DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 69849

Title Page

Correlation of cytochrome P450 oxidoreductase expression with the expression of 10 isoforms of cytochrome P450 in human liver

Hai-Feng Zhang, Zhi-Hui Li, Jia-Yu Liu, Ting-Ting Liu, Ping Wang, Yan Fang, Jun Zhou, Ming-Zhu Cui, Na Gao, Xin Tian, Jie Gao, Qiang Wen, Lin-Jing Jia, Hai-Ling Qiao

Institute of Clinical Pharmacology, Zhengzhou University, Zhengzhou, People's Republic of China

Running Title Page

Running title: Correlation of POR and CYP expression in human liver

Corresponding author

Hai-Ling Qiao, Professor

Institute of Clinical Pharmacology, Zhengzhou University

40 Daxue Road, Zhengzhou, 450052, China

Tel: 86-371-6665-8363; Fax: 86-371-6665-8363

Email: qiaohl@zzu.edu.cn

Number of text pages: 32

Number of tables: 3

Number of Figures: 7

Number of references: 37

Number of words in *Abstract*: 249

Number of words in *Introduction*: 749

Number of words in *Discussion*: 1493

Abbreviations: HLM, human liver microsomes; SNP, single nucleotide polymorphism; POR, cytochrome P450 oxidoreductase; CYP, cytochrome P450; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; PXR, pregnane X receptor; CAR, constitutive androstane receptor; HNF4α, hepatocyte nuclear factor 4 alpha.

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Human cytochrome P450 oxidoreductase (POR) provides electrons for all microsomal cytochromes P450 (CYP) and plays an indispensable role in drug metabolism catalyzed by this family of enzymes. We evaluated 100 human liver samples and found that POR protein content varied 13-fold, from 12.59 to 160.97 pmol/mg, with a median value of 67.99 pmol/mg; POR mRNA expression varied by 26.4-fold. POR activity was less variable with a median value of 56.05 nmol/min/mg. Cigarette smoking and alcohol consumption clearly influenced POR activity. Liver samples with a *2286822 TT* genotype had significantly higher POR mRNA expression than samples with *CT* genotype. Homozygous carriers of POR*2286822 C>T*, *2286823 G>A*, and *3823884 A>C* had significantly lower POR protein levels compared with the corresponding heterozygous carriers. Liver samples from individuals homozygous at *286823G>A*, *1135612 A>G*, and *10954732 G>A* generally had lower POR activity levels than those from heterozygous or wild-type samples, whereas the common variant POR**28* significantly increased POR activity. There was a strong association between POR and the expression of all 10 CYP isoforms at the mRNA and protein level, whereas the relationship at the activity level, as well as the effect of POR protein content on CYP activity, was less pronounced. POR transcription was strongly correlated with both HNF4α and PXR mRNA levels. In conclusion, we have elucidated some potentially important correlations between POR SNPs and POR expression in the Chinese population and have developed a database that correlates POR expression with the expression and activity of 10 CYPs important in drug metabolism.

Introduction

Human cytochrome P450 oxidoreductase (POR) is a 78-kDa di-flavin protein anchored to the cytoplasmic side of endoplasmic reticular membranes (Xia et al., 2011). POR provides electrons to a variety of substrates including all the microsomal cytochromes P450 (CYP), heme oxygenase, squalene monooxygenase, and cytochrome b_5, as well as reducing some therapeutic prodrugs (Miller et al., 2011; Huang et al., 2015).

To date, few studies have characterized the variation in the expression and activity of POR in human liver samples, with only four studies measuring POR content in human samples. A study by Gomes *et al*. measured mRNA, protein and activity of POR in a cohort of liver samples, but the distribution and range of POR mRNA and protein were not reported (2009). A study by Gan *et al*. provided both spectral and western blot analysis of absolute POR content, but there was a 3-fold difference in the mean value as measured by these two methods (2009). Recently, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)-based proteomics for absolute quantification of protein has been developed (Picotti et al., 2009). Two studies reported the absolute content of POR using LC-MS/MS, but one assessed only a small number of liver samples (Ohtsuki et al., 2012) and the other determined the POR protein expression in a CYP2C19 genotype-defined pooled of human liver microsomal (HLM) samples (Shirasaka et al., 2015). Therefore, determination of the mRNA and protein content and activity of POR across a large number of normal human liver samples is needed in order to provide reliable physiological parameters for POR expression in physiological, pharmacological, and toxicological research.

Although humans have only a single POR gene, it is highly polymorphic. More than 200

mutations and polymorphisms in the POR gene have been reported (Pandey and Flück, 2013). However, very little is known about the effects of POR SNPs on its expression and activity in HLM. Of the 34 identified POR polymorphisms in HLM, only two (L577P and SNP *41301427 G>A*) were associated with altered POR activity (Hart et al., 2008). Gomes *et al*. reported 46 POR SNPs in HLM and found that only one SNP (*17148944G>A*) directly affected POR mRNA levels and none affected POR protein levels or activity (Gomes et al., 2009).

CYP is responsible for the metabolism of many endogenous and exogenous substances including 70–80% of all drugs currently in clinical use (Zhang et al., 2015a), and there are large individual variations in CYP-mediated drug metabolism. All microsomal CYPs require the input of two electrons for catalysis and POR is the sole donor for the first electron (Bridges et al., 1998). Thus, POR is indispensable in metabolic reactions catalyzed by CYP. Several *in vitro* and *in vivo* studies have revealed that polymorphisms that affect POR activity can have differing effects on CYP activities, depending on the specific POR mutation, CYP isoform, and the substrate used to assay activity, and hence the activity of a POR variant with one CYP does not predict its activity with other CYPs (Yang et al., 2011; Chen et al., 2012). Therefore, the impact of a particular POR mutant needs to be studied individually with each CYP. However, the effect of POR protein content on CYP protein content or activities has not been reported to date.

Though POR plays a vital role in drug metabolism, the transcriptional regulation of the POR gene by xenobiotic receptors has not been fully described. Receptor-selective agonists for the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) induced

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

POR mRNA expression in mouse liver, whereas in human hepatocytes only PXR activators could upregulate POR expression (Maglich et al., 2002). One study has reported that POR expression was associated with the expression level of CAR and hepatocyte nuclear factor 4 alpha (HNF4α) in human livers (Wortham et al., 2007). Thus, it is appropriate to characterize the expression levels of various regulatory factors and determine to what extent they correlate with POR expression.

In this study, 125 liver samples were collected and used to determine the absolute POR protein content by LC-MS/MS, POR mRNA expression, and POR activity. POR SNPs occurring with a frequency of >1% in Chinese populations were used to analyze the effect of these SNPs on POR protein content, mRNA levels, and activity. The distribution of POR protein and mRNA was assessed and relationships between POR expression and the expression of 10 CYPs involved in drug metabolism at the protein, mRNA, and activity levels were analyzed. In addition, the regulation of POR expression in human livers was explored.

Materials and Methods

Human liver samples and liver microsomes. Human liver samples were obtained from 125 Chinese patients undergoing hepatic surgery during 2012 and 2014 at the First Affiliated Hospital of Zhengzhou University, the People's Hospital of Henan Province and the Tumors' Hospital of Henan Province. Detailed information for each patient was obtained including gender, age, body height, body weight, smoking habits, alcohol consumption, clinical diagnosis, regular drug intake before surgery, previous history, allergic history, pathological diagnosis, imaging examination and laboratory test data as described previously (Zhang et al., 2015b). The study was approved by the ethics committees of Zhengzhou University and written informed consent was obtained from each patient. All experiments were performed in accordance with the Declaration of Helsinki. Samples from normal livers were collected, with liver health confirmed by liver function tests, histopathological analysis, and imaging examination by ultrasonography or CT. All liver samples were frozen immediately after removal and stored in liquid nitrogen until use. HLMs were prepared by differential centrifugation, and total HLM protein concentration was determined by the Bradford method.

Measurement of POR, HNF4*α* and PXR mRNA levels in human liver. Primers for POR was designed by the TaKaRa Bio Inc. (Japan) and other primers were from the literature (Table 1) (Wang et al., 2011). mRNA levels were measured as described previously (Zhang et al., 2015a). Briefly, total RNA was isolated from human liver samples using an RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions. The cDNA for qRT-PCR was synthesized from 1 μg total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time; TaKaRa). CYP mRNA expression was detected by two-step qRT-PCR

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). GAPDH was used as a reference gene, and expression of target mRNA was calculated using the $2^{-\Delta CT}$ method (ΔCT equals the difference between target gene and GADPH).

Quantification of POR protein content in HLM.

Preparation of a QconCAT protein. Protein quantitation of POR was performed by nano-LC-MS/MS using our previously established quantitative concatemer (QconCAT) strategy combined with stable isotope dilution-multiple reaction monitoring (SID-MRM) (Wang et al., 2015). Briefly, two signature peptides (GVATNWLR and FAVFGLGNK) were selected to quantify POR based on a genome wide BLAST search. QconCAT proteins were designed as a concatemer of all the stable isotope-labeled signature peptides. After prokaryotic expression, the QconCAT protein was purified using affinity chromatography and evaluated by matrix-assisted laser desorption ionization-time-of-flight MS (Beynon et al., 2005).

Protein digestion. HLM proteins were denatured, reduced, alkylated, diluted with 7 volumes of 50 mM NH_4HCO_3 solution and digested with trypsin at a trypsin: substrate ratio of 1:50 at 37 °C for 26 h. The digested QconCAT protein was examined by Fourier Transform-Linear Ion Trap (LTQ) Ion Cyclotron Resonance MS (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Quantification of the QconCAT protein. The peptides ASGNLIPQEK and TILDELVQR that composed the QconCAT protein were used to determine the protein using a nano-high performance LC coupled to multiple reaction monitoring MS analysis. The limit of quantitation, linear range, and concentration of the QconCAT protein were calculated and

reported in our previous work (Wang et al., 2015).

Nano-LC-MS/MS quantitative analysis. The concentration of CYP protein was determined by nano-LC-multiple reaction monitoring MS using an easy nano-LC coupled to a TSQ Vantage™ Triple Quadrupole mass spectrometer (Thermo Fisher Scientific Inc.). Samples were first loaded separately on a trap column (100 μm × 20 mm) packed with SP-300-ODS-AP

(5 μm particle diameter; 100 nm pore size in house), then each sample was eluted into an analytical column (75 μm ×11 mm) also packed with SP-300-ODS-AP and separated at a flow rate of 300 $nl \cdot min^{-1}$ with an elution gradient consisting of mobile phase B (99.9% acetonitrile, 0.1% formic acid) and mobile phase A (99.9% H_2O, 0.1% formic acid). Elution gradient solutions were added as follows: B was increased from 2% to 10% in 5 min, 10% to 40% in 60 min, and 40% to 95% in 5 min, followed by automatic equilibration of the LC system with mobile phase A for approximately 10 min before the next analysis. Fractions were continuously directed into the TSQ Vantage™ Triple Quadrupole mass spectrometer with a nanoelectro-spray ionization source at a capillary temperature of 240 °C and spray voltage of 1900 V. Three transitions were selected per peptide for the quantification of each protein with the following MS conditions: Q1 and Q3 resolution, 0.7 Da full width at half maximum; Q2 pressure, 1.5 mTorr (Ar); cycle time, 1.5 s; collision energy, 0.034 × precursor ion m/z + 3.314.

Determination of POR activity in HLM. The POR activity assay is based on the rate of cytochrome C reduction by liver microsomes (Guengerich et al., 2009). The reaction was conducted in a 200 μl volume with 0.3 M potassium phosphate buffer (pH 7.7), 0.2 mM horse

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

cytochrome C, and 5 μg microsomal protein. Reactions were initiated by the addition of 20 μl of 10 mM NADPH to a 200 μl assay mixture for a total volume of 220 μl. The rate of cytochrome C reduction was determined from the rate of increase in absorbance at 550 nm produced by the reduced form of cytochrome C using a BioTek Synergy H1MD Multi-Mode microplate reader in the kinetic mode before and after the addition of NADPH (0-5 min).

Genotypes of POR. Genomic DNA was isolated from human liver tissue using a genomic DNA purification kit (Beijing ComWin Biotech Co.,Ltd., China). Polymorphisms in POR with frequencies of > 1% in the Chinese population were genotyped in this study sample. A total of 18 SNPs in the POR gene were detected. All the POR SNPs were determined by Sequenon method except SNP 3823884*A*>*C* and SNP 2302433*C*>*T* (by PCR-sequencing).

Determination of CYP metabolic activities in HLMs. Incubation mixtures contained single concentration of substrate (400μM phenacetin for CYP1A2, 20μM coumarin for CYP2A6, 500μM bupropion for CYP2B6, 40μM paclitaxel for CYP2C8, 1500μM tolbutamide for CYP2C9, 250μM omeprazole for CYP2C19, 320μM dextromethorphan for CYP2D6, 500μM chlorzoxazone for CYP2E1 and 50μM midazolam for CYP3A4/5), HLMs (0.3 mg protein/ml for CYP1A2, CYP2A6, and CYP2E1; 0.2 mg protein/ml for CYP2D6 and CYP3A; 0.5 mg protein/ml for CYP2B6, CYP2C8, CYP2C9, and CYP2C19), and 1 mM NADPH. The mixture was pre-incubated for 5 min at 37 ℃. Optimal incubation times for each substrate were as follows: 30 min for phenacetin O-deethylation, coumarin 7-hydroxylation, and chlorzoxazone 6-hydroxylation; 60 min for bupropion 1-hydroxylation, and tolbutamide 4-hydroxylation; 90 min for omeprazole 4-hydroxylation, 120 min for paclitaxel 6-hydroxylation; 20 min for dextromethorphan O-demethylation; and 5 min for midazolam

1′-hydroxylation.Each reaction was terminated after specified incubation period by adding 20μl ice-cold acetonitrile or 1ml ethyl acetate or perchloric acid and metabolite concentrations were determined by HPLC-UV or HPLC-FLD. The detailed analysis and results of CYP activities were reported in our previous work (Zhang et al., 2015b).

Relationships between POR and 10 CYPs at the mRNA, protein content, and activity levels. CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were the most important members of CYP family. The mRNA levels (by qPCR), protein content (by LC-MS/MS), and activities of the 10 CYPs were determined in the same set of human liver samples. These data and the analytical methods have been submitted in detail separately (Wang et al., 2015). In this study we used simple linear regression approaches to evaluate the relationship between POR and the 10 CYPs at the mRNA, protein, and activity levels with the goal of identifying possible contributions of POR to CYP phenotypic variation. In addition, we evaluated the effect of POR protein content on the 10 CYP activities.

Statistical Analyses. The normality of the data distribution was checked using Kolmogorov-Smirnov and Shapiro-Wilk methods. Since most data sets were not normally distributed, nonparametric methods were generally used for statistical analyses. The Mann-Whitney U test was used for pairwise comparison and the Kruskal-Wallis H test was used for multiple pairwise comparisons. Non-parametric Spearman rank correlation analysis was performed to calculate the correlation coefficient (r). A P value < 0.05 was considered statistically significant (two-tailed). SPSS statistics 17 software was used for data management and statistical analyses. Graphs were generated using GraphPad Prism 5.04

software.

Results

1. mRNA, protein and activity of POR in human livers

1.1 POR mRNA

The values for POR mRNA expression in 107 liver samples were not normally distributed (Figure. 1A). The relative POR mRNA expression level varied 26.4-fold (range: 0.05-1.25), with a median value of 0.28. The levels of POR mRNA at the 2.5th and the 97.5th percentiles were 0.05 and 0.83, respectively, exhibiting about a 16-fold variation. As shown in Figure. 1A, there was one liver sample that had an extremely high POR mRNA level (1.25).

1.2 POR protein content

Similar to POR mRNA, the protein content of POR in 100 liver samples also was not normally distributed, with a median of 67.99 pmol/mg. The lowest and highest content of POR protein was 12.59 and 160.97 pmol/mg, displaying a 12.8-fold variation (Figure. 1B). The variation in POR protein levels at the 95% prediction interval (PI) was 10.6-fold. Two samples exhibited extreme POR protein content (12.59 and 160.97 pmol/mg, respectively).

1.3 Activity of POR

Compared with mRNA and protein, POR activity was less variable: a 4.7-fold range (26.91-125.77 nmol/min/mg) was observed across 125 human liver microsomes, with a median value of 56.05 nmol/min/mg (Figure. 1C). The variation in POR protein content at 95% PI was 3.6-fold.

1.4 Correlations in POR mRNA, protein and activity in human livers

Spearman correlation analysis was used to identify correlations and the results showed that

POR protein content was significantly correlated with POR activity (r=0.368, *P*=0.000). However, we failed to detect significant correlations between POR mRNA and protein level, or POR mRNA and POR activity (Figure. 2).

1.5 Effect of demographic factors on POR mRNA, protein and activity

POR mRNA, protein and activity data were stratified by liver donor age, gender, smoking habit, alcohol consumption and tissue resource and then analyzed by Mann-Whitney U test or Kruskal-Wallis test. As shown in Figure 3A, POR activity was significantly higher in nonsmokers than in smokers (*P*=0.041). Statistically significant differences were also observed between drinkers and nondrinkers (*P* =0.012) (Figure. 2B). The other demographic factors had no noticeable effect on POR mRNA, protein or activity levels (*P*>0.05) (**Supplemental Table 1)**.

2. Influence of POR gene polymorphisms on POR mRNA, protein and activity in human livers

2.1 Gene polymorphisms of POR

As shown in Table 2, the variant frequencies of 18 SNPs in the POR gene ranged from 0.91% (for *17148944G>A*) to 88.2% (for *2302431T>C*), in which the frequency of the common mutant *1057868 C>T (*28)* was 35.5%. This is consistent with previously published results on the Chinese population (Huang et al., 2008). All 18 POR SNPs were evaluated for their effect on POR mRNA, protein and activity levels with the positive results shown in Figure. 4-6.

2.2 Influence on mRNA level

Only SNP *2286822C>T* of POR had an impact on mRNA expression. Samples with *2286822*

TT genotype had significantly higher POR median mRNA levels than samples with the *CT* genotype (*P*=0.025). (Figure. 4).

2. 3 Influence on protein content

Three POR SNPs (*2286822 C>T*, *2286823 G>A*, and *3823884 A>C*) had an influence on POR protein content with similar effects. The homozygous carriers of POR *2286822 C>T*, *2286823 G>A*, and *3823884 A>C* had significantly lower protein levels compared with the corresponding heterozygous carriers (Figure. 5).

2. 4 Influence on activity

As shown in Figure. 6, individuals who exhibited the POR *2286822 TT* (*C>T*) genotype had lower hepatic POR activity as compared to *2286822 CC* carriers. Individuals genotyped as *286823 AA* (*G>A*) had lower POR activity than those carrying the *286823 GG* and *GA* genotypes. Similarly, *1135612 GG* (*A>G*) and*10954732 AA* (*G>A*) carriers also showed significantly decreased POR activity as compared to corresponding wild-types as well as heterozygous individuals. However, POR activity in the *1057868 CT* (*C>T*) group was higher than that of wild-type group. Meanwhile, there was a tendency toward increased POR activity in *1057868 TT* carriers compared with wild-type and heterozygous carriers, but it did not reach statistical significance.

3. Correlation between POR and CYP at the mRNA, protein and activity levels

The mRNA, protein and activity levels of 10 CYPs were simultaneously quantified with POR expression and activity in the same set of 100 HLMs (data published separately). Spearman correlation analysis was used to determine the correlation between POR and the 10 CYPs at the mRNA, protein, and activity levels. As shown in Table 3, significant correlations were

observed between POR and all 10 CYPs at the mRNA level (P<0.05). There also were significant associations between POR protein content and all CYP isoform content except with CYP2B6. Strong correlations were found between POR protein content and CYP protein content for CYP2C8 and CYP2C9 (r>0.8, P<0.001). For CYP2E1 and CYP3A4 the correlation coefficient reached 0.6. However, the association between POR and CYPs at the activity level was relatively poor. POR activity was positively associated with CYP2C19 and negatively associated with CYP2C8 activity. In addition, significant associations were found between POR content and the activities of 4 CYPs (CYP2B6, CYP2C8, CYP2C19, and CYP2E1) (P< 0.05).

4. Expression of HNF4α and PXR and their relationship with POR in human livers

Both HNF4α and PXR mRNA were determined together with POR by qPCR in the same set of 107 human liver samples. Neither HNF4α nor PXR mRNA was normally distributed among the 107 patient samples, with an overall variation of 62.8- and 85.3-fold, respectively. The mRNA levels of both HNF4α and PXR were strongly correlated to the POR transcript levels in all samples (r=0.707, P=0.000; r=0.718, P=0.000; Figure. 7).

Discussion

This is the first extensive study to quantify the absolute content of POR protein by LC-MS/MS in a large number of normal Chinese liver samples. The not-normally distributed POR values in 100 samples varied from 12.59 to160.97 pmol/mg with a median of 67.99 pmol/mg. The mRNA expression level and activity of POR was measured in liver tissues and microsomes, respectively, and showed 26.4- and 4.7-fold variations, respectively. Cigarette smoking and alcohol consumption clearly influenced POR activity. Several of the 18 SNPs

analyzed had a significant impact on POR expression or activity, and there were significant associations between the POR mRNA level and the 10 CYP mRNA levels, between POR protein content and the protein content of 9 CYPs, and between POR protein content and the activity of 4 CYPs. In addition, POR transcription was strongly correlated with both HNF4α and PXR mRNA levels in these samples.

Although POR is an enzyme important to physiology, pharmacology, and toxicology, the information concerning POR expression and activity is limited. Gomes *et al*. (2009) reported a mean POR content of 60.0±29.6 pmol/mg for female and 51.9±29.9 pmol/mg for male Caucasian human liver samples by Western blotting, although there was no statistical difference in the expression between genders. In Gan *et al*.'s study (2009), POR concentration as measured by immunoblotting was 28±15 pmol/mg, whereas in the same set of HLMs mean POR content was 86±35 pmol/mg as measured by spectrophotometry. The average POR content of 70.67±35.79 pmol/mg based on LC-MS/MS from 100 liver tissues in our study was consistent with the result of Ohtsuki *et al* (71.6±17 pmol/mg) (2012) by LC-MS/MS. However, due to the relatively small sample size (n=17), Ohtsuki *et al.* only observed a 2.4-fold variation in POR content, while the variation in our study was as large as 12.8-fold. The mean POR protein content for pooled HLMs from donors with different CYP2C19 diplotypes by LC-MS/MS was 21.8±11.4 pmol/mg to 31.6±16.4 pmol/mg (Shirasaka et al., 2015), which is much smaller than the results we obtained here. It is possible that the discrepancy is due to the different ethnicities or the pooled HLMs used in that study.

In two studies on POR mRNA levels (Wortham et al., 2007; Gomes et al., 2009), the largest variation was 18.3-fold, whereas a 26.4-fold difference was observed by our group

(Figure. 1). A linear trend analysis displayed no significant correlation between POR mRNA and protein levels (Figure. 2), indicating that POR expression could be regulated post-transcriptionally, consistent with a study showing that miR-214 down-regulates POR expression (Dong *et al*., 2014).

Two earlier studies reported mean POR activities of 139.0 nmol/min/mg and 177±53 nmol/min/mg in Caucasian human liver samples (Gomes et al., 2009; Hart et al., 2008). In the present study, we measured POR activity in 125 Chinese human liver samples and found that POR activity was not normally distributed. The median POR activity was 56.05 nmol/min/mg (Figure. 1C), which is lower than the abovementioned results. Compared to mRNA and protein expression, POR activity was less variable with only a 4.7-fold difference in this study cohort, which is consistent with the result of Gomes *et al.* (2009).

Although POR is highly polymorphic, the effect of common polymorphisms on POR expression and activity are minimal. Of the 46 SNPs analyzed, only POR *17148944*G>A was related to a decrease in POR mRNA (Gomes et al., 2009). We failed to detect such an effect, but we found that POR *2286822C>T* mutant was associated with increased POR mRNA expression (Figure. 4). We found that POR mutants correlated with altered POR protein content.

The mutant homozygous carriers of POR *2286822C>T*, *2286823G>A*, and *3823884A>C* had significantly lower protein levels compared with the corresponding heterozygous carriers (Figure. 5). In addition, the effect of SNPs *286823G>A*, *1135612A>G* and *10954732G>A* on POR activity were consistent. Homozygous *286823G>A*, *1135612A>G* and *10954732G>A* mutants generally had lower POR activity than heterozygous mutants and wild-type samples.

However, the common SNP*1057868 C>T* (also known as POR**28* mutant) increased POR activity, although the activity difference between *TT* and *CC* groups was not statistically significant (Figure. 6). Both Gomes *et al*. and Hart *et al* measured the effect of SNPs *286823G>A*, *1135612A>G*, and *286823G>A* on POR activity in Caucasian populations (Hart et al., 2008; Gomes et al., 2009), but the differences were not statistically significant. However, Hart *et al.* found that L577P and SNP *41301427G>A* were associated with decreased POR activity. No L577P mutant was found in our or in Gomes *et al.* studies and we conclude that L577P is a rare mutant.

Of the five SNPs that affected POR expression and activity, both *2286822C>T* and *2286823 G>A* are intronic mutations, whereas *3823884A>C* is located in the 5'-untranslated region. Emerging evidence indicates that non-coding genetic variants play an important role in gene regulation by influencing the transcriptional activity, splicing efficiency, or altering the splicing site of their host genes (Cooper et al., 2010). We also found that a common nonsynonymous variant of POR SNP *1057868 C>T* increased POR activity, whereas no significant association of this polymorphism with microsomal POR activity was identified in either Gome *et al.*'s or Hart *et al.*'s studies. The frequency of POR SNP*1057868 C>T* was 35.5% in this cohort, while relatively lower frequencies (19.1%, 21.9%, 26.4%, and 30.3%) were observed in a Western population (Huang et al., 2008). Therefore the discrepancy may be due to the different frequencies of the POR *1057868 C>T* variant in different ethnic groups. An *in vivo* study in healthy Chinese volunteers showed that individuals with the *POR 1057868 C>T* variant were associated with 1.6-fold higher hepatic CYP3A activity, which is in agreement with our result (Yang et al., 2011).

We found that strong correlations existed between POR and 9 CYPs at the level of protein expression. POR mRNA levels were correlated with all 10 CYP mRNA levels. The strong association between POR and CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 at the mRNA level was also observed in an earlier study (Wortham et al., 2007). POR, together with all microsomal CYPs, constitutes a complex system responsible for the biosynthesis and degradation of endogenous and exogenous substances. The high correlation between POR and CYPs at the protein and mRNA levels provides compelling evidence that the expression of POR and CYPs is co-regulated. The expression of metabolic enzymes is frequently regulated coordinately by a network of transcription factors such as HNF4α, PXR, and CAR. The strong association between POR and HNF4α and PXR in this study cohort (Figure. 7) suggests that POR expression is controlled, at least in part, by these transcription factors and, importantly, may partly explain the co-regulation of POR and CYPs.

Though the expression of POR and CYPs was highly correlated at protein and mRNA levels, the correlation at the level of activity was less pronounced. POR activity was significantly associated with two CYP activities (CYP2C19 and CYP2C8). This is inconsistent with the result by Hart *et al*., in which POR activity was significantly associated with all the analyzed CYP activities including CYP 2C19 and CYP1A2 (2008). However, it should be noted that the marker substrates we used for CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 and CYP3A4/A5 were different from those used by Hart *et al*. It may be that the effect of POR activity on microsomal CYP activities is CYP- and substrate-dependent. In addition to POR, other factors such as demography, genetic polymorphisms, protein-protein interactions, and epigenetic regulation can also affect the activity of CYPs.

The weaker correlation between POR and CYP activities hints that POR is not the sole factor that determines CYP activities and the correlation between POR and a specific CYP cannot be extrapolated to other CYPs or even the same CYP with a different substrate.

POR content was positively correlated with CYP2C19 and CYP2E1 activities (Table 3). However, a negative correlation was observed between POR content and CYP2B6 and CYP2C8. As only a single POR protein is required to donate electrons to all the microsomal CYPs, the protein-protein interaction likely differs between POR and different CYPs, and therefore a negative correlation may be reasonable and deserves further investigation.

In conclusion, this is the first report to comprehensively analyze the protein content, mRNA level, and enzyme activity, as well as the effects of gene polymorphisms, on POR expression and activity in a Chinese population. There are large individual variations at the mRNA and protein level, whereas POR activity is less variable. Some SNPs have a significant effect on POR expression and activity, including the common variant POR**28*. Strong correlations between POR and 10 CYPs at the protein and mRNA levels in human livers hint that POR and CYPs are co-regulated, and this can be explained at least in part by the collinearity of expression of POR and HNF4α and PXR. A poor correlation between POR and the 10 CYPs at the level of activity indicates that POR is not the sole determinant of CYP activity. These findings provide important physiological and functional database for POR expression and highlight the complex role POR plays in CYP-mediated metabolism.

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Qiao, Zhang.

Conducted the experiments: Zhang, Jia-Yu Liu, Ting-Ting Liu, Wang, Fang, Na Gao, Tian.

Contributed new reagents or analytic tools: Zhou, Cui, Jie Gao, Wen, Jia.

Performed data analysis: Zhang, Li, Jia-Yu Liu.

Wrote or contributed to the writing of the manuscript: Zhang, Li.

References

Beynon RJ, Doherty MK, Pratt JM, and Gaskell SJ (2005) Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. *Nat Methods* **2:**587-589.

Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G, and Waskell L (1998) Identification of the binding site on cytochrome P450 2B4 for cytochrome b5 and cytochrome P450 reductase. *J Biol Chem* **273:**17036-17049.

Chen X, Pan LQ, Naranmandura H, Zeng S, and Chen SQ (2012) Influence of various polymorphic variants of cytochrome P450 oxidoreductase (POR) on drug metabolic activity of CYP3A4 and CYP2B6. *PLoS One* **7:**e38495.

Cooper DN (2010) Functional intronic polymorphisms: Buried treasure awaiting discovery within our genes. *Hum Genomics* **4:**284-288.

Dong X, Liu H, Chen F, Li D, and Zhao Y (2014) MiR-214 promotes the alcohol-induced oxidative stress via down-regulation of glutathione reductase and cytochrome P450 oxidoreductase in liver cells. *Alcohol Clin Exp Res* **38:**68-77.

Gan L, von Moltke LL, Trepanier LA, Harmatz JS, Greenblatt DJ, and Court MH (2009) Role of NADPH-cytochrome P450 reductase and cytochrome-b5/NADH-b5 reductase in variability of CYP3A activity in human liver microsomes. *Drug Metab Dispos* **37:**90-96.

Gomes AM, Winter S, Klein K, Turpeinen M, Schaeffeler E, Schwab M, and Zanger UM (2009) Pharmacogenomics of human liver cytochrome P450 oxidoreductase: multifactorial

analysis and impact on microsomal drug oxidation. *Pharmacogenomics* **10:**579-599.

Guengerich FP, Martin MV, Sohl CD, and Cheng Q (2009) Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nat Protoc* **4:**1245-1251.

Hart SN, Wang S, Nakamoto K, Wesselman C, Li Y, and Zhong XB (2008) Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsomal P450-catalyzed drug metabolism. *Pharmacogenet Genomics* **18:**11-24.

Huang N, Agrawal V, Giacomini KM, and Miller WL (2008) Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. *Proc Natl Acad Sci U S A* **105:**1733-1738.

Huang R, Zhang M, Rwere F, Waskell L, and Ramamoorthy A (2015) Kinetic and structural characterization of the interaction between the FMN binding domain of cytochrome P450 reductase and cytochrome c. *J Biol Chem* **290:**4843-4855.

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, and Kliewer SA (2002) Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* **62:**638-646.

Miller WL, Agrawal V, Sandee D, Tee MK, Huang N, Choi JH, Morrissey K, and Giacomini KM (2011) Consequences of POR mutations and polymorphisms. *Mol Cell Endocrinol* **336:**174-179.

Ohtsuki S, Schaefer O, Kawakami H, Inoue T, Liehner S, Saito A, Ishiguro N, Kishimoto W, Ludwig-Schwellinger E, Ebner T, and Terasaki T (2012) Simultaneous absolute protein

quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. *Drug Metab Dispos* **40:**83-92.

Pandey AV and Flück CE (2013) NADPH P450 oxidoreductase: structure, function, and pathology of diseases. *Pharmacol Ther* **138:**229-254.

Picotti P, Bodenmiller B, Mueller LN, Domon B, and Aebersold R (2009) Full dynamic range proteome analysis of S. cerevisiae by targeted proteomics. *Cell* **138:**795-806.

Shirasaka Y, Chaudhry AS, McDonald M, Prasad B, Wong T, Calamia JC, Fohner A, Thornton TA, Isoherranen N, Unadkat JD, Rettie AE, Schuetz EG, and Thummel KE (2015) Interindividual variability of CYP2C19-catalyzed drug metabolism due to differences in gene diplotypes and cytochrome P450 oxidoreductase content. *Pharmacogenomics J*.

Tomková M, Marohnic C.C, Gurwitz D, Šeda O, Masters B.S.S, and Martásek P (2012) Identification of six novel P450 oxidoreductase missense variants in Ashkenazi and Moroccan Jewish populations. *Pharmacogenomics* **13:** 543-554.

Wang D, Jiang Z, Shen Z, Wang H, Wang B, Shou W, Zheng H, Chu X, Shi J, and Huang W (2011) Functional evaluation of genetic and environmental regulators of p450 mRNA levels. *PLoS One* **6:**e24900.

Wang H, Zhang H, Li J, Wei J, Zhai R, Peng B, Qiao H, Zhang Y, and Qian X (2015) A new calibration curve calculation method for absolute quantification of drug metabolizing enzymes in human liver microsomes by stable isotope dilution mass spectrometry. *Analytical Methods* **7:**5934-5941.

Wortham M, Czerwinski M, He L, Parkinson A, and Wan YJ (2007) Expression of constitutive androstane receptor, hepatic nuclear factor 4 alpha, and P450 oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. *Drug Metab Dispos* **35:**1700-1710.

Xia C, Hamdane D, Shen AL, Choi V, Kasper CB, Pearl NM, Zhang H, Im SC, Waskell L, and Kim JJ (2011) Conformational changes of NADPH-cytochrome P450 oxidoreductase are essential for catalysis and cofactor binding. *J Biol Chem* **286:**16246-16260.

Yang G, Fu Z, Chen X, Yuan H, Yang H, Huang Y, Ouyang D, Tan Z, Tan H, Huang Z, and Zhou H (2011) Effects of the CYP oxidoreductase Ala503Val polymorphism on CYP3A activity in vivo: a randomized, open-label, crossover study in healthy Chinese men. *Clin Ther* **33:**2060-2070.

Zhang H, Gao N, Liu T, Fang Y, Qi B, Wen Q, Zhou J, Jia L, and Qiao H (2015a) Effect of Cytochrome b5 Content on the Activity of Polymorphic CYP1A2, 2B6, and 2E1 in Human Liver Microsomes. *PLoS One* **10:**e0128547.

Zhang H, Gao N, Tian X, Liu T, Fang Y, Zhou J, Wen Q, Xu B, Qi B, Gao J, Li H, Jia L, and Qiao H (2015b) Content and activity of human liver microsomal protein and prediction of individual hepatic clearance in vivo. *Sci Rep* **5:**17671.

Footnotes

*This work was supported by the National Natural Science Foundation of China [No.81473279], Science and Technology Innovative Scholar Program of Henan Province [No.134200510019] and Scientific and Technical Innovation Team of Zhengzhou City [131PCXTD604].

Legends of Figures

Figure. 1 Frequency distribution of POR mRNA levels (as measured by qPCR, relative to GAPDH, n= 107) (A), POR protein content (as measured by LC-MS/MS, n = 100) (B) and POR activity as measured by the spectral method, n = 125) (C). The data are presented as the means of three independent experiments.

Figure.2 Correlations in POR mRNA, protein and activity inhuman livers. (A) Correlation between POR protein content and POR mRNA level; (B) correlation between POR protein content and POR activity; and (C) correlation between POR activity and mRNA level. The data are presented as the means of three independent experiments.

Figure. 3 Effect of cigarette smoking (A) and alcohol consumption (B) on POR activity in HLM. The black horizontal line represents the median value with 2.5^{th} to 95^{th} percentile values.

Figure. 4 Effect of SNP on POR mRNA level in human livers. Median mRNA level of the genotype variant of POR*2286822 C>T* was statistically significant (P<0.05). Data are shown as box plots representing medians with 2.5^{th} to 97.5^{th} percentile values.

Figure. 5 Effect of SNPs on POR protein content in HLM. Differences in the median protein levels of the different genotype variants of POR *2286822C>T* (A), *286823G>A* (B) and 3823884*A>C* (C) were statistically significant (P<0.05). Data are shown as box plots

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

representing medians with 2.5th to 95th percentile values.

Figure. 6 Effects of SNPs on POR activity in HLM. Differences in the median activity level of the different genotype variants of POR *2286822 C>T* (A), *286823G>A* (B), *1135612 A>G* (C), and *1057868 C>T* (D) were statistically significant (P<0.05). Data are shown as box plots representing medians with 2.5thto 95thpercentile values.

Figure. 7 Correlation between POR expression and transcriptional factors. (A) Correlation between POR mRNA and HNF4α mRNA levels; (B) correlation between POR mRNA and PXR mRNA levels. The data were determined by qPCR in 107 liver samples and are presented as the means of three independent experiments.

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Primers for quantitative real-time polymerase chain reaction

Gene	Forward Primer (5´→3´)	Reverse Primer (5´→3´)
GAPDH	AACAGGGTGGTGGACCTCAT	GGAGGGGAGATTCAGTGTGG
POR	TTTCGCTCATCGTGGGTCT	TCCTCCCCGTTTTCTTCATCT
PXR	ACAGCTGGCTAGCATTCCTCA	CTTGCCTCTCTGATGGTCCTG
HNF4α	AGCGATCCAGGGAAGATCAAG	AGCAGCAGCAGCTCTCCAA

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2 POR variants detected in this cohort

SNP ID	Genomic position	Coding position	Location	Amino acid change	Genotype frequency			Variant allele Frequency (%)
					Genotype	N	Frequency (%)	
rs3823884	5036A>C	-47A>C	5'-UTR		AA	55	52.4	27.1
					AC	43	41	
					CC	7	6.6	
rs17148944	62448G>A	237+88G>A	Intron 2		GG	108	98.2	0.91
					GA	2	1.8	
					AA	0	0	
rs10239977	69567C>T	366+89C>T	Intron 3		CC	94	90.4	4.8
					CT	10	9.6	
					TT	0	0	
rs1135612	70258A>G	387A>G	Exon 4	Pro129=	AA	32	28.6	46.9
					AG	55	49.1	
					GG	25	22.3	
rs10954732	71730G>A	931+225G>A	Intron 6		GG	27	24.1	48.2
					GA	62	55.4	
					AA	23	20.5	
rs3815455	72337C>T	830+116C>T	Intron 7		CC	43	41.3	34.6
					CT	50	48.1	
					TT	11	10.6	
rs41301394	73384C>T	831-35C>T	Intron 7		CC	46	41.8	35.5
					CT	50	45.5	
					TT	14	12.7	
rs4732515	74610T>C	1067-66T>C	Intron 9		TT	2	1.8	85.1
					TC	29	26.1	
					CC	80	72.1	
rs4732516	74663C>G	1067-13C>G	Intron 9		CC	2	1.8	84.5
					CG	30	27.3	
					GG	78	70.9	

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

rs2286822	74869C>T	1248+12C>T	Intron 10		CC	26	25	51.4
					CT	49	47.1	
					TT	29	27.9	
rs2286823	74877G>A	1248+20G	Intron 10		GG	23	22.3	51.5
					GA	54	52.4	
					AA	26	25.3	
rs41301427	75138G>A	1398+32G>A	Intron 11		GG	100	97.1	1.46
					GA	3	2.9	
					AA	0	0	
rs2302432	75445G>T	1399-33G>T	Intron 11		GG	0	0	87.6
					GT	25	24.8	
					TT	76	75.2	
rs2302431	75444T>C	1399-34T>C	Intron 12		TT			88.2
					TC	24	23.5	
					CC	78	76.5	
rs1057868	75587C>T	1508C>T	Exon 12	Ala503Val	CC	46	41.8	35.5
					CT	50	45.5	
					TT	14	12.7	
rs2228104	75534T>C	1455T>C	Exon 12	Ala485=	TT	0	0	88.1
					TC	24	23.8	
					CC	77	76.2	
rs2302433	75781C>T	1669+33C>T	Intron 12		CC	98	89.1	5.9
					CT	11	10.0	
					TT	1	0.9	
rs1057870	75868G>A	1716G>A	Exon 13	Ser572=	GG	94	91.3	4.4
					GA	9	8.7	
					AA	0	0	

POR SNP ID, genomic position, coding position, amino acid change, and location were compiled from Hart et al., 2008; Huang et al., 2008; Gomes et al., 2009 and Tomková et al., 2012. SNPs that do not result in amino acid changes are indicated by '='. 5'-UTR, 5'-untranslated region.

DMD Fast Forward. Published on June 6, 2016 as DOI: 10.1124/dmd.116.069849
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3 Correlation between POR and CYP at mRNA, protein and activity levels

(n=100)

	POR mRNA vs. CYP mRNA	POR protein vs. CYP protein	POR activity vs. CYP activity	POR protein vs. CYP activity
CYP1A2	0.561**	0.576**	-0.01	-0.007
CYP2A6	0.476**	0.457**	0.163	0.102
CYP2B6	0.787**	0.172	0.067	-0.247*
CYP2C8	0.450**	0.818**	-0.233*	-0.329**
CYP2C9	0.212*	0.849**	0.132	-0.118
CYP2C19	0.602**	0.474**	0.507**	0.438**
CYP2D6	0.246*	0.535**	-0.062	-0.207
CYP2E1	0.477**	0.618**	0.197	0.224*
CYP3A4	0.494**	0.661**	0.158	0.017
CYP3A5	0.324**	0.532**	0.158	0.017

For CYP2B6, n=91; CYP2C19, n=54.

Figure 1

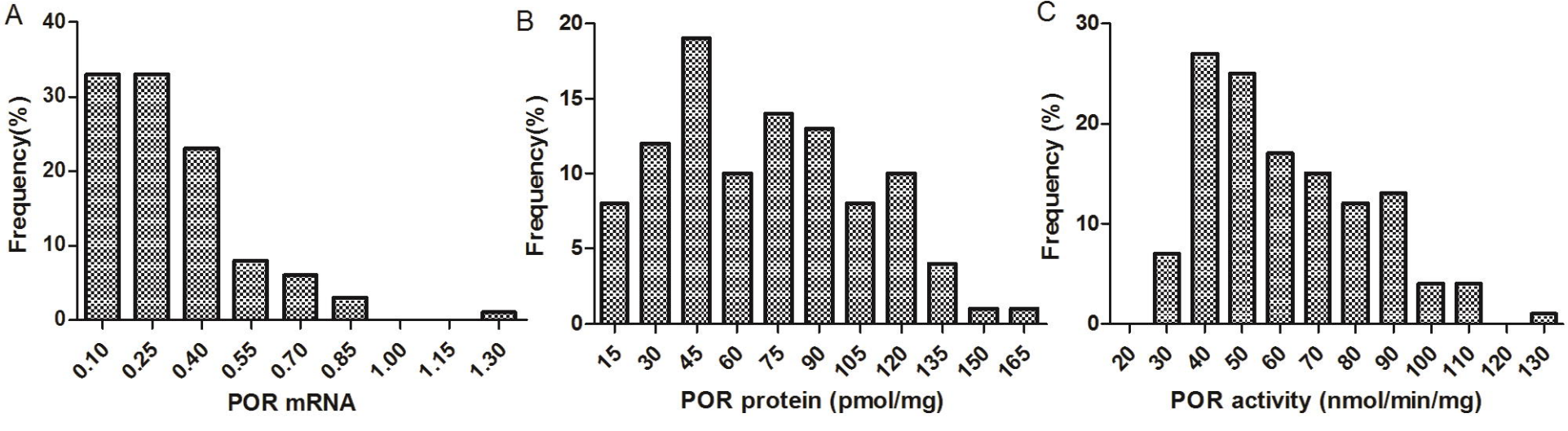

A
Frequency(%)
40
30
20
10
0
0.10
0.25
0.40
0.55
0.70
0.85
1.00
1.15
1.30
POR mRNA
B
Frequency(%)
20
15
10
5
0
15
30
45
60
75
90
105
120
135
150
165
POR protein (pmol/mg)
C
Frequency (%)
30
20
10
0
20
30
40
50
60
70
80
90
100
110
120
130
POR activity (nmol/min/mg)

Figure 2

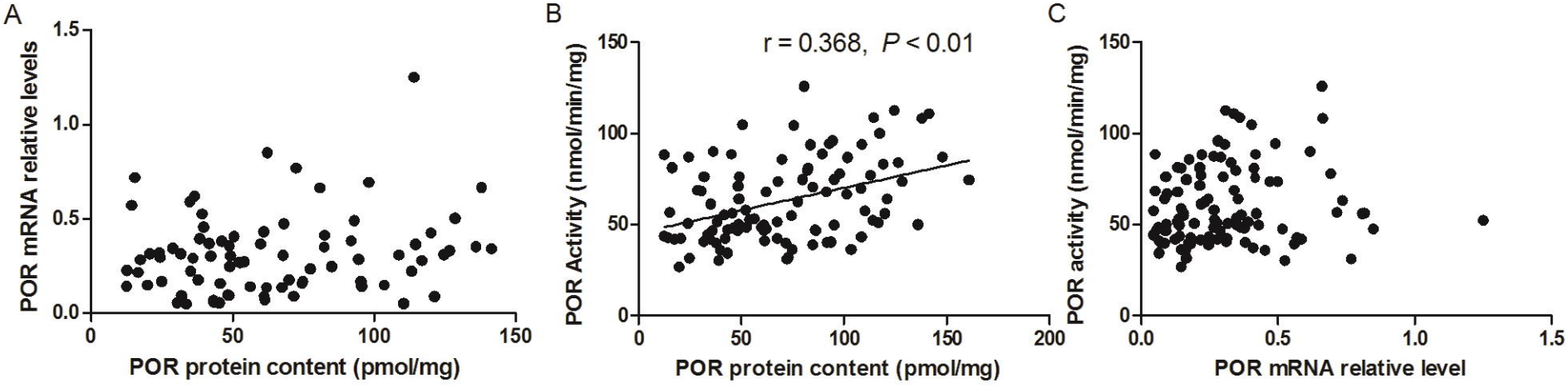

A
POR mRNA relative levels
1.5
1.0
0.5
0.0
0
50
100
150
POR protein content (pmol/mg)
B
r = 0.368, P < 0.01
POR Activity (nmol/min/mg)
150
100
50
0
0
50
100
150
200
POR protein content (pmol/mg)
C
POR activity (nmol/min/mg)
150
100
50
0
0.0
0.5
1.0
1.5
POR mRNA relative level

Figure 3

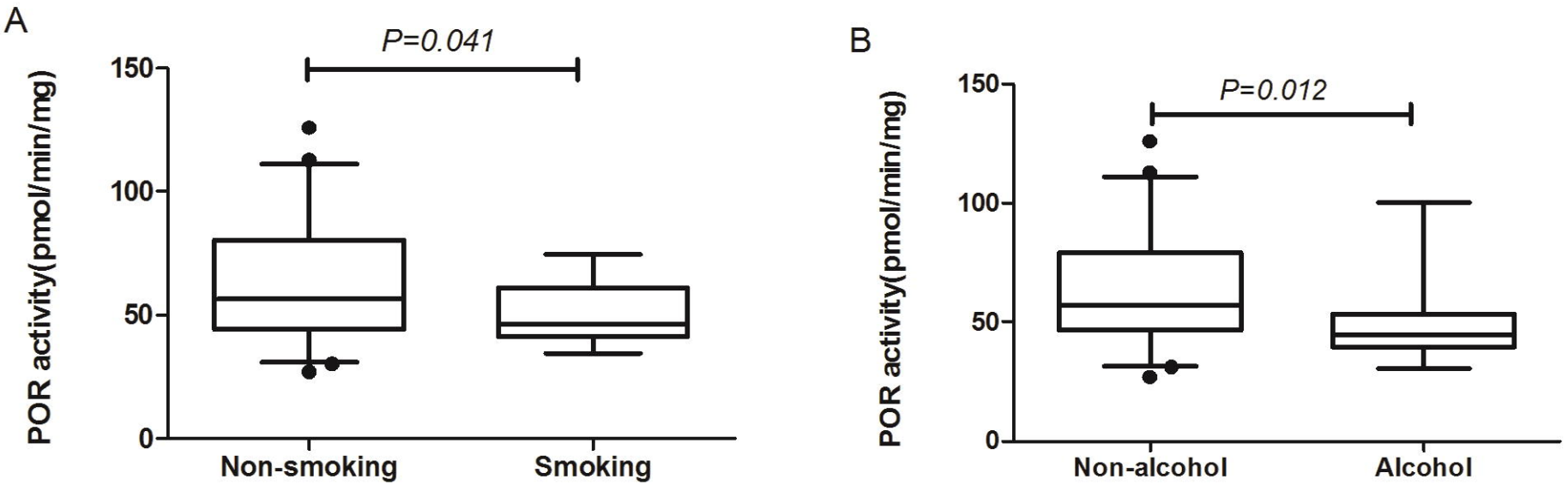

A
P=0.041
150
100
50
0
POR activity(pmol/min/mg)
Non-smoking
Smoking
B
P=0.012
150
100
50
0
POR activity(pmol/min/mg)
Non-alcohol
Alcohol

Figure 4

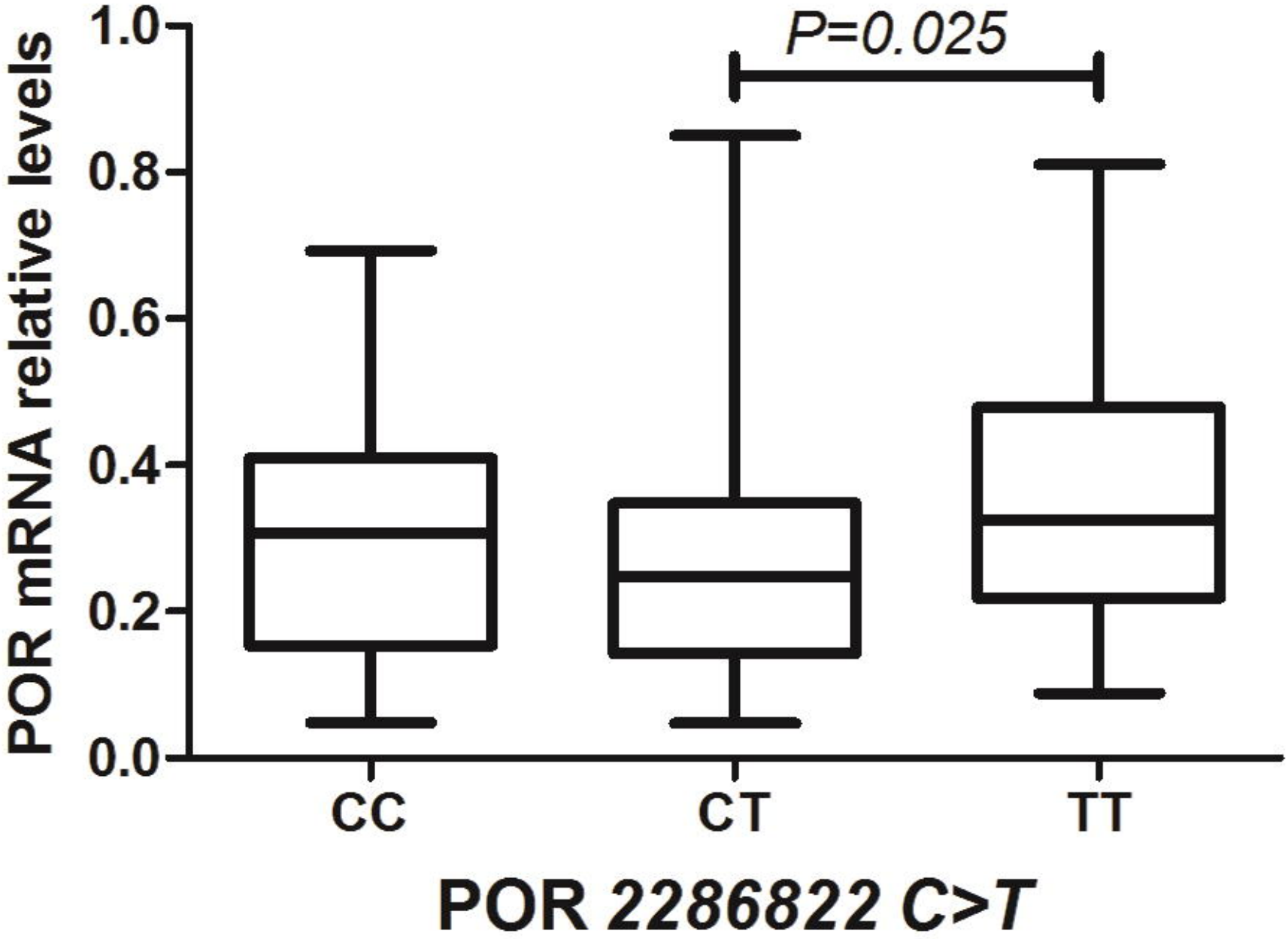

P=0.025
POR mRNA relative levels
1.0
0.8
0.6
0.4
0.2
0.0
CC
CT
TT
POR 2286822 C>T

Figure 5

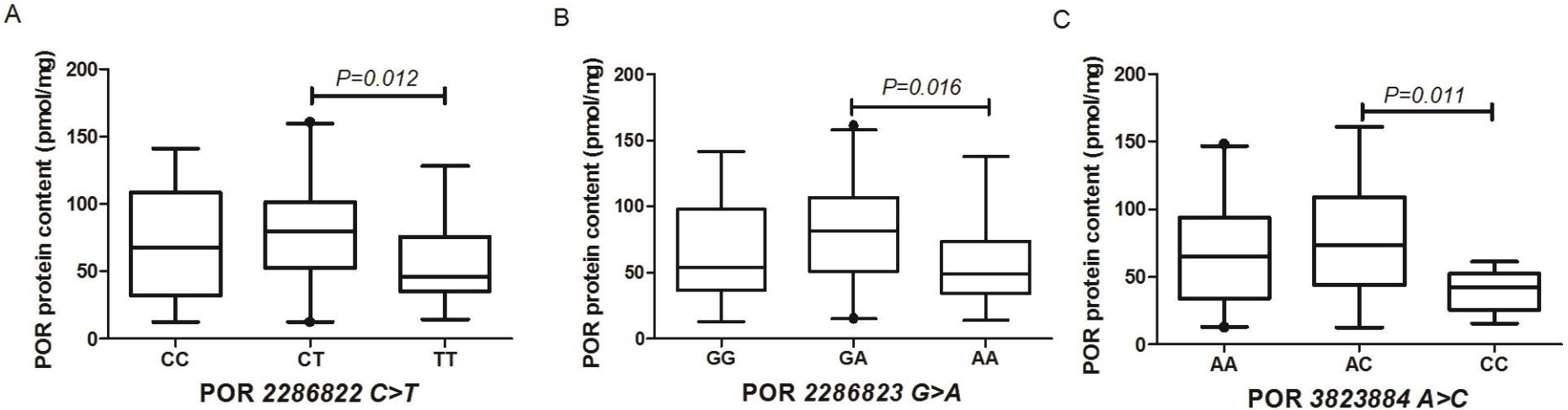

A
POR protein content (pmol/mg)
200
150
100
50
0
P=0.012
CC
CT
TT
POR 2286822 C>T
B
POR protein content (pmol/mg)
200
150
100
50
0
P=0.016
GG
GA
AA
POR 2286823 G>A
C
POR protein content (pmol/mg)
200
150
100
50
0
P=0.011
AA
AC
CC
POR 3823884 A>C

Figure 6

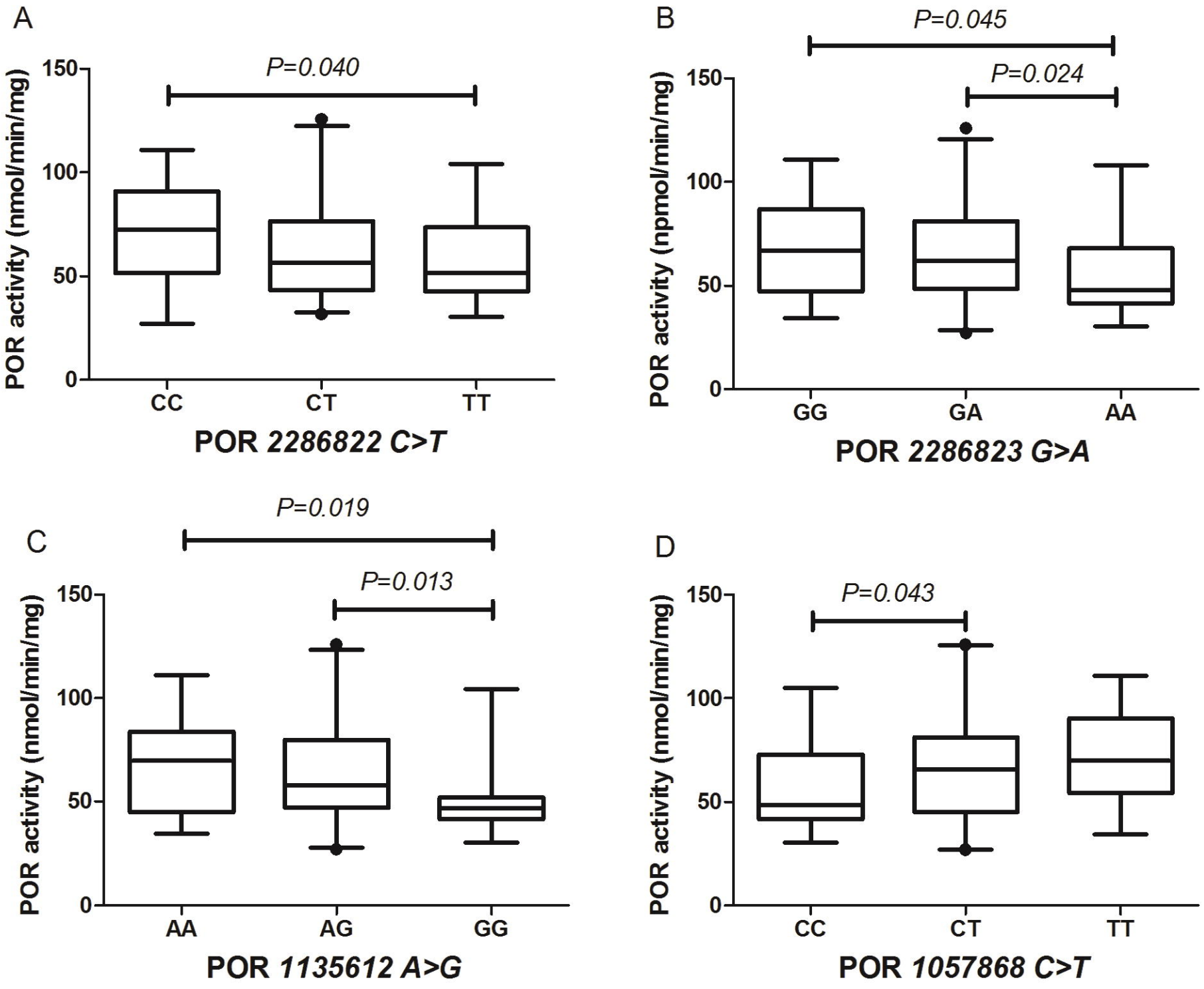
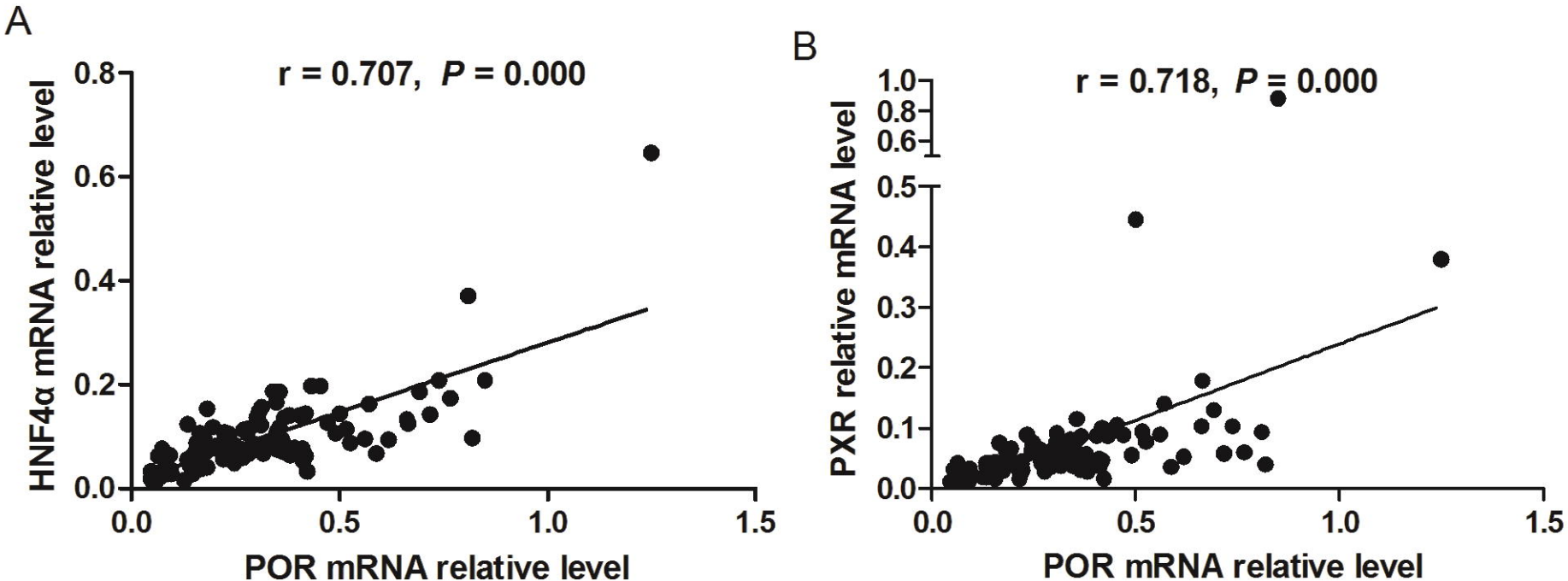

A
P=0.040
150
100
50
0
POR activity (nmol/min/mg)
CC
CT
TT
POR 2286822 C>T
B
P=0.045
P=0.024
150
100
50
0
POR activity (npmol/min/mg)
GG
GA
AA
POR 2286823 G>A
C
P=0.019
P=0.013
150
100
50
0
POR activity (nmol/min/mg)
AA
AG
GG
POR 1135612 A>G
D
P=0.043
150
100
50
0
POR activity (nmol/min/mg)
CC
CT
TT
POR 1057868 C>T

Figure 7


A
r = 0.707, P = 0.000
HNF4α mRNA relative level
0.0
0.2
0.4
0.6
0.8
0.0
0.5
1.0
1.5
POR mRNA relative level
B
r = 0.718, P = 0.000
PXR relative mRNA level
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.8
1.0
0.0
0.5
1.0
1.5
POR mRNA relative level

Supplemental Data

Journal Title

Drug Metabolism and Disposition

Article Title

Correlation of cytochrome P450 oxidoreductase expression with the expression of 10 isoforms of cytochrome P450 in human liver

Hai-Feng Zhang, Zhi-Hui Li, Jia-Yu Liu, Ting-Ting Liu, Ping Wang, Yan Fang, Jun Zhou, Ming-Zhu Cui, Na Gao, Xin Tian, Jie Gao, Qiang Wen, Lin-Jing Jia, Hai-Ling Qiao*

Institute of Clinical Pharmacology, Zhengzhou University, Zhengzhou, People's Republic of China

Supplemental Table

Supplementary Table 1

Donor characteristics of human liver samples and POR data in donor subgroups

Variables	Group	N	POR (Median)		
			Protein (pmol/mg) (N)	mRNA (N)	Activity (nmol/min/mg)
Gender	Male	40	76.23 (36)	0.27 (36)	50.99
	Female	85	67.65 (64)	0.28 (71)	56.49
Age (years)	20-45	49	73.50 (36)	0.28 (45)	61.25
	46-60	62	60.55 (52)	0.28 (51)	52.03
	61-75	14	93.44 (12)	0.22 (11)	65.56
Smoking	Non-smoking	112	71.72 (87)	0.29 (94)	56.57
	smoking	13	52.40 (13)	0.26 (13)	46.53*
Drinking	Non-drinking	112	70.90 (88)	0.29 (94)	57.01
	drinking	13	50.35 (12)	0.26 (13)	44.45$
Medical Diagnosis	cavernous hemangioma of liver	90	56.26 (69)	0.28 (76)	53.44
	cholelithiasis	9	72.35 (9)	0.32 (9)	56.05
	gallbladder cancer	5	85.34 (4)	0.28 (4)	43.41
	hepatocellular carcinoma	4	58.03 (4)	0.23 (4)	62.07
	hepatic cholangiocarcinoma	7	86.90 (6)	0.23 (6)	68.09
	metastatic carcinoma	10	88.44 (8)	0.15(8)	61.99

For POR protein and mRNA, the number of liver samples was 100 and 107, respectively. *P<0.05 VS nonsmoking; $ P<0.05 VS non-drinking.