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Original Paper

Effect of P450 Oxidoreductase Polymorphisms on the Metabolic Activities of Ten Cytochrome P450s Varied by Polymorphic CYP Genotypes in Human Liver Microsomes

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Key Words

Por • Gene polymorphism • Cytochrome P450 • CYP genotypes • Human liver microsomes • Drug metabolism

Abstract

Background/ Aims: Little is known about the effect of P450 oxidoreductase (*POR*) gene polymorphisms on the activities of CYPs with multiple genotypes. ***Methods:*** We genotyped 102 human livers for 18 known *POR* single nucleotide polymorphisms (SNPs) with allelic frequencies greater than 1% as well as for 27 known SNPs in 10 *CYPs*. CYP enzyme activities in microsomes prepared from these livers were determined by measuring probe substrate metabolism by high performance liquid chromatograph. ***Results:*** We found that the effects of the 18 *POR* SNPs on 10 CYP activities were *CYP* genotype-dependent. The *POR* mutations were significantly associated with decreased overall K_m for CYP2B6 and 2E1, and specific genotypes within *CYP1A2, 2A6, 2B6, 2C8, 2D6* and *2E1* were identified as being affected by these *POR* SNPs. Notably, the effect of a specific *POR* mutation on the activity of a *CYP* genotype could not be predicted from other *CYP* genotypes of even the same CYP. When combining one *POR* SNP with other *POR* SNPs, a hitherto unrecognized effect of multiple-site *POR* gene polymorphisms (MSGP) on CYP activity was uncovered, which was not necessarily consistent with the effect of either single *POR* SNP. ***Conclusions:*** The effects of *POR* SNPs on CYP activities were not only *CYP*-dependent, but more importantly, *CYP* genotype-dependent. Moreover, the effect of a *POR* SNP alone and in combination with other *POR* SNPs (MSGP) was not always consistent, nor predictable. Understanding the impact of *POR* gene polymorphisms on drug metabolism necessitates knowing the complete SNP complement of *POR* and the genotype of the relevant *CYPs*.

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Introduction

P450 oxidoreductase (POR) is located on the smooth endoplasmic reticulum where it donates electrons to several oxygenase enzymes, including cytochrome P450 (CYP) [1]. CYPs, especially those in families 1-3 account for the oxidation of approximately 70-80% of the clinically used drugs [2]. As the principal electron donor to the CYPs, POR is essential for CYP activity [3].

The human *POR* gene is highly polymorphic, with 48 alleles identified on the *POR* allele nomenclature web page (http://www.cypalleles.ki.se/por.htm), and 140 *POR* single nucleotide polymorphism (SNPs) were detected among 842 individuals from four ethnicities [4]. The majority of early studies on POR mutations were derived from on patients with P450 oxidoreductase deficiency in association with disordered steroidogenesis [5, 6]. Only recently have *POR* polymorphisms been studied in healthy humans. Two studies [7, 8] carried out with heterologously expressed proteins revealed that *POR A503V* impaired CYP2D6 activity by 40-50% and CYP3A4 activity by 61-77%. In contrast, two further studies [9, 10] conducted *in vivo* reported significantly higher CYP3A activity in association with the *POR A503V* mutation. However, these studies just focused on *POR A503V* mutation, and the reconstituted system used may not accurately model some aspects of enzyme function in a whole cell, making extrapolation to an *in vivo* situation difficult. Moreover, they determined CYP activity based on a single substrate concentration and could not generate complete kinetic parameters (K_m, V_{max} and CL_{int}).

It is well known that some human drug metabolizing CYPs are highly polymorphic. Our previous studies [11-14] performed with microsomes from 105 normal and 102 hepatocellular carcinoma liver tissue samples revealed substantial inter-individual variations in CYP content [15] and activity, also in predicted *in vivo* hepatic clearance. In addition, our earlier studies have confirmed the significant associations between POR content and the activities of four CYPs (CYP2B6, 2C8, 2C19, and 2E1) [16], and between cytochrome b5 content and activity of CYP1A2, 2B6 and 2E1 [17]. However, other factors that may underly the large inter-individual variation remain unknown. Several previous *in vitro* and *in vivo* studies have revealed that the effect of *POR* gene polymorphisms differed, depending on the specific *POR* mutation, the CYP isoform, and the substrate used to assay activity [9, 18, 19]. Most of these reports [18, 20] were concerned with the effect on the overall activity of CYP and did not examine the effects on different *CYP* genotypes. Only one study [10] has characterized the impact of a single common *POR* SNP, *A503V*, on the activity of the *CYP3A5*1/*1* and *CYP3A5*3/*3* genotypes *in vivo.* Two studies in reconstituted systems revealed different effects on *CYP2C8* variants [21] and *CYP2C9* variants [22]; however, these studies were performed with a very limited number of *POR* mutations and CYP isoforms. Human liver microsomes (HLMs) provide a valuable means to test the effect of POR polymorphisms on CYP activity because they contain the enzymes in their native environment.Unfortunately, no studies on the activities of different *CYP* genotypes have been conducted with HLMs. Whether *POR* polymorphisms result in substantially different activities with the specific polymorphic genotypes of each CYP remains to be determined.

The presence of multiple mutations in the *POR* gene lead us to hypothesize that these *POR* mutations might exert synergistic or compensating effects on CYP activity. Moreover, no studies on the effect of compounded mutations in *POR* have been conducted.

To this end, we performed a comprehensive study on the effect of POR gene polymorphisms on 10 CYP isoforms. Eighteen *POR* SNPs and the activities of 10 CYP isoforms determined by metabolism of appropriate probe drugs were carried out with HLMs from102 subjects. In addition, the effects of multiple *POR* SNPs in combination (multiple site gene polymorphisms, MSGP) on the activities of the various CYP genotypes were determined.

Materials and Methods

Human liver microsomes

Human liver tissue samples (n=102) were acquired from Chinese patients [17] undergoing hepatic surgery in the first affiliated hospital of Zhengzhou University and the People's Hospital of Henan Province (Table 1) and frozen in liquid nitrogen within 1~4 hrs after being collected. All liver samples were tested for and declared free of infectious agents, including human immunodeficiency virus (HIV), hepatitis B (HBV), and hepatitis C (HCV). Well-documented demographic information such as age, sex, body weight, smoking habits, alcohol consumption, and pre-surgical medication for was obtained for each patient.

Ethical statement

This study was carried out in accordance with approved guidelines of the ethics committees of Zhengzhou University with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by Ethics Committee of the Zhengzhou University (Zhengzhou, China).

Preparation of human liver microsomes

Human liver microsomes (HLMs) were prepared by differential centrifugation. After being thawed on ice and weighed, tissue samples were homogenized on ice in Tris-HCl (pH 7.0) buffer containing 1.12% KCl (w/v) and 1.12% EDTA (v/v). The homogenate then was subjected to centrifugation at 9, 000 x g for 20 min at 4°C.The supernatant fraction was collected and submitted for a second centrifugation at 100, 000 x g for 1 h at 4°C. After resuspension in 0.15M Tris-HCl (pH 7.6) buffer, the resulting microsomal fraction was pelleted at 100, 000 g at 4°C for an additional hour. The microsomal pellet was finally suspended in 0.25 M suspension buffer, frozen in liquid nitrogen, and stored at -80°C until use. Microsomal protein concentration was measured by the Bradford method [23].

Genotyping of POR and CYP isoforms

Genomic DNA was isolated from 102 liver tissues using the Charge Switch Gdna Mini Tissue Kit from Invitrogen. Eighteen polymorphisms in the *POR* gene with frequencies greater than 1% in the Chinese population were genotyped. Apart from rs3823884, rs2286822, rs2302432, rs2228104, which were genotyped by PCR-sequencing, all the remaining *POR* SNPs were determined by the Sequenom method [24, 25] and a total of 27 CYP gene polymorphisms (displayed as their genomic positions) including five mutations for *CYP1A2* (*-3860G>A*, *-3113A>G*,*-163C>A*, *2159G>A* and *5347T>C*), three for *CYP2A6* [**1B*, **4* and **9*(*-48T>G*)], two for *CYP2B6* (*15631G>T* and *18053A>G*), two for *CYP2C8* [**1B*(*-271C>A*) and **1C*(*-370T>C*)], one for *CYP2C9*3*(*42614A>C*), two for *CYP2C19* [**2 (19154G>A)* and **3*(*17948G>A*)], three for *CYP2D6* (*100C>T*, *1661G>C* and *2850C>T*), six for *CYP2E1* (*-1293G>C*, *-1053C>T*, *-352A>G*, *-333T>A*, *-71G>T* and *7632T>A*), two for *CYP3A4* (*20070T>C* and *20230G>A*) and one for *CYP3A5*3* (6986A>G*)* were detected by the Sequenom method, a two-step PCR method or a PCR-sequencing method, as described previously [12]. Genotyping errors were detected by re-genotyping with a sub-sample and reproducibility was routinely greater than 99%.

Table 1. Demographic information on the human liver cohort (n=102)

	Subgroup	Number	Percentage(%)
Gender	Males	35	34.3
	Females	67	65.7
Age	Age≥20 and ≤45 years	36	35.3
	Age>46 and ≤60 years	59	57.8
	Age ≥61 and≤75years	7	6.86
Smoking	Nonsmoker	92	90.2
	smoker	10	9.80
Drinking	Nondrinker	93	91.2
	Drinker	9	8.82
Medical diagnose	Cavernous hemangioma of liver	73	71.6
	Metastaic carcinoma	8	7.84
	Cholelithiasis	7	6.86
	Gallbladder cancer	4	3.92
	Hepatic cholangiocarcinoma	6	5.88
	Hepatocellular carcinoma	4	3.92
Drug exposure	For all donors, only regular drugs intake preceding liver surgery, no exposure to known CYP-inducing or –inhibiting agents.		

CYP Enzyme Activity Assays

For each liver sample, the activities of ten CYPs were determined by measuring the rates of each of the following reactions by high performance liquid chromatography according to previously described procedures [12] with seven or eight substrate concentrations within the following substrate concentration ranges: 6.25-800 μM phenacetin *O*-deethylation (CYP1A2), 0.156-20 μM coumarin 7-hydroxylation (CYP2A6), 7.8-500 μM bupropion1-hydroxylation (CYP2B6), 2.5-80 μM paclitaxel 6-hydroxylation (CYP2C8), 31.25-2000 μM tolbutamide 4-hydroxylation (CYP2C9), 3.9-500 μM omeprazole 4-hydroxylation (CYP2C19), 0.625-960 μM dextromethorphan *O*-demethylation (CYP2D6),7.8-1000 μM chlorzoxazone 6-hydroxylation (CYP2E1), and 3.9-200 μM midazolam 1′-hydroxylation (CYP3A4/5). Incubation conditions for each substrate were optimized in preliminary experiments to determine linearity with respect to incubation time, substrate concentration and protein concentration. Incubation mixtures contained HLMs (0.3mg protein/ml for CYP1A2, 2A6, and 2E1; 0.2mg protein/ml for CYP2D6 and 3A4/5; 0.5mg protein/ml for CYP2B6, 2C8, 2C9 and 2C19), 100 mM phosphate buffer (pH 7.4), and 1mM NADPH. Optimal incubation times were as follows: 30 min for CYP1A2, 2A6, 2E1; 60 min for CYP2B6, 2C9; 90 min for CYP2C19; 120 min for CYP2C8; 20 min for CYP2D6 and 5 min for CYP3A4/5. All the incubations were linear within incubation time. Metabolites were determined by HPLC-UV or HPLC-FLD. All experiments included two replicates. The K_m and V_{max} of each microsomal CYP was determined by nonlinear regression analysis using GraphPad Prism 5 and the CL_{int} was calculated from the ratio of V_{max} to K_m.

Statistical Analysis

The χ^2 test for goodness-of-fit was used to check whether the distribution of genotypes in HLMs deviated from the Hardy–Weinberg equilibrium. The initial rate of metabolite formation for each CYP Michaelis constant (K_m) and maximum velocity (V_{max}) values were determined by nonlinear regression analysis using GraphPad Prism5. Intrinsic clearance (CL_{int}) was calculated from the ratio of V_{max} to K_m. The Shapiro-Wilk test of normality was used to check the distribution shape of CL_{int}, K_m and V_{max} for each individual CYP enzyme. Mean-rank' post hoc multiple comparisons were carried out with the Kruskal-Wallis H test using SPSS statistics 21 software. An adjusted *P* value<0.05 was considered statistically significant (two-tailed).

Results

POR Gene Polymorphisms

Frequency of POR SNPs. A total of 18 *POR* SNPs were genotyped in 102 HLMs with allelic frequencies ranging from 0.98% to 88.3% (Table 2). There were 13 SNPs with allele frequencies greater than 10%. All *POR* SNPs were in Hardy-Weinberg equilibrium (*P*>0.05). For rs3815455, rs41301394 and rs1057868, the genotype could be determined in 98, 96 and 100 of the samples, respectively; 83 of these samples yielded a genotype for all three SNPs. Genotypes for rs4732515, rs4732516 and rs2302431 could be determined in 99, 96 and 98 samples, respectively, and all three genotypes could be determined in 80 samples. For rs2302432 and rs2228104, 99 and101 samples could be genotyped, respectively, and both SNP genotypes could be determined in 99 samples. All the remaining polymorphisms were found in all 102 samples.

Linkage Disequilibrium Analysis. Linkage disequilibrium (LD) analysis was performed by the $r^2$and $|D'|$ statistics. For r^2 values, perfect linkage (r^2=1) was detected between rs10239977 and rs1057870, among rs3815455, rs41301394 and rs1057868, and among rs4732515, rs4732516 and rs2302431 (Fig.1). Relatively strong linkage ($r^2 \geq 0.95$) was observed between rs2302432 and rs2228104, with the two variants differing in just one sample.

Effect of POR single nucleotide polymorphisms (SNPs)

Effect on CYP overall Activity. When assessing the effect of *POR* SNPs on CYP activities, we ruled out SNPs rs17148944, rs41301427, rs10239977, rs1057870 for their low allele frequencies and took rs1057868 and rs4732515 to represent rs3815455-rs41301394-

Table 2. Gene polymorphisms of POR in human liver microsomes. Genomic positions, coding position, amino acid change, location and allele were compiled from studies Hart et al., 2008; Huang et al., 2008; Gomes et al., 2009, Tomková et al., 2012 and Sim et al., 2009. =: Silent mutation; 5'-UTR, 5'-untranslated region. *, #, ▲refer to perfect linkage (r^2=1) observed between rs10239977 and rs1057870, among rs3815455, rs41301394 and rs1057868, among rs4732515, rs4732516 and rs2302431. ◆refer to strong linkage (r^2≥0.95) observed between rs2302432 and rs2228104, with the two variants differing in just one sample. For rs3815455, rs41301394 and rs1057868, the genotype could be determined in 98, 96 and 100 of the samples, respectively; 83 of these samples yielded a genotype for all three SNPs. For rs4732515, rs4732516 and rs2302431, genotypes could be determined in 98, 96 and 100 of the samples, respectively; and all three genotypes could be determined in 80 samples. For rs2302432 and rs2228104, 99 and101 samples could be genotyped, respectively, and both SNP genotypes could be determined 99 samples. For the rest polymorphisms, all the 102 samples could be detected. To support readability of the following analysis, POR SNP1, SNP2, SNP3, SNP4, SNP5, SNP6, SNP7, SNP8, SNP9 were used to represent rs3823884, rs1135612, rs10954732, rs4732515, rs2286822, rs2286823, rs2302432, rs1057868, rs2302433, respectively, for the latter analysis

(rs number/ SNP ID)	Allele	Location	Genomic position	Coding position	Amino acid change	Genotype Frequency Genotype n	Frequency(%)	Variant allele Frequency(%)
rs3823884		5'-UTR	5036A>C	-47A>C		AA	54	52.9	27.0
(SNP1)						AC	41	40.2	
						CC	7	6.9	
rs17148944		Intron 2	62448G>A	237+88G>A		GG	100	98.0	0.98
						GA	2	2.0	
						AA	0	0	
rs10239977		Intron 3	69567C>T	366+89C>T		CC	93	91.2	4.4*
						CT	9	8.8	
						TT	0	0	
rs1135612		Exon 4	70258A>G	387A>G	Pro129=	AA	28	27.4	47.1
(SNP2)						AG	52	51.0	
						GG	22	21.6	
rs10954732		Intron 6	71730G>A	931+225G>A		GG	23	22.5	49.0
(SNP3)						GA	58	56.9	
						AA	21	20.6	
rs3815455		Intron 7	72337C>T	830+116C>T		CC	36	43.4	33.7#
						CT	38	45.8	
						TT	9	10.8	
rs41301394		Intron 7	73384C>T	831-35C>T		CC	36	43.4	33.7#
						CT	38	45.8	
						TT	9	10.8	
rs4732515		Intron 9	74610T>C	1067-66T>C		TT	0	0	87.5▲
(SNP4)						TC	20	25	
						CC	60	75	
rs4732516		Intron 9	74663C>G	1067-13C>G		CC	0	0	87.5▲
						CG	20	25	
						GG	60	75	
rs2286822		Intron 10	74869C>T	1248+12C>T		CC	25	25	52.0
(SNP5)						CT	46	46	
						TT	29	29	
rs2286823		Intron 10	74877G>A	1248+20G		GG	23	22.5	51.5
(SNP6)						GA	53	52.0	
						AA	26	25.5	
rs41301427		Intron 11	75138G>A	1398+32G>A		GG	99	97.1	1.47
						GA	3	2.9	
						AA	0	0	
rs2302431		Intron 11	75444T>C	1399-34T>C		TT	0	0	87.5▲
						TC	20	25	
						CC	60	75	
rs2302432		Intron 11	75445G>T	1399-34T>C		GG	0	0	88.3◆
(SNP7)						GT	23	23.2	
						TT	76	76.8	
rs2228104		Exon 12	75534T>C	1455T>C	Ala485=	TT	0	0	87.9◆
						TC	24	24.2	
						CC	75	75.8	
rs1057868	POR*28	Exon 12	75587C>T	1508C>T	Ala503Val	CC	36	43.4	33.7#
(SNP8)						CT	38	45.8	
						TT	9	10.8	
rs2302433		Intron 12	75781C>T	1669+33C>T		CC	90	88.2	6.37
(SNP9)						CT	11	10.8	
						TT	1	1.0	
rs1057870		Exon 13	75868G>A	1716G>A	Ser572=	GG	93	91.2	4.4*
						GA	9	8.8	
						AA	0	0	

rs1057868 and rs4732515-rs4732516-rs2302431, respectively, given their perfect linkages. We also let rs2302432 represent rs2302432-rs2228104 due to their relatively strong linkage noted above. To support readability of the following analysis, we use *POR* SNP1, SNP2, SNP3, SNP4, SNP5, SNP6, SNP7, SNP8, SNP9 (Fig. 1) to represent rs3823884, rs1135612, rs10954732, rs4732515, rs2286822, rs2286823, rs2302432, rs1057868, and rs2302433, respectively. We only report results with a significance level of $P<0.05$.

None of the tested *POR* SNPs showed a significant impact on overall activities of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6 and 3A4/5. Conversely, CYP2B6 and 2E1 were significantly influenced by some *POR* SNPs (Table 3). SNP2 and SNP8 were associated with significantly decreased substrate K_m for CYP2E1 and CYP2B6, respectively. K_m for CYP2E1 decreased to 82% in the POR SNP2 GG group compared with SNP2 wild type. As for CYP2B6, when compared with POR SNP8 CT, K_m decreased to 66% in TT group.

Effect on the Activity of Polymorphic CYPs. The effect of *POR* SNPs on CYP enzyme activities varied with *POR* SNP and *CYP* genotype. SNP7 showed a significant impact on the activities of the highest number of CYPs. Significantly altered activities of CYPs, including CYP1A2, 2B6 and 2D6 were associated with *POR* SNP7. SNP2, SNP3 and SNP8 showed a significant impact on the activities of 2 CYPs. SNP2 showed significant impact on activities of CYP2D6 and 2E1. SNP3 revealed a significant impact on activities of CYP2A6 and 2E1. Moreover, activities of CYPs including CYP2A6 and 2C8 were significantly affected by SNP8. SNP1, SNP4, SNP5 and SNP9 only elicited a significant effect on one CYP (Table 4). For the 10 polymorphic *CYP* isoforms, only the genotypes for which the K_m, V_{max} and/or CL_{int} was significantly affected by the *POR* SNPs are shown.

SNP1 was significantly associated with dramatically decreased enzyme activities for samples genotyped as *CYP1A2-3860GA*, as evidenced by the CL_{int} for *CYP1A2 -3860GA* genotype in the SNP1 AC group decreasing by 53% when compared with the AA group.

SNP2 was associated with a significantly increased K_m for *CYP2D6 1661GG* and decreased K_m for *CYP2E1 -333TA*. The K_m of the *CYP2D6 1661GG* genotype increased to 228% in SNP2 GG group as compared with AA group. Moreover, SNP2 was also associated with decreased K_m by 44% for CYP2E1-333TA in SNP2 GG when compared with AA group (P=0.009).

SNP3 was significantly associated with profoundly

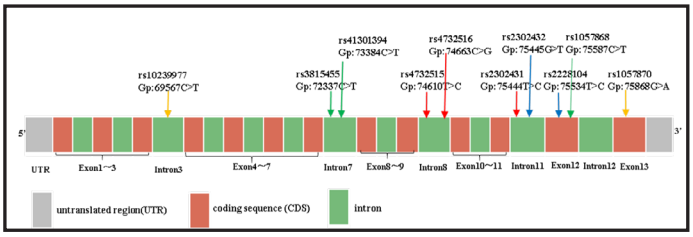

Fig. 1. Linkage disequilibrium of SNPs in POR gene. Gp, Genomic position. Arrow in red, green and yellow refer to perfect linkage ($r^2=1$) observed among rs4732515, rs4732516 and rs2302431, among rs3815455, rs41301394 and rs1057868, between rs10239977 and rs1057870. Blue arrow refer to strong linkage ($r^2\geq0.95$) observed between rs2302432 and rs2228104, with the two variants differing in just one sample.

Table 3. Effect of POR SNPs on overall K_m, V_{max}, and CL_{int} of 10 CYPs. For all CYPs, only CYP of which the overall K_m, V_{max} and/or CL_{int} are/is significantly affected by POR SNPs are shown. SNP, Single Nucleotide Polymorphism; K_m values are in μM; V_{max} values are in pmol·min^{-1}·mg^{-1} protein; CL_{int} values are in μl·min^{-1}·mg^{-1} protein. SNP2, rs1135612; SNP8, rs1057868; [a]P=0.022 vs SNP2 AA ; [b]P=0.018 vs SNP8 CT. All the P value are the adjusted one after correction

POR SNP	CYP	POR genotype	n	Km(μM)	Vmax (pmol·min-1·mg-1 protein)	CLint(μl·min-1·mg-1 protein)
SNP2	CYP2E1	AA	28	60(42~177)	485(297~1844)	9.1(1.9~39.0)
(A>G)		AG	52	53(29~96)	544(163~1982)	11.5(3.3~31.5)
		GG	22	49(27~72)[a]	505(267~856)	11.1(6.4~14.9)
SNP8	CYP2B6	CC	36	66(27~185)	57(22~173)	0.98(0.16~2.79)
(C>T)		CT	38	82(41~195)	51(14~95)	0.68(0.18~2.26)
		TT	9	54(17~99)[b]	55(13~100)	1.14(0.13~4.07)

decreased K_m and increased CL_{int} for some genotypes of *CYP2E1*. Km for CYP2E1 -333TA decreased to 54% and 78% in SNP3 AA group when compared with GG and GA group, respectively (P=0.006, 0.04). Moreover, Clint of CYP2E1 7632TA increased to 270% in POR SNP3 GA carriers as compared with GG carriers. SNP3 also showed significantly lower V_{max} and CL_{int} for *CYP2A6*1/*1*, as indicated by the most obvious case where the V_{max} for *CYP2 A6*1/*1* decreased to 37% and Clint to 76% in SNP3 (G>A) GA carriers as compared with GG group.

Only individuals with *CYP2B6-785AG* genotypes were significantly influenced by POR SNP4, with a slightly higher K_m and lower CL_{int} in POR SNP4 (T>C) CC carriers as compared with TC group.

Table 4. Effect of POR SNPs on K_m, V_{max}, and CL_{int} of 10 polymorphic CYPs. For all polymorphic CYPs, only the genotypes for CYPs in which the overall K_m, V_{max} and/or CL_{int} are/is significantly affected by POR SNPs are shown. SNP, Single Nucleotide Polymorphism; K_m values are in μM; V_{max} values are in pmol·min^{-1}·mg^{-1} protein; CL_{int} values are in μl·min^{-1}·mg^{-1} protein. SNP1, rs3823884; SNP2, rs1135612; SNP3, rs10954732; SNP4, rs4732515; SNP5,rs2286822; SNP6, rs2286823;SNP7,2302432; SNP8, rs1057868; SNP9, rs2302433. All the P value are the adjusted one after correction

POR SNP	CYP genotype	POR genotype	n	K_m(μM)	V_{max}(pmol·min^{-1} mg^{-1} protein)	CL_{int}(μl·min^{-1}· mg^{-1} protein)
POR SNP1	1A2 -3860GA	AA	20	49(32~134)	804(333~1440)	15.0(6.0~31.9)
(A>C)		AC	12	77(41~182)	544(216~3154)	7.0(2.8~28.4)[a]
		CC	3	56(36~65)	784(581~987)	16.2(12.1~17.8)
[a]P=0.038 vs POR SNP1 AA						
POR SNP2	2D6 1661GG	AA	11	29(11~160)	54(24~214)	1.93(0.65~12.23)
(A>G)		AG	21	66(20~261)[a]	68(32~322)	1.18(0.20~8.75)
		GG	11	105(19~171)	113(41~255)	1.50(0.27~1.92)
	2E1 -333TA	AA	9	78(43~177)	457(297~618)	7.27(1.90~11.1)
		AG	22	58(29~92)	633(163~1982)	11.1(3.30~22.4)
		GG	7	44(28~64)[aa]	503(318~721)	11.6(7.30~14.7)
[a]P= 0.036 vs POR SNP2 AA, [aa]P=0.009 vs POR SNP2 AA						
POR SNP3	2A6*9 *1/*1	GG	5	5.69(1.70~10.09)	800(291~1430)	164(141~184)
(G>A)		GA	37	2.29(0.90~7.88)	293(223~332)[a]	125(107~143)[b]
		AA	12	2.60(1.33~3.37)	391(285~499)	150(112~189)
	2E1 -333TA	GG	7	84(43~177)	453(297~618)	6.97(1.90~11.10)
		GA	23	58(29~92)	627 (163~1982)	10.95(3.30~22.40)
		AA	8	45(28~64)[cd]	496(318~721)	11.37(7.30~14.70)
	2E1 7632TA	GG	5	97(53~177)	388(297~590)	5.15(1.90~11.10)
		GA	16	52(29~89)[e]	739 (163~1982)	13.92(3.30~39.00)[f]
		AA	7	50(28~77)[g]	555(318~1237)	10.76(7.30~16.10)
[a]P=0.029 vs GG in POR SNP3, [b]P=0.025 vs GG in POR SNP3,[c]P=0.006 vs GG in POR SNP3, [d]P=0.04 vs GA in POR SNP3,[e]P=0.037 vs GG in POR SNP3, [f]P=0.026 vs GG in POR SNP3, [g]P=0.012 vs GG in POR SNP3.						
POR SNP4	2B6 785AG	TC	5	66(56~83)	51(41~1430)	0.78(0.61~1.76)
(T>C)		CC	19	87(51~185)[a]	361(50~903)	0.49(0.18~1.70)[b]
[a]P=0.041 vs TC in POR SNP4, [b]P=0.036 vs TC in POR SNP4.						
POR SNP5	2D6 2850CT	CC	11	28(13~58)	100(48~140)	3.56(0.89~10.33)
(C>T)	CT	20	29(11~237)	136(24~1041)	4.64(0.50~39.51)	TT 16 26(13~141) 128(54~222)[a] 4.70 (0.80~12.23)
[a]P=0.042 vs TT in POR SNP vs CC in POR SNP5.						
POR SNP7	1A2 -3860GG	GT	13	38.1(4.7~79.6)	848(95~1430)	17.2(5.9~45.3)
(G>T)		TT	45	59.6(29.7~160.1)[a]	731(328~2505)	13.0 (3.5~57.2)[b]
	1A2 5347CC	GT	16	50.5 (4.7~134.0)	852(95~1440)	16.1(6.0~31.9)
		TT	59	59.6(32.4~181.6)	727(180~3154)	11.5 (2.8~42.8)[c]
	2B6 516GT	GT	9	70(35~94)	55(22~132)	0.78(0.23~1.88)
		TT	20	82(51~185)	42(13~94)	0.52(0.18~1.21)[d]
	2D6 1661GG	GT	13	40.3(11.2~80.5)	87.2(23.5~322)	2.93(0.50~8.75)
		TT	39	72.2(6.54~261)[e]	120.8(36.7~255.3)	1.47(0.20~11.92)
[a]P= 0.017 vs POR SNP7 GT, [b]P= 0.047 vs POR SNP7 GT, [c]P= 0.026 vs POR SNP7 GT, [d]P= 0.043 vs POR SNP7 GT, [e]P= 0.021 vs POR SNP7 GT.						
POR SNP8	2A6 *1A/*1B	CC	32	2.20 (0.87~3.90)	396(102~702)	159(107~545)
(C>T)		CT	25	2.52(0.78~7.88)	325(50~974)	135(27~190)[a]
		TT	5	2.85(1.63~5.79)	300(180~873)	138(110~171)
	2C8 -271CC	CC	29	14.4(8.7~38.7)	51(3~175)	3.43(0.09~6.19)
		CT	36	14.0(7.7~30.9)	33(5~120)[b]	2.48(0.62~5.33)[bb]
		TT	8	16.0(8.3~22.9)	53(16~58)	3.15(0.68~3.92)
	2C8 -370TC	CC	18	14.1(8.7~22.9)	44(19~97)	3.17(0.84~6.19)
		CT	14	13.0(7.7~18.2)	14(4~88)[cc]	1.38(0.24~5.33)[cc]
		TT	2	16.0(15.9~16.1)	55(53~58)	3.46 (3.33~3.59)
[a]P= 0.022 vs POR SNP8 CC, [b]P= 0.026 vs POR SNP8 CC, [bb]P= 0.009 vs POR SNP8 CC, [cc]P= 0.004 vs POR SNP8 CC.						
POR SNP9	1A2 -163CA	CC	31	49.0(28.4~141.3)	772(395~3154)	16.1(3.5~42.8)
(C>T)		CT	5	41.2(4.7~124.2)	265(95~2763)[a]	11.6(3.6~38.8)
[a]P=0.037 vs CC in POR SNP9.						

A markedly higher V_{max} for *CYP2D6 2850CT* was found in samples with POR SNP5 mutations, the V_{max} increased to 128% for *CYP2D6 2850CT* in POR SNP5(C>T) TT group as compared with CC group.

SNP7 appeared to have a significant effect on activities of CYP1A2, CYP2B6 and CYP2D6. In this study only *POR* SNP7 GT and TT were detected, while allele GG was not found. POR SNP7 TT carriers showed an increased K_m in samples genotyped as *CYP1A2 -3860GG* as well as *CYP2D6 1661GG* and a decreased CL_{int} in samples genotyped as *CYP1A2 -3860GG, 5347CC* and *CYP2B6 516GT*. For the *CYP2D6 1661GG* genotype, the results indicated higher activities (CL_{int}) in individuals with homozygous mutations for the *POR* SNP7 genotype as compared with heterozygotes.

POR SNP8 carriers showed a significantly decreased V_{max} for samples genotyped as *CYP2C8 -271CC* and *-370TC* as well as a decreased CL_{int} for *CYP2A6*1A/*1B*, *CYP2C8 -271CC*, and *-370TC* genotypes. Obviously, for *CYP2C8 -271CC,* livers carrying *POR* SNP8 mutant heterozygotes had a decrease in V_{max} by 35% and CL_{int} decreased by 28%, as compared with wild-type (P=0.026, 0.009). For *CYP2C8 -370TC*, livers carrying *POR* SNP8 mutant heterozygotes had a decrease inV_{max} by 68% and CL_{int} decreased by 56%, as compared with wild-type(P=0.004).

In this study, only *POR* SNP9 CC and CT were detected, and no TT allele was discovered. For livers genotyped as *CYP1A2-163CA*, a significantly decreased K_m (16%) and V_{max} (66%) was noted in *POR* SNP9 CT carriers compared with CC carriers.

Effect of Multiple-Site Gene Polymorphisms (MSGP). There are 38 *POR* MSGPs; however, only 5 MSGPs (listed from MSGP1 to MSGP5) had a sufficient number of individuals (more than or equal to 5, Table 5) to be analyzed for the effect of MSGP on CYPs activities. The effect of these POR MSGPs were determined by the comparison of K_m, V_{max}, CL_{int} of CYPs between different MSGPs according to nonparametric methods.

MSGP Positive with SNP Negative

When combining *POR* SNP1 with other SNPs, livers carrying SNP1 showed diminished activity for CYP2B6, indicated by the V_{max} and CL_{int} in MSGP5 group decreasing by 48% (P<0.01) and 54%, respectively, as compared with MSGP4 group (Fig. 2). Here, MSGP5 contained an SNP1 mutation while MSGP4 did not. It is somewhat surprising that SNP1 alone did not show any significant influence on the overall activity of CYP2B6 nor on the activity of polymorphic *CYP2B6*.

MSGP Negative with SNP Positive

In contrast to the results above, the *POR* SNP1 alone was associated with significantly reduced CL_{int} for *CYP1A2 -3860GA*. However, no significant difference was observed in the kinetic parameters of CYP1A2 between the MSGP4 and MSGP5 groups, which differed in the presence of the SNP1 polymorphism (MSGP5). Similar examples were identified in the effect

Table 5. POR multiple-site gene polymorphisms (MSGP) in human liver microsomes.MSGP, multiple-site gene polymorphism of POR; SNP1, rs3823884; SNP2, rs1135612; SNP3,rs10954732; SNP4, rs4732515; SNP5, rs2286822; SNP6, rs2286823; SNP7, rs2302432; SNP8, rs1057868; SNP9, rs2302433. Only 5 MSGPs (listed from MSGP1 to MSGP5) had a sufficient number of individuals (more than or equal to 5). These 5 MSGPs accounted for a number of 32 livers. "Others" mean that there were 45 livers left which belong to the MSGPs with sample numbers less than 5

	POR SNP alleles									
MSGP genotype	SNP1 (A>C)	SNP2 (A>G)	SNP3 (G>A)	SNP4 (T>C)	SNP5 (C>T)	SNP6 (G>T)	SNP7 (G>T)	SNP8 (C>T)	SNP9 (C>T)	n
MSGP1	AC	AG	GA	TC	CT	GT	GT	CC	CC	6
MSGP2	AA	AG	GA	CC	CC	GT	TT	CT	CC	5
MSGP3	AA	AG	GA	CC	CT	GT	TT	CT	CC	9
MSGP4	AA	GG	AA	CC	TT	TT	TT	CC	CC	6
MSGP5	AC	GG	AA	CC	TT	TT	TT	CC	CC	6
Others	-	-	-	-	-	-	-	-	-	45

of SNP7 alone on some genotypes of *CYP1A2*, while no effect on activities was seen between MSGP5 and MSGP1 groups, where MSGP5 contains the SNP7 homozygote mutation and MSGP1 does not.

Different Effect between MSGP and SNP

Another finding was that the magnitude of the differences in the effect between one SNP alone and an MSGP on enzyme activities of CYPs differed. The K_m and CL_{int} of CYP2D6 increased and decreased by 90% and 60%, respectively, with the MSGP3 genotype which contained the SNP5 mutation as compared with MSGP2 group. As for SNP5 alone, the V_{max} for *CYP2D6 2850CT* increased by 28%, implying a more profound effect of the MSGP than the SNP alone on activity of CYP2D6.

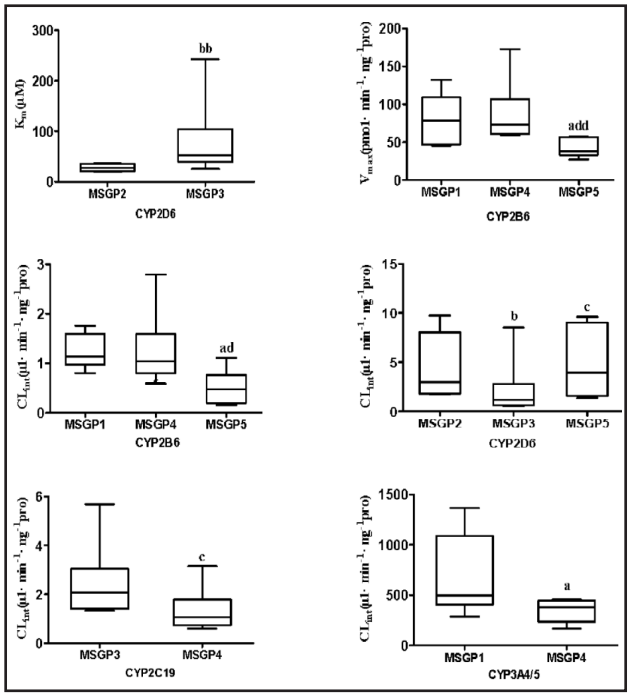

Fig. 2. Effect of POR MSGP on activities of CYPs. MSGP, multiple-site gene polymorphism. Only CYPs for which the K_m, V_{max} and/or CL_{int} are/is significantly affected by POR multiple-site gene polymorphism (MSGP) are displayed. K_m, V_{max} and/or CL_{int} are displayed in Scatter dot plot, with a line at the median and inter-quartile range. (MSGP1, n=6; MSGP2, n=5; MSGP3, n=9; MSGP4, n=6; MSGP5, n=6). [a]P=0.034 vs MSGP1 group; [b]P=0.042,[bb]P=0.005vs MSGP2 group; [c]P=0.012 vs MSGP3 group; [d]P=0.03, [dd]P=0.007 vs MSGP4 group. All the P value are the adjusted one after correction.

Discussion

Precision medicine_ENREF_1, proposing "providing the right patient with the right drug at the right dose at the right time", represents a major goal for 21st-century medicine [26, 27]. It is well recognized that inter-individual variations in drug response, including a lack of efficacy and adverse drug reactions represent the major challenges for personalized medicine. Although a drug effect is complex and depends on many factors, some human gene polymorphisms already have been associated with substantial differences in the metabolism or effects of drugs [28], and some are now being used to predict toxic metabolite-related disease or a clinical response [29-35]_ENREF_1. _ENREF_1However, as the principal electron donor for the drug metabolizing CYPs, little is known about the effect of *POR* gene polymorphisms on the activities of CYPs with different genotypes.

The perfect linkage between rs4732515, rs4732516 and rs2302431 has not been reported previously. Perfect linkage (r^2=1) was detected between rs3815455, rs41301394 and rs1057868, and between rs10239977 and rs1057870, and a relatively strong linkage (r^2≥0.95) was observed between rs2302432 and rs2228104 (Fig. 1). Some other perfect linkages observed in previous studies [36-38] were _ENREF_7not detected in our study. These disparities may stem from the different number of specimens and the ethnicity of the populations studied. Our present study confirmed several of the already reported common *POR* single nucleotide polymorphisms (SNPs) and allele frequencies and are consistent with the data in Chinese population obtained in a previous report [38].

It should be emphasized that effect of *POR* SNPs on the overall activity of CYPs differed from that exerted on polymorphic CYPs. *POR* SNP2 and SNP8 showed significantly decreased overall K_m of CYP2E1 and CYP2B6, respectively. The impact of *POR* SNPs on the activity of a given CYP depended on the genotype. When CYP activities were characterized by *CYP* genotypes, several additional CYPs, including *CYP1A2*, *2A6*, *2C8* and *CYP2D6* contained specific genotypes that exhibited significantly altered kinetic parameters in association with *POR* SNPs, as did specific genotypes for *CYP2B6*, and *2E1*. *POR* SNP2 showed a significantly decreased K_m for CYP2E1 by 18%; when CYP2E1 activity was analyzed for the different *2E1* genotypes, only samples genotyped as *CYP2E1 333TA* showed a significant decrease in CL_{int} by 26%. No significant effect on the activity of other *CYP2E1* genotypes was found with POR SNP2.

We found that the effect of multiple-site gene polymorphisms (MSGPs) on CYP activity did not always correspond to the effect seen with a single POR SNP. The effect of the *POR* SNP1 on CYP2B6 activity was not significant when it was the only POR mutation, but when it was combined with other *POR* SNPs, as in MSGP5, it showed significantly decreased activity of CYP2B6; note the difference in CYP2B6 activity between MSGP5 and MSGP4 (Fig. 2), the latter of which lacks the SNP1 mutation. A second example is with CYP1A2, where SNP1 alone was significantly associated with altered CYP1A2 activity but had no effect when combined with other *POR* SNPs, as in MSGP5. The magnitude of an effect can also differ between SNPs and MSGPs: SNP5 showed significantly increased CYP2D6 activity but had a greater effect when combined with other SNPs in MSGP3 compare with MSGP2. Taken together, the co-occurrences of mutations in *POR* might exert synergistic or compensating effects on CYPs activities.

Interestingly, our study indicates that the intronic *POR* SNPs resulted in dramatically altered activities of polymorphic CYPs, which was in partial agreement with an *in vitro* study [19] assessing more globally the influence of 46 POR mutations and an *in vivo* study [39] on effect of 5 intronic and one exonic POR mutations on CYP1A2 activity. However, *CYP* genotypes were not examined in these studies. We note that SNP1, which is a mutation in the 3'-untranslated gene region, was significantly associated with reduced activities of CYP1A2, suggesting that this SNP play a role in CYP activities despite being in a non-coding region of the gene. Consistent with our study, studies focusing on common intronic and silent polymorphisms in the *CYP2D6* and *CYP2C19* genes [40, 41] both indicated that intronic *CYP* SNPs were significantly associated with CYP activity. Studies on possible mechanisms are currently in progress. Emerging evidence [42] 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. Significantly altered POR expression and activity associated with *POR* mutations in introns and 5'-untranslated region, leading to altered CYP activity, according to our previous study [16] may help demonstrate this.

In conclusion, our study is the first to investigate systematically the effect of *POR* single nucleotide polymorphisms (SNPs) alone and multiple-site gene polymorphisms (MSGP) on the activities of 10 highly polymorphic CYPs in a large collection of normal Chinese liver samples. The effect of *POR* gene polymorphisms on CYP activities varied not only in a CYP-dependent manner, but more importantly in a *CYP* genotype-dependent manner. Moreover, the effect of *POR* MSGPs on CYP activities was not always consistent with that of single *POR* SNPs, and *POR* MSGP analysis appears to be more appropriate for accurately evaluating the effect of *POR* mutations on CYP activity. This work may have important implications for providing a convincing demonstration of the potential functional impact of *POR* gene polymorphisms on activities of polymorphic CYPs while at the same time revealing a hitherto-unrecognized effect of MSGPs on CYP drug metabolism, which may help elucidate the genetic basis underlying substantial changes in drug metabolism and facilitate the development of personalized medicine.

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Disclosure Statement

The authors declare no competing financial interests.

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