMSAT2-D. Arrows indicate the positions of an amino acid change.

Figure 5a shows a 3D structure of the human POR homolog and interactions with its cofactors of NADPH, FAD, and FMN predicted by the EsyPred3D server. Although the L420M polymorphism occurs in the K416–E425 α helix, compared with the L420 residue (Fig. 5b), the M420 does not disrupt the helix formation (Fig. 5c). Neither the L420 nor M420 residues participate in hydrophilic, hydrophobic, or hydrogen bonding with FAD or any other cofactor. Figure 5d shows hydrogen bonding between the backbone nitrogen of L577 and a water molecule, which likely also dynamically bonds to several other surrounding residues and stabilizes NADPH binding. When L577 is replaced with P577 (Fig. 5e), a hydrogen bond between the backbone nitrogen of P577 and the water molecule no longer forms due to the conformation and properties of proline. Such change may destabilize NADPH binding.

Correlation of single nucleotide polymorphisms in the cytochrome P450 oxidoreductase gene with cytochrome P450 oxidoreductase activity

SNPs in the POR gene had significant influence on the POR activity in this liver cohort. Assuming a dominant genetic model, we performed a multiple linear regression analysis on each SNP adjusting for sex, age, ethnicity, reason for death, CMV infection, smoking, and drinking. As an example, Table 5a summarizes the regression coefficients, standard error, *t* values, and *P* values for SNP7, which causes the L577P amino acid change. Sex, age, ethnicity, CMV infection, reason for death, smoking, and drinking were not associated with POR activity ($P > 0.05$). After adjusting for all confounding factors, we found that samples with L577/P577 had significantly lower POR activity than samples with L577/L577 (coefficient estimate = -101.152 and $P = 0.003$). Table 5b summarizes

Fig. 5



Effects of the polymorphisms on cytochrome P450 oxidoreductase structure. (a) EsysPred3d predicted 3D structure using rat *Por* (PDB: 1AMO chain 'A') as a template and displayed by PyMOL. Functional domains are distinguished by coloration. The FMN-binding domain is in red, FAD-binding domain is green, the flexible hinge domain is colored blue, and the NADPH-binding domain is pink. FAD, FMN, and NADPH cofactors are tan, brown, and orange, respectively. (b and c) Magnification around wild-type L420 and mutant M420 residues (respectively) shows this mutation occurs in an exposed region that is not involved in interactions with FAD or other cofactors. (d) The backbone nitrogen of residue L577 is potentially involved in stabilizing a water molecule that interacts with several other residues and NADPH. (e) In the case of the P577 mutation, its backbone nitrogen is unable to participate in this interaction with the water molecule due to the unique structure of proline. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate.

the effects of all 34 POR SNPs on POR activity after adjusting for all the above-mentioned confounders. In addition to SNP7, samples with GA and AA genotypes of rs41301427 (a G > A change in intron 12 with a minor allele frequency of 0.096) was associated with decreased POR activity compared to samples with GG genotype (coefficient estimate = -32.409 and $P = 0.030$) without influence from the confounders.

Correlation of single nucleotide polymorphisms in the cytochrome P450 oxidoreductase gene with P450 activities

POR SNP7 had significant influence not only on the POR activity but also on most P450 enzyme activities. The influence of L577P is shown in Fig. 6. Four samples (L099, L078, L059, and L080) had heterozygous genotype of SNP7 encoding heterozygous L577/P577 POR protein. The POR activity in the four samples with heterozygous L577/P577 was significantly lower than that in the 95 samples with homozygous L577/L577

($P = 0.003$, Fig. 6a). For 10 drug-metabolizing P450 enzymes, the corresponding activities were lower for CYP2A6 and CYP2E1 at significant levels of $P < 0.01$, lower for CYP2B6, CYP2C9, CYP3A4, and CYP4A9 at significant levels of $P < 0.05$, and lower for CYP1A2 at $P = 0.09$, but not significantly lower for CYP2C8, CYP2C19, and CYP2D6 (Fig. 6b) when Student's t -tests were applied. Although rs41301427 correlated to decreased POR activity (coefficient estimate = -32.409 and $P = 0.030$), Student's t -tests revealed this SNP did not influence all P450 activities (data are not shown).

Correlation of cytochrome P450 oxidoreductase gene expression with cytochrome P450 oxidoreductase activity

The POR activity was significantly associated with POR gene expression at mRNA levels in this liver cohort. The POR mRNA levels were quantified by branch DNA (bDNA) technology. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was

Table 5a Association of SNP7 (L577P) with POR activity after adjusting for possible confounders

Factor	Coefficient estimate	Standard error	t value	P value
Intercept**	176.331	36.679	4.807	0.000
Sex male vs. female	5.698	12.180	0.468	0.641
Age	-0.658	0.366	-1.799	0.076
Ethnicity AA vs. A	26.271	34.492	0.762	0.449
Ethnicity C vs. A	26.754	31.321	0.854	0.396
Ethnicity H vs. A	56.797	35.211	1.613	0.111
Death AA vs. A	59.706	55.452	1.077	0.285
Death CVA vs. A	9.483	17.911	0.529	0.598
Death HT vs. A	-10.238	20.234	-0.506	0.614
Death MI vs. A	10.241	36.448	0.281	0.779
Death MVA vs. A	-60.224	40.609	-1.483	0.142
CMV positive vs. negative	-5.779	12.976	-0.445	0.657
Smoker yes vs. no	-0.500	12.459	-0.040	0.968
Alcohol yes vs. no	9.773	12.277	0.796	0.428
L577/P577 vs. L577/L577**	-101.152	32.776	-3.086	0.003

Ethnicity: A, Asian; AA, African-American; C, Caucasian; H, Hispanic.

Death: A, anoxia; AA, aortic aneurysm; CVA, cerebrovascular aneurysm; HT, head trauma; MI, myocardial infarction; MVA, motor vehicle accident; POR, cytochrome P450 oxidoreductase; SNP, single nucleotide polymorphism.

**Significant level $P < 0.01$.

Table 5b Association of POR SNPs with POR activity^a

SNP	Coefficient estimate	Standard error	t value	P value
rs10262966	39.997	25.690	1.557	0.124
rs412952381	53.519	41.036	1.304	0.196
SNP1	41.628	55.631	0.748	0.457
rs1135612	18.700	12.465	1.500	0.138
rs2286819	-4.208	20.492	-0.205	0.838
rs2286820	-43.708	40.479	-1.080	0.284
SNP2	72.665	58.322	1.246	0.217
SNP3	66.196	40.190	1.647	0.104
rs41299517	26.616	26.629	1.000	0.321
rs3815455	-15.296	12.055	-1.269	0.208
rs13223707	0.332	26.344	0.013	0.990
rs13240147	4.096	22.362	0.183	0.855
rs41301394	-6.677	12.923	-0.517	0.607
rs4732514	-16.875	13.353	-1.264	0.210
rs6971082	79.195	39.998	1.980	0.051
rs4732515	11.014	23.103	0.477	0.635
rs4732516	5.153	26.652	0.193	0.847
rs2286822	13.340	11.823	1.128	0.263
rs2286823	9.860	11.961	0.824	0.412
SNP4	34.617	19.824	1.746	0.085
SNP5	74.489	56.321	1.323	0.190
SNP6	-25.56	49.912	-0.533	0.595
rs41301427*	-32.409	14.685	-2.207	0.030
rs2302431	2.129	20.504	0.104	0.918
rs2302432	-7.039	23.962	-0.294	0.770
rs6950661	1.008	19.355	0.052	0.959
rs1057868	-2.467	13.634	-0.181	0.857
rs1057870	6.380	12.217	0.522	0.603
SNP7**	-101.152	32.776	-3.086	0.003
rs41302345	131.300	67.637	1.941	0.056
SNP8	131.300	67.637	1.941	0.056
SNP9	86.951	56.308	1.544	0.127
rs41302348	-11.587	46.209	-0.251	0.803
rs17685	-17.435	11.956	-1.458	0.149

^aAdjusted for age, sex, ethnicity, reason for death, smoking history, drinking history, and cytomegalovirus infection.

POR, cytochrome P450 oxidoreductase; SNP, single nucleotide polymorphism. * $P < 0.05$; ** $P < 0.01$.

simultaneously quantified by bDNA technology. Relative POR mRNA levels were defined by normalizing with GAPDH. Interindividual variations of POR mRNA were

observed in this liver cohort with a mean of 0.195 and an SD of 0.109. Correlation analysis of POR mRNA with POR activity was performed using the same multiple linear regression adjusting for all previously mentioned confounders. None of the confounders had a significant effect on correlation between POR mRNA and POR activity in this liver cohort (Table 6). A significant association (coefficient estimate = 123.653 and $P = 0.041$) was found between POR mRNA and POR activity.

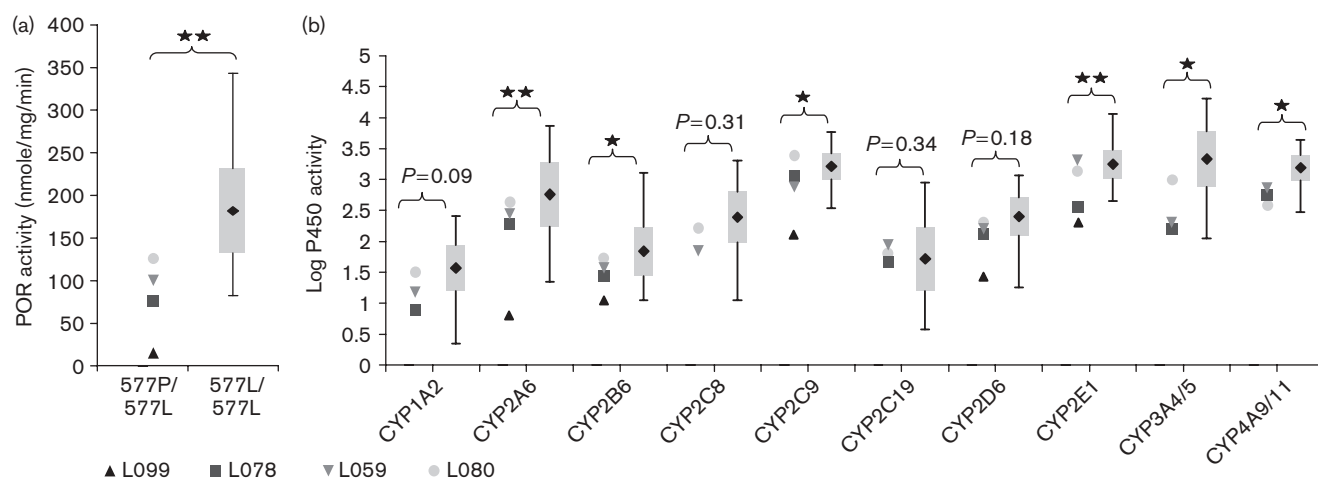
Four samples (L099, L078, L059, and L080), which have the same heterozygous genotype of SNP7 and can produce both wild-type L577 and mutant P577 POR proteins in their livers, had varied POR activities from 505 to 139 000 pmol/mg protein/min (Table 7). If the average of POR activity (181 000 pmol/mg protein/min) in the samples ($n = 95$) with the wild-type genotype of L577/L577 is considered as 100%, POR activity in L099, L078, L059, and L080 is 0.2, 45, 61, and 77% of the average, respectively. An increasing trend from extremely low in L099 to close to the average in L080 maintains same in almost all examined P450 enzymes with P value all less than or close to 0.1 in a linear regression analysis (Table 7). This trend was also observed between the POR activity and the POR mRNA level ($P = 0.083$). The liver of L099 was from a 58-year-old Caucasian man who did not smoke or drink, was not infected by CMV, and died as a result of myocardial infarction. The liver was removed after 5 hrs of death and recorded having normal morphology. His total P450 protein content and GAPDH RNA were normal. His extremely low POR activity (0.2%) and either no detectable or less than 10% of the average P450 activities may suggest a combinational effect of P577 mutation and low POR gene expression (9% of the average POR mRNA level). The influence of P577 mutation on POR and P450 activities in the sample L080 might be overcome by high POR gene expression (240% of the average POR mRNA level).

Discussion

We have demonstrated that POR activity correlates with most drug-metabolizing P450 enzyme activities in the study samples. This finding suggests that these P450 enzymes are sensitive to the amount of POR available. This phenomenon was also observed in microsomal P450 enzymes that are involved in steroid hormone biosynthesis (P450C17 and P450C21) [42,43].

We observed less significant correlations between POR activity and CYP2D6, CYP2C19, or CYP1A2 activity in the study population, which may be due to the known genetic polymorphisms in these P450 enzyme genes. Pharmacogenomic aspects of CYP2D6 and CYP2C19 have been well documented [44-47]. To date, more than 60 alleles for CYP2D6 and 20 alleles for CYP2C19 have been named by the Human Cytochrome P450 Allele Nomenclature Committee [48]. Genetic polymorphisms

Fig. 6



Effect of the L577P amino acid change on POR and P450 activities. (a) Comparison of the POR activity between samples ($n=4$) with L577/P577 (left) and samples ($n=95$) with L577/L577 (right). (b) Comparison of the P450 activities between L577/P577 and L577/L577 samples using Student's t -tests. To better meet normality assumption, logarithm transformation was applied to all P450 enzyme activities. The samples with no detected enzyme activities therefore were removed for being undefined to take logarithm transformation. There are one for CYP1A2, two for CYP2C8, one for CYP2C19, one for CYP3A4/5, and one for CYP4A9/11. A black diamond and a grey bar in each right column represent the mean and standard deviation of the enzyme activity in 95 samples with L577/L577. * $P \leq 0.05$, ** $P \leq 0.01$. POR, cytochrome P450 oxidoreductase.

Table 6 Association of POR mRNA level with POR activity after adjusting for possible confounders

Factor	Coefficient estimate	Std. error	t value	P value
Intercept*	225.483	45.175	4.991	4.21×10^{-6}
Sex male vs. female	10.246	13.036	0.786	0.434
Age	-0.470	0.385	-1.218	0.227
Ethnicity AA vs. A	32.475	35.765	0.908	0.366
Ethnicity C vs. A	24.908	32.306	0.771	0.443
Ethnicity H vs. A	49.679	36.936	1.345	0.183
Death AA vs. A	11.164	62.369	0.179	0.858
Death CVA vs. A	-12.372	19.392	-0.638	0.525
Death HT vs. A	-22.850	21.618	-1.057	0.294
Death MI vs. A	-28.250	35.535	-0.795	0.429
Death MVA vs. A	-59.316	41.950	-1.414	0.162
CMV positive vs. negative	5.725	13.376	0.428	0.700
Smoker yes vs. no	1.128	12.966	0.087	0.931
Alcohol yes vs. no	10.455	12.703	0.823	0.413
POR mRNA vs. POR activity*	123.653	59.391	2.082	0.041

Ethnicity: A, Asian; AA, African-American; C, Caucasian; H, Hispanic.
 Death: A, anoxia; AA, aortic aneurysm; CVA, cerebrovascular aneurysm; HT, head trauma; MI, myocardial infarction; MVA, motor vehicle accident; POR, cytochrome P450 oxidoreductase; SNP, single nucleotide polymorphism.
 *Significant ($P < 0.05$).

in CYP2D6 and CYP2C19 are found in all ethnic populations [49], though some are specific for only one or a few populations. When individuals carry genetic mutations that decrease their CYP2D6 or CYP2C19 activity, these individuals in an examined population will likely have low CYP2D6 or CYP2C19 activities regardless of how high their POR activity is. In Fig. 2b and 2c, there are numerous individuals who have normal range, or even high POR activity, but very low CYP2D6 or CYP2C19 activity. These samples are from individual potentially

carrying genetic mutations in CYP2D6 or CYP2C19 gene which need to be analyzed.

Our data show that the POR gene is quite polymorphic in the study population. Within the 7.7 kb genomic area covering all POR exons and surrounding introns, we identified 34 SNPs, in which nine are common with a minor allele frequency of $> 10\%$, 11 are rare with a minor allele frequency of $< 1\%$, and 14 are in-between ($1-10\%$) (Table 4). The common SNPs include three exonic polymorphisms (rs1135612 A $>$ G P129P, rs1057863 C $>$ T A503V, and rs1057870 G $>$ A S572S), but only rs1057863 results in an amino acid change, from alanine to valine, at position 503. A503V is a conservative change in an unstructured loop of the FAD-binding domain. The amino acid replacement from alanine to valine at 503 results in a minor decrease in cytochrome c reduction and P450C17 hydroxylase [17]. Because of the functional importance of the POR protein, we would not expect there to be common SNPs existing in the POR gene that could significantly affect POR functions. All the common SNPs ($> 10\%$) identified in the study samples are not associated with decreased POR activity.

Naturally existing POR mutations have been identified in POR deficiency patients [15–18], including the missense or frameshift mutations of T142A, Q153R, Y181D, M263V, A287P, R457H, Y459H, V492E, G539R, L565P, C569Y, Y578C, V608F, R616X, F646del, I444fsX449, and L612W620delinsR. Site-mutagenesis experiments demonstrated that mutations in the FMN (Q153R, Y181D), FAD (A287P, R457H, Y459H, V492E), and

Table 7 Comparison of POR activity with P450 activities and POR mRNA level in wild-type samples with L577/L577 and mutant samples with L577/P577

Enzyme activity (pmol/mg protein/min)	Ninety-five samples with wild-type L577/L577	Sample L099 L577/P577	Sample L078 L577/P577	Sample L059 L577/P577	Sample L080 L577/P577	Linear regression
	Activity mean (%)	Activity (%)	Activity (%)	Activity (%)	Activity (%)	<i>P</i> value ^a
POR activity	181 000 (100)	505 (0.2)	82 000 (45)	110 000 (61)	139 000 (77)	0
CYP1A2	52 (100)	NPD	8 (16)	15 (29)	33 (63)	<0.001
CYP2A6	1016 (100)	6 (0.6)	209 (21)	306 (30)	402 (40)	0.036
CYP2B6	110 (100)	10 (9.1)	29 (26)	40 (36)	48 (43)	0.006
CYP2C8	350 (100)	NPD	NPD	82 (24)	186 (53)	0.036
CYP2C9	1826 (100)	127 (7.0)	1540 (84)	1350 (74)	2750 (151)	0.032
CYP2C19	100 (100)	NPD	91 (91)	119 (119)	95 (95)	0.078
CYP2D6	312 (100)	25 (8.0)	274 (88)	270 (87)	292 (94)	0.071
CYP2E1	2146 (100)	201 (9.4)	358 (17)	2400 (112)	1900 (89)	0.120
CYP3A4/5	3504 (100)	NPD	207 (6)	268 (8)	1800 (51)	0.014
CYP4A9/11	1726 (100)	NPD	805 (47)	987 (57)	710 (41)	0.087
POR mRNA	0.195 (100)	0.018 (9.2)	0.279 (143)	0.128 (66)	0.468 (240)	0.083

^a*P* value for linear regression between POR activity and each P450 activity or between POR activity and POR mRNA. Owing to abnormal distribution of P450 activities in this liver cohort, the linear regression was conducted with logarithm transformation of the P450 activities. Samples with NPD are considered as 0 after logarithm in the linear regression.

NPD, no product detected; POR, cytochrome P450 oxidoreductase.

NADPH (G539R, L565P, C569Y, Y578C, V608F, R616X) binding domains had the most influence on POR functions [17,50]. Some mutations ablated virtually all measurable POR activity and caused serious human diseases. These mutations, however, occur at very low frequency. As expected, none of these POR deficiency mutations were detected in the study population.

Three novel nonsynonymous polymorphisms, which result in amino acid changes at K49N, L420M, and L577P, are identified in this study. K49N resides in the amino-terminal tail, L420M in the connecting domain, and L577P in the NADPH-binding domain. Molecular modeling predicts that L420M does not change secondary or 3D structure of POR protein, but replacement of P577 prevents Y576 from participating in an α helix and may disrupt hydrogen bonding with a water molecule, destabilizing NADPH binding in POR 3D configuration. The 3D configuration is very important for electron flow from NADPH through POR to P450. Crystal structure of rat *Por* [42] shows that *Por* contains two distinct regions, one containing the NADPH-binding site and the FAD-binding domain, and the other containing the FMN domain that eventually interacts with the redox-partner binding site of P450. NADPH attaches to the NADPH-binding site where it releases a hydride ion to FAD and becomes NADP⁺. The NADPH-binding site and FAD-binding domain must be within 4Å for this reaction to proceed [41]. Kinetics of NADPH binding, electron pass, and NADP⁺ release in POR are important for both POR and P450 enzyme functions. Genetic mutations in the NADPH-binding site (G539R, L565P, Y578C, and V608F), which decrease cytochrome *c* reduction and P450 (CYPC17 and CYPC21) activities, have been identified in the POR deficiency patients [17,18]. In this report, we demonstrate that L577P, which is adjacent to Y578C, correlates with decreased POR activity (cytochrome *c*

reduction) and influences most drug-metabolizing P450 enzymes, except CYP2D6, CYP2C19, and CYP2C8 (Fig. 6). Less correlation in CYP2D6 and CYP2C19 may be again due to genetic polymorphisms in these P450 genes. We have also shown that the POR mutation that decreases POR activity does not necessarily lead to the POR deficiency disease. The mechanism as to how L5577P decreases POR activity merits further investigation.

L577P has a minor allele frequency of 0.02 in the study population. Its allele frequency in the general population needs to be investigated. We expect that individuals carrying the homozygous mutation of P577/P577 have decreased ability to metabolize almost all drugs primarily catalyzed by microsomal P450 enzymes. This hypothesis, however, needs to be confirmed in future studies. It is difficult to predict POR activity in an individual who carries heterozygous L577/P577, because one copy of the POR gene encodes normal POR and another encodes mutant POR. Large interindividual variations of POR activity were observed in four samples with heterozygous L577/P577 in this study (Table 7). Sample L099 had extremely low POR activity (< mean - 2 SD), samples L078 and L059 had low POR activity (between - 2 SD and - 1 SD), and sample L080 had normal POR activity (between - 1 SD and + 1 SD). In these samples, most P450 enzyme activities correlated with POR activity.

The variation in POR activity among the samples with same L577/P577 genotype lead us to hypothesize that the interindividual variations of POR activity are not solely dependant on genotype but may partially be due to variation of POR gene expression either among or within individuals. Among individuals in a group, total POR expression may vary considerably, as POR mRNA varied 26 f in our four samples with the P577 mutation. Additionally, imbalanced allelic variation of POR gene

expression may exist between the L577 and P577 alleles in the individuals. Imbalanced allelic variation of gene expression is a common phenomenon existing in more than 50% of human genes [51,52]. The expression ratio of normal to mutant alleles may vary from person to person. An individual who expresses higher level of the normal allele will ultimately have a greater enzyme activity than one who expresses more of the mutant allele. Therefore, quantification of POR gene expression from the normal L577 allele will provide more accurate correlation with the POR activity in comparison to quantification of total POR mRNA levels from both L577 and P577 alleles. Genetic polymorphisms in the promoter, 5'-untranslated region, 3'-untranslated region, and introns, all can affect gene expression at levels of transcription, splicing, translation, RNA stability or protein modification and contribute to imbalanced allelic gene expression. A comprehensive analysis of genetic polymorphisms in the entire POR gene is needed to further understand influence of genetic polymorphisms on gene expression and functions of POR.

An intronic polymorphism, rs41301427, was found to be associated with a decrease in POR activity. The mechanism for the decrease of POR activity is unclear. Degree to which it associates with decreasing POR activity (coefficient estimate = -32.409 and $P = 0.030$), however, is much less significant than the L577P (coefficient estimate = -101.152 and $P = 0.003$). Such a small change may help to explain why rs41301427 did not influence P450 activities.

Overall, our data demonstrate several novel findings important for studying drug metabolism. First, cytochrome P450 activities are significantly correlated with POR activity, and we propose that POR can be a rate-limiting step in P450-mediated catalysis. Second, we have predicted the impact of novel nonsynonymous polymorphisms identified in the study population on protein structure and 3D configuration. Finally, we have identified and characterized several known and novel polymorphisms, including L577P, which decreases POR activity and subsequent P450-catalyzed drug metabolism, but is not associated with the POR deficiency disease. Such discoveries highlight the importance of POR on drug metabolism and suggest a potential biomarker for pharmacogenomic screening.

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