From Genotype to Phenotype: Content and Activities of Cytochromes P450 2A6 in Human Liver In Vitro and Predicted In Vivo^S

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ABSTRACT

Unraveling the molecular mechanisms by which genetic variants of cytochrome P450 2A6 lead to different metabolic phenotypes remains a long-standing but important challenge. CYP2A6 is an enzyme involved in the metabolism of several clinical drugs as well as the metabolic activation of carcinogenic nitrosamines. Herein, CYP2A6 genotypes and phenotypes, as indicated by protein content [by liquid chromatography–mass spectrometry (MS)/MS] and metabolic activities [$V_{\max}$, clearance (CL)], were determined for 90 human liver samples. We determined the median, range, and interindividual and intraindividual variation of CYP2A6 content and activity at the microsomal, liver tissue, and whole liver level and predicted hepatic in vivo clearance by in vitro–in vivo extrapolation based on CYP2A6-mediated coumarin metabolism by each CYP2A6 genotype. These results reveal how different CYP2A6 genotypes yield different phenotypic traits in protein content and enzyme activity. For relative $V_{\max}$, CL, and protein content, the intraindividual percentage coefficients of variation (ICVs) were 41.0% (18.8%–125.1%), 28.5% (2.39%–133.5%), and 27.8% (2.68%–88.0%), respectively. The high ICVs implied large intraindividual variation at different levels, sometimes in a genotype-dependent manner.

Intergenotype analysis revealed that the CYP2A6*4 allele demonstrated the most obvious effect on phenotypic outcomes, both in protein content and in metabolic activity. Indeed, decreased CYP2A6 protein content with the CYP2A6*4 genotype might explain the decreased metabolic activity from the molecular to the organismal level. These findings may allow useful predictions for CYP2A6-mediated drug metabolism on an individual patient basis in accord with the goal of achieving personalized medicine.

SIGNIFICANCE STATEMENT

We provide the median, range, and interindividual and intraindividual variation in CYP2A6 content at the microsomal, liver tissue, and whole liver level by liquid chromatography–mass spectrometry (MS)/MS as well as activities at the protein, microsomal, liver tissue, and whole liver level both in vitro and at the organismal level based on CYP2A6-mediated coumarin metabolism with each CYP2A6 genotype, thereby allowing us to elucidate how different CYP2A6 genotypes yield differing phenotypic traits (protein content and enzyme activity), facilitating the development of personalized medicine.

Introduction

A recent study (Tennessen et al., 2012) yielding the sequence of 15,585 human protein-coding genes showed that 2.3% of the 13,595 single nucleotide variants involving ~313 genes were predicted to impact protein function. Cytochrome P450 enzymes are characterized by abundant gene polymorphisms and great interindividual variations in drug-metabolizing activity (Zanger and Schwab, 2013). Our previous studies carried out with human liver samples derived from both individuals who were normal or had hepatocellular carcinoma indicated that various genetic variants of CYP450

(Gao et al., 2016b; Fang et al., 2018a,b) combined with other factors, including CYP450 reductase (Tian et al., 2016; Fang et al., 2018b) and CYPb5 (Zhang et al., 2015a), generated significant variation in metabolic phenotypes (Zhang et al., 2016; Gao et al., 2017c).

More importantly, differences in CYP activity (Gao et al., 2016a; Zhou et al., 2016) are associated with increased risk for diseases like hepatofibrosis (Gao et al., 2017b) and hepatocarcinogenesis (Gao et al., 2018a,b). Thus, studies on CYP genotype-phenotype associations may have important disease implications. However, with regard to metabolic phenotypes for CYPs, research in this area has focused primarily on the effect of CYP genetic variants on enzyme-catalyzed drug metabolism in vitro (Palma et al., 2010; Mürdter et al., 2012; Dai et al., 2014; Werk and Cascorbi, 2014) or on drug clearance in vivo (Mega et al., 2009; Zordoky and El-Kadi, 2010; Zhu

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ABBREVIATIONS: BW, body weight; CL, clearance; HLM, human liver microsome; ICV, intraindividual percentage coefficient of variation; LC, liquid chromatography; LW, liver weight; MPPGL, microsomal protein per gram of liver; MS, mass spectrometry.

et al., 2011; Shuker et al., 2016). It has long been known that genetic effects on physiologic phenotypes often depend on the cellular and environmental context, such as culture media, cell type, or tissue. For this reason, a more comprehensive process for CYP genotype-phenotype association is needed.

In the past several years, CYP2A6 has gained importance in numerous studies on the more than 45 known allelic variants (<http://www.imm.ki.se/CYPalleles/cyp2a6.htm>) and their roles in the metabolism of coumarin and a number of pharmaceuticals. Along with these pharmaceuticals, toxins and procarcinogens, such as aflatoxin B1 and tobacco-specific nitrosamines, may exhibit differences in blood concentration and duration of effect as a result of CYP2A6 polymorphisms (Sadeque et al., 1997; White et al., 2001; Xu et al., 2002) that are metabolically activated by CYP2A6. Development of a comprehensive genotype-phenotype model for CYP2A6 has the potential to improve our understanding of phenotypic variation and enhance the prediction of clinical outcomes for drugs metabolized by this cytochrome P450.

Clinically, studies on CYP2A6 genotype-phenotype associations may have important clinical implications because of CYP2A6's major role in the metabolism of nicotine. However, studies on CYP2A6 genotypes determined in vitro (Fukami et al., 2005; Wang et al., 2006) (e.g., by site-directed mutagenesis and liver microsomes) and/or in vivo (e.g., by phenotyping using substrates) are not always consistent. Take CYP2A6*1B as example: It was characterized with either elevated catalytic activity (Yoshida et al., 2003; Wang et al., 2006) or normal activity (Ujjiin et al., 2002; Peamkrasatam et al., 2006; Wang et al., 2011), making it difficult to determine the genotype-phenotype association and predict phenotypic outcomes.

One recent report focused on the CYP2A6 genotype-phenotype correlation and showed large interindividual variation in phenotypes (Tanner et al., 2017). Unfortunately, insufficient background information for more than half of the donors made accurate and rigorous evaluation of the genotype-to-phenotype association difficult. Moreover, phenotypes including protein content and activity measured in that study were only determined at one level in vitro. The extrapolation from phenotype in vitro to phenotype in vivo is complex and may involve multiple intermediates. Simple genotype-to-phenotype prediction may not be sufficient to accurately determine the phenotype. As a result, efforts to identify the influential intermediate steps in the process from genotype to ultimate phenotype for CYP2A6 are still an urgent need.

With regard to the characterization of CYP2A6 phenotypes, as mentioned in a previous study (Gao et al., 2017a), they can be divided into content phenotypes and metabolic phenotypes, as evidenced by CYP2A6 protein expression and activity from the molecular level to the cellular level and then to organismal level.

We generated a genotype-phenotype model for CYP2A6 based on three gene polymorphisms of CYP2A6 (CYP2A6*1B, *4, and *9) with frequencies greater than 1% in Chinese subjects. We determined protein expression and metabolic activity from the molecular to the cellular (microsomal, liver tissue, and liver) and organismal level and presented the intraindividual and interindividual variation in metabolic activity for CYP2A6 with different genotypes. Consideration of differences in enzyme activity at the molecular level and the effect on clearance at the organismal level may provide insight into how CYP2A6 molecular genetic variation influences phenotypic variation.

Material and Methods

Human Liver Samples

Normal human liver tissues were obtained from 90 Chinese patients undergoing liver surgical resection at Henan Provincial People's Hospital or Henan Provincial Tumor Hospital during 2012 and 2014. This research was performed in accordance with the Declaration of Helsinki. All experiments were approved by the Medical Ethics Committee of Zhengzhou University, and written informed consent was obtained from all patients. All liver samples were obtained from patients who were undergoing hepatic surgery and free of infectious disease, with medical diagnoses mostly of cavernous hemangioma of liver (61 cases); a few had metastatic cancers (seven cases), cholelithiasis (eight cases), gallbladder cancer (four cases), hepatic cholangiocarcinoma (six cases), or hepatocellular carcinoma (four cases). Detailed demographic information for the liver samples are presented in Supplemental Table 1. For patients who were tumor-bearing, normal liver samples were obtained 2 cm distant from the tumor tissue. Liver function tests, histopathological analysis, and imaging by ultrasonography or computed tomography were used to confirm that only liver tissue with normal liver function and normal histologic appearance was collected. Liver samples were frozen immediately after removal and stored in liquid nitrogen until use. There was no history of exposure to known CYP-inducing or CYP-inhibiting agents, and only routine anesthetics were used with the patients. Detailed donor characteristics of the human liver samples and human liver microsomes (HLMs) and the method of preparation by differential centrifugation are described in a previous study (Zhang et al., 2015b). Total HLM protein concentration was measured by the Bradford method (Bradford, 1976).

Genotyping of CYP2A6

Genomic DNA was isolated from human liver tissues using a genomic DNA purification kit (Beijing ComWin Biotech Co., Ltd., China). A two-step polymerase chain reaction method (Oscarson et al., 1999) was used to detect CYP2A6*1B and CYP2A6*4. Another single nucleotide polymorphism CYP2A6*9 (rs28399433) was genotyped by polymerase chain reaction sequencing. Genotyping errors were detected by resequencing with a subsample, and reproducibility was routinely greater than 99%.

Quantification of CYP2A6 Protein

Protein quantification of CYP2A6 was performed by nano-liquid chromatography (LC)-mass spectrometry (MS)/MS as previously reported (Zhang et al., 2016). A QconCAT protein consisting of 57 stable isotope-labeled peptides from 21 drug-metabolizing enzymes (including CYP2A6) in which two or three peptides were selected for each targeted protein was employed to quantify these proteins in HLMs (Li et al., 2015). The concentration of CYP2A6 protein was determined by nano-LC-multiple reaction monitoring MS using an easy nano-LC (Thermo Fisher Scientific Inc., Waltham, MA) coupled to a TSQVantage Triple Quadrupole mass spectrometer (Thermo Fisher Scientific Inc.).

Determination of Phenotype at Different Levels

Molecular phenotype is defined as protein content (Content_P) and activity [V_{max-P}, clearance (CL)_P] at the protein level. Cellular phenotype can be divided into microsomal, tissue, and organ levels; accordingly, protein expression at the cellular level in the liver is defined as Content_M, Content_{LT}, and Content_L, and activity is defined as V_{max-M}, V_{max-LT}, and V_{max-L} and CL_M, CL_{LT}, and CL_L, respectively. For organismal phenotype, this refers to drug clearance from the body at the whole organism level (in vivo).

Microsomal Phenotype. The subcellular phenotype, termed the microsomal phenotype, is defined as protein content (CYP_M), V_{max} (V_{max-M}), and clearance (CL_M). V_{max-M} and CL_M were determined by measuring the rate of coumarin 7-hydroxylation by high-performance liquid chromatography with eight coumarin concentrations (0.156–20 μM) according to a previously described method (Zhang et al., 2016).

Incubation mixtures contained HLMS, 100 mM phosphate buffer (pH 7.4) with 1 mM NADPH, and substrate. Optimal protein concentration was 0.3 mg/ml protein, and incubation time was 30 minutes. Perchloric acid (10 μ l) was added to terminate the reaction. The metabolite was identified by high-performance LC–UV. All experiments included two replicates. The Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max-M}) of CYP2A6 were determined by nonlinear regression analysis using GraphPad Prism 6.0, and CL_M was calculated based on the ratio of V_{max-M} to K_m .

$$CL_M = V_{max-M} / K_m \quad (1)$$

Molecular Phenotype. The metabolic phenotype at the molecular level is defined as V_{max} (V_{max-P}) and clearance (CL_P) of drug per picomole of CYP2A6. The V_{max-P} and CL_P for each individual were obtained by dividing each individual microsomal phenotype parameter by the corresponding individual CYP2A6 protein content ($Content_M$).

$$CL_P = CL_M / Content_M \quad (2)$$

Tissue Phenotype. The tissue phenotype is defined as protein content ($Content_{LT}$), V_{max} (V_{max-LT}), and clearance (CL_{LT}). The $Content_{LT}$, V_{max-LT} , and CL_{LT} for each individual were obtained by multiplying each individual microsomal phenotype parameter ($Content_M$, V_{max-M} , and CL_M) by the corresponding individual microsomal protein per gram of liver (MPPGL). MPPGL is the microsomal protein per gram of liver and determined as previously reported (Zhang et al., 2015b). The mean MPPGL value was 42.0 (9.9–127.9) mg/g in this cohort of 90 liver samples.

$$CL_{LT} = CL_M \times MPPGL \quad (3)$$

Organ Phenotype. The organ phenotype is defined as content ($Content_L$), V_{max} (V_{max-L}), and clearance (CL_L) in liver. $Content_L$, V_{max-L} , and CL_L were obtained by multiplying each individual liver weight (LW)/body weight (BW) by the individual tissue phenotype parameter, also known as $Content_{LT}$, V_{max-LT} , and CL_{LT} , respectively. Detailed values of LW and BW were previously reported (Gao et al., 2017a). BW is the actual body weight for each individual, and LW is the liver weight, which is calculated by multiplying the liver volume (LV) by the liver density, wherein LV (ml) = $12.5 \times BW$ (kg) + 536.4 (Wang et al., 2008), and the liver density is 1.001 g/ml (Yuan et al., 2008). The mean LW/BW value was 21.2 (18.4–30.4) g/kg in this cohort of 90 liver samples.

Organismal Phenotype. The organismal phenotype is defined as clearance (CL_H) in vivo. According to our previous report, the Bias-Corrected Conventional in vitro–in vivo extrapolation method (Gao et al., 2016a, 2017a) was adopted for extrapolating the CL_H for different genotypes on the basis of Q_H , $f_{u,p}$, and R_B . C_O , the cardiac output (C_O), determined based on age and gender, was 5.45 (4.92–6.65) l/min. Q_H , the hepatic blood flow, determined as 24.5% of the C_O (Barter et al., 2013), was 1334.9 (1205.4–1629.3) ml/min. The plasma unbound fraction, $f_{u,p}$, determined as 0.055 for coumarin and R_B , ratio of the drug concentration in blood to plasma, and as 1 for coumarin, was obtained from our previous study (Gao et al., 2016a). Detailed data and the computing method for Q_H , $f_{u,p}$, and R_B were reported in our previous studies (Zhang et al., 2015b; Gao et al., 2016a). The clearance for CYP2A6 in vivo (CL_H) was calculated using the well-stirred model:

$$CL_H = (Q_H \times CL_L \times f_{u,p} / R_B) / (Q_H + CL_L \times f_{u,p} / R_B) \quad (4)$$

The average fold error (AFE) was introduced to assess the overall accuracy of prediction, which was calculated as follows:

$$AFE = 10^{\frac{1}{N} \sum \log(\text{predicted mean} / \text{observed overall mean})} \quad (5)$$

The individual fold error (IFE) was introduced to evaluate the individual accuracy, which was calculated as:

$$IFE = 10^{\frac{1}{N} \sum \log(\text{predicted individual value} / \text{observed overall mean})} \quad (6)$$

N refers to the number of separate reports in the literature concerning coumarin intravenous clearance.

The definitions and corresponding units for parameters at each level were listed in Table 1.

Intraindividual Variation of CYP2A6 at Different Levels

To make CYP2A6 phenotypes at different levels comparable, relative V_{max} (R_{Vmax}), relative CL (R_{CL}), and relative Content ($R_{Content}$) were computed. For one specific level, R_{Vmax} , R_{CL} , and $R_{Content}$ were calculated as the individual V_{max} , CL , and Content divided by the median for that level. Variation in R_{Vmax} , R_{CL} , and $R_{Content}$ among different levels for each person was expressed as the intraindividual percentage coefficient of variation (ICV).

$$\text{Relative phenotypes}(R_{Vmax}, R_{CL} \text{ and } R_{Content}) = (\text{individual value}) / (\text{median}) \quad (7)$$

$$\text{ICV for relative phenotypes} = (\text{standard deviation}) / (\text{average value}) \quad (8)$$

For one specific relative phenotype (R_{Vmax} , R_{CL} or $R_{Content}$), the ICV was calculated as the S.D. divided by the corresponding average value of the relative phenotype among different levels.

To compare the variation trend of V_{max} and CL from the molecular to the organismal level, the percentage rank change of individual CYP2A6 phenotypes was developed. Ninety samples were ranked in ascending order according to the value of each metabolic phenotype parameter: V_{max-P} , V_{max-M} , V_{max-LT} , V_{max-L} , CL_P , CL_M , CL_{LT} , CL_L , and CL_H . The rank change for each individual was determined by comparison with phenotype at the molecular level (V_{max-P} or CL_P), calculation of the absolute difference value between the rank of V_{max} or CL at different levels (V_{max-M} , V_{max-LT} , V_{max-L} , or CL_M , CL_{LT} , CL_L , and CL_H), and the rank of corresponding V_{max-P} or CL_P . The percentage of rank change was computed for the sum of the 90 samples divided by the rank change absolute value of each individual. For CYP2A6 content, content phenotype at the molecular level could not be obtained, and so analysis of its rank change could not be determined.

Intergenotype Variation of CYP2A6 at Different Levels

V_{max-P} , V_{max-M} , V_{max-LT} , V_{max-L} , $Content_M$, $Content_{LT}$, $Content_L$, CL_P , CL_M , CL_{LT} , CL_L , and CL_H were sorted by CYP2A6 genotype. As compared with wild type, fold changes of genotype were calculated by dividing the absolute value of the median for the phenotype by the absolute value of median for the corresponding wild type. The fold change for all phenotypes with wild type was defined as one.

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software (SPSS Inc., Chicago, IL). Kolmogorov-Smirnov and Shapiro-Wilk tests were introduced to assess the normality of the data distribution. One-way ANOVA and Bonferroni tests were used for the comparison of relative phenotype potency. Since most data sets of phenotypes, including kinetic parameters and protein content, were not normally distributed, nonparametric methods were generally used. Mann-Whitney U and Kruskal-Wallis H tests were used for pairwise comparison and multiple pairwise comparisons of phenotypes with different genetic polymorphisms. Nonparametric Spearman rank correlation analysis was conducted to calculate the correlation coefficient (r) among different phenotypes. $P < 0.05$ (two-tailed) was considered statistically significant. Graphs were generated using Graph Pad Prism software version 6.02. Both the genotyping and phenotyping tests were derived from three separate and independent replicates to obtain

TABLE 1

Definitions of phenotypes for CYP2A6 at different levels

Molecular level, or phenotype at the protein level, $V_{\max-P}$ (pmol/min per picomole), CL_{-P} (μ l/min per picomole). Subcellular level, or phenotype at microsomal level, $V_{\max-M}$ (pmol/min per milligram), CL_{-M} (μ l/min per milligram), $Content_{-M}$ (pmol/mg). Tissue level, or phenotype at liver tissue level, $V_{\max-LT}$ (pmol/min per gram), CL_{-LT} (μ l/min per gram), $Content_{-LT}$ (pmol/g). Organ level, or phenotype at liver level, $V_{\max-L}$ (nmol/min per kilogram), CL_{-L} (μ l/min per kilogram), $Content_{-L}$ (nmol/kg). Organism level, or phenotype (clearance) at in vivo level, CL_{-H} (ml/min).

Level	$V_{\max}$		CL_{int}		Content	
	Def	Units	Def	Units	Def	Units
Molecular (protein)	$V_{\max-P}$	mol/min per picomole	CL_{-P}	μ l/min per picomole	—	—
Subcellular (microsomal)	$V_{\max-M}$	mol/min per milligram	CL_{-M}	μ l/min per milligram	$Content_{-M}$	pmol/mg
Tissue (liver tissue)	$V_{\max-LT}$	mol/min per gram	CL_{-LT}	μ l/min per gram	$Content_{-LT}$	pmol/g
Organ (liver)	$V_{\max-L}$	mol/min per kilogram	CL_{-L}	μ l/min per kilogram	$Content_{-L}$	nmol/kg
Organism (in vivo)	—	—	CL_{-H}	ml/min	—	—

def, definitions.

an accurate value. Experimental results were averaged to obtain a single value for that experimental series.

Results

Genotype of CYP2A6

The three most common genetic variants with an allele frequency of more than 1% in the Chinese population were analyzed for a total of 90 samples. The allele *CYP2A6*1B* has a gene conversion located in the 3'-untranslated region, with the frequency of the wild-type, heterozygous, and homozygous genotypes being 12.22%, 56.67%, and 17.78%, respectively. *CYP2A6*4* is a *CYP2A6* gene-deletion variant, with the frequency of the wild type and heterozygotes being 86.67% and 13.33%, respectively, and with no homozygous genotype detected. The *CYP2A6*9* (−48T>G) is located in the TATA box, with the frequency of the wild-type, heterozygous, and homozygous genotypes being 51.11%, 32.22%, and 3.33%, respectively.

Phenotype of CYP2A6

Phenotypes at Different Levels. Substantial interindividual variations in the metabolic phenotypes ($V_{\max}$, CL) and protein phenotypes (Content) for CYP2A6 from the molecular

to cellular level and then to organismal levels are depicted in Table 2, with the greatest difference demonstrated in CL (454-fold) at microsomal level, 144.8 (1.20~544.7) μ l/min per milligram. The CL phenotype exhibited the most remarkable variations, followed by $V_{\max}$, and CYP2A6 content was relatively smaller at most levels. Variations of $V_{\max}$ showed an increased trend from molecular (63.8-fold) to the tissue level (247-fold) and then to the organ level (134-fold).

To make CYP2A6 phenotypes at different levels comparable, relative $V_{\max}$ ($R_{-V_{\max}}$), CL (R_{-CL}), and Content ($R_{-Content}$) were computed. Each level's $R_{-V_{\max}}$, R_{-CL} , and $R_{-Content}$ were calculated as the individual $V_{\max}$, CL , and Content divided by the median for that level. To sort each level's relative phenotypes in a strong-to-weak sequence, we evaluated the relative potencies at each level ($R_{-V_{\max}}$, R_{-CL} , and $R_{-Content}$) in 90 samples. It is noteworthy that the $R_{-V_{\max}}$ potencies at the organ (liver) level, compared with other levels, were always higher. Analysis for the values of $R_{-V_{\max}}$ of four different levels revealed that the $R_{-V_{\max}}$ for the organ level was significantly higher than that of the other three levels (Table 2). For values of R_{-CL} and $R_{-Content}$, statistically significant differences were not demonstrated among different levels.

Intraindividual Variation among Different Levels. The relative $V_{\max}$ ($R_{-V_{\max}}$), CL (R_{-CL}), and Content ($R_{-Content}$) values for different levels are depicted in Fig. 1. The ICVs of

TABLE 2

CYP2A6 phenotypes at different levels ($n = 90$)

Relative phenotypes, $R_{-V_{\max}}$, R_{-CL} , $R_{-Content}$, referring to relative $V_{\max}$, CL , and Content, respectively, were computed to make CYP2A6 phenotypes at different levels comparable. Each level's relative $V_{\max}$, CL , and Content ($R_{-V_{\max}}$, R_{-CL} , and $R_{-Content}$) were calculated as the individual $V_{\max}$, CL , and Content divided by the median of corresponding parameter for that level. Molecular level, or phenotype at the protein level, $V_{\max-P}$ (pmol/min per picomole), CL_{-P} (μ l/min per picomole). Subcellular level, or phenotype at microsomal level, $V_{\max-M}$ (pmol/min per milligram), CL_{-M} (μ l/min per milligram), $Content_{-M}$ (pmol/mg). Tissue level, or phenotype at liver tissue level, $V_{\max-LT}$ (pmol/min per gram), CL_{-LT} (μ l/min per gram), $Content_{-LT}$ (pmol/g). Organ level, or phenotype at liver level, $V_{\max-L}$ (nmol/min per kilogram), CL_{-L} (μ l/min per kilogram), $Content_{-L}$ (nmol/kg). Organism level, or phenotype (clearance) at in vivo level, CL_{-H} (ml/min).

Level	$V_{\max}$			CL			Content		
	Range	Ratio	$R_{-V_{\max}}$	Range	Ratio	R_{-CL}	Range	Ratio	$R_{-Content}$
Molecular (protein)	M 19.79 R 1.08–68.89	63.8	1.16 ± 0.71	8.73 0.83–43.89	52.9	1.19 ± 0.86	—	—	—
Subcellular (microsomal)	M 354.4 R 9.47–1430	151	1.07 ± 0.78	144.8 1.20–544.7	454	0.97 ± 0.43	14.60 1.44–54.89	38.1	1.22 ± 0.75
Tissue (liver tissue)	M 12.27 R 0.25–61.74	247	1.28 ± 1.14	5.19 0.06–17.03	284	1.12 ± 0.68	637.6 71.17–3031	42.6	1.09 ± 0.56
Organ (liver)	M 267 R 6.22–1337	214	$2.08 \pm 1.86^{a,b,c}$	109.2 1.36–371.2	273	1.13 ± 0.70	893.2 58.43–5497	94.1	1.14 ± 0.89
Organism (in vivo)	M — R —	—	—	287.3 3.87–653.5	169	1.05 ± 0.48	—	—	—

M, median; R, range.

^a $P < 0.05$ vs. $V_{\max-P}$ at molecular level.^b $P < 0.05$ vs. $V_{\max-M}$ at microsomal level.^c $P < 0.05$ vs. $V_{\max-LT}$ at tissue level.

$R_{V_{\max}}$, R_{CL} , and $R_{Content}$ within different levels were 41.0% (18.8%–125.1%), 28.5% (2.39%–133.5%), and 27.8% (2.68%–88.0%) for 90 cases (Fig. 1A). ICVs with the values >100%, 50%–100%, and <50% are indicative of high, moderate, and low variation, respectively. In these 90 cases, the number (percentage) of high, moderate, and low ICVs for the values of $R_{V_{\max}}$ within four levels was 4 (4.44%), 10 (11.1%), and 76 (84.4%); for R_{CL} , they were 4 (4.44%), 24 (26.7%), and 62 (68.9%); and for $R_{Content}$, they were 15 (16.7%) and 75 (83.3%) for moderate and low ICV, respectively, with no high ICV detected. The results indicated surprisingly high intraindividual variation for CYP2A6 phenotypes among different levels. When relative phenotypes were classified by CYP2A6 genotypes, the ICVs of $R_{V_{\max}}$, R_{CL} , and $R_{Content}$ within different levels varied (Fig. 1, B–D). One notable example was for the CYP2A6*4 polymorphism, with $R_{V_{\max}}$ being 39.1% (18.8%–100.8%) and 50.0% (25.9%–125.0%) and R_{CL} being 26.7% (2.39%–102.9%) and 41.6% (11.1%–133.5%) for *1/*1 and *1/*4 genotypes, respectively. For $R_{Content}$, the genotype-dependent effect was less obvious.

To obtain more detailed information on how the alteration of CYP2A6 phenotypes among different levels originated, values for MPPGL, LW/BW, and Q_H (hepatic blood flow) were determined for individuals with CYP2A6 genetic differences (Fig. 2). Metabolic phenotypes at the liver tissue level ($V_{\max-LT}$, $CL-LT$) were obtained by multiplying each individual MPPGL by the corresponding individual $V_{\max}$ or CL at the microsomal level. LW/BW and Q_H were introduced to determine phenotypes at the organ and organismal levels, respectively. However, no significant effect of CYP2A6 genotype was found.

Genotype-Phenotype Association

No significant effect of demographic factors, including gender, age, smoking status, drinking habit, and medical

diagnoses (Supplemental Table 1), on phenotypes was detected (Supplemental Figs. 1–5).

CYP2A6*1B. No significant differences were found for $V_{\max}$ and CL at different levels among genetically different individuals with respect to CYP2A6*1B polymorphisms, nor was a significant effect on the K_m value detected. As for the content of CYP2A6 at different levels, the effect varied, with a minor impact on $Content_{LT}$ and $Content_L$, as demonstrated by significantly higher $Content_{LT}$ and $Content_L$ for individuals carrying CYP2A6*1B/*1B homozygote as compared with *1A/*1B heterozygotes; however, there was no significant difference as compared with wild type. It is worth mentioning that strongly significant correlations were charted between K_m and $V_{\max}$ at different levels among most of the genetic variants and were dependent upon CYP2A6 variant. For instance, for $V_{\max}$ at microsomal level ($V_{\max-M}$), extremely high correlation was detected with K_m for all CYP2A6*1B-related variants (Fig. 3); however, in terms of $V_{\max}$ at the isoform level ($V_{\max-P}$), no significant correlation was obtained with K_m for CYP2A6*1A/*1A wild type, and moderate correlation was obtained for *1A/*1B and *1B/*1B subjects. The mechanism underlying this observation remained to be determined.

CYP2A6*4. CYP2A6*4 was significantly associated with decreased $V_{\max}$, and the effect on phenotype at the cellular level was more markedly significant than that at the molecular level. The $V_{\max-P}$ values of CYP2A6*1/*4 individuals (15.24 pmol/min per picomole) were dramatically lower than *1/*1 (26.45 pmol/min per picomole), and the $V_{\max-M}$, $V_{\max-LT}$, and $V_{\max-L}$ of CYP2A6*1/*4 individuals were significantly less than a third of *1/*1 genotypes (Fig. 4). Subjects carrying CYP2A6*1/*4 genotypes showed lower $content_M$ (10.62 pmol/mg) and $content_{LT}$ (370 pmol/g) when compared with $content_M$ (18.92 pmol/mg) and $content_{LT}$ (629 pmol/g) for subjects with *1/*1. Observably lower values of $content_M$ and $content_{LT}$ for *1/*4 individuals than those in *1/*1 individuals might explain significantly greater lowered $V_{\max-M}$ and $V_{\max-LT}$ in

Fig. 1. ICVs of relative $V_{\max}$, CL, and Content of CYP2A6 at different levels ($n = 90$). ICVs of relative $V_{\max}$, CL, and Content of CYP2A6 for total samples (A) and ICVs of relative $V_{\max}$ (B), CL (C), and Content (D) sorted by different CYP2A6 genotypes. ICVs were calculated as the S.D. of relative $V_{\max}$, CL, and Content divided by the corresponding average value for that case. Each level's relative $V_{\max}$, CL, and Content were calculated as the individual $V_{\max}$, CL, and Content divided by the median for that level. Horizontal lines represent medians with the interquartile range. *1A/*1A, $n = 11$; *1A/*1B, $n = 51$; *1B/*1B, $n = 16$. *1/*1, $n = 78$; *1/*4, $n = 12$. *1/*1, $n = 46$; *1/*9, $n = 29$; *9/*9, $n = 3$.

Fig. 2. Effect of *CYP2A6* genotypes on MPPGL (A), LW/BW (B), and Q_H (C). Q_H, hepatic blood flow. Horizontal lines represent medians with the interquartile range. *1A/*1A, *n* = 11; *1A/*1B, *n* = 51; *1B/*1B, *n* = 16. *1/*1, *n* = 78; *1/*4, *n* = 12. *1/*1, *n* = 46; *1/*9, *n* = 29; *9/*9, *n* = 3.

*1/*4 individuals than *1/*1 subjects. Consistent with this, we found that the $V_{\max-L}$ values of *1/*4 individuals (110.2 nmol/min per kilogram) were significantly lower than *1/*1 (366.2 nmol/min per kilogram) individuals.

It should be stated that individuals with the *CYP2A6**1/*4 genotype showed lower K_m than those with the *1/*1 genotype. No statistical difference was found for CL_P , which was obtained by dividing each individual $V_{\max-P}$ by the individual K_m , between the *1/*1 (10.76 μ l/min per picomole) and *1/*4 (10.83 μ l/min per picomole) genotypes, and this might be due to the simultaneously lowered $V_{\max-P}$ and K_m in individuals and the effect on $V_{\max-P}$ partially counteracting the negative effect on K_m , as evidenced by significant correlation between K_m and $V_{\max-P}$ ($r = 0.457$, $P = 2.570 \times 10^{-5}$) for *1/*1 individuals; however, no such correlation was seen for *1/*4 individuals. The CL_M (75.05 μ l/min per milligram),

CL_{LT} (3.70 ml/min per gram), and CL_L (76.97 ml/min per kilogram) of *CYP2A6* for subjects with *1/*4 were dramatically lower than those for *1/*1 individuals (150.11 μ l/min per milligram, 6.12 ml/min per gram, and 130.12 ml/min per kilogram, respectively). This could be due to the simultaneously lowered $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$, and K_m in individuals, as evidenced by a significant correlation between K_m and $V_{\max}$ at different levels, especially for the extremely strong correlation between K_m and $V_{\max-M}$ ($r = 0.908$, $P = 2.092 \times 10^{-30}$), K_m and $V_{\max-LT}$ ($r = 0.751$, $P = 2.285 \times 10^{-15}$), and K_m and $V_{\max-L}$ ($r = 0.751$, $P = 1.024 \times 10^{-13}$) for *1/*1 individuals; however, no significant correlation was seen for *1/*4 individuals.

CYP2A6*9. Consistent with *CYP2A6**1B, the effect of *CYP2A6**9 on phenotypes was not as significant as with *CYP2A6**4 (Fig. 5). Only subjects with *CYP2A6**1/*1 wild type and *1/*9 heterozygotes were taken into analysis,

Fig. 3. Genotype-phenotype associations varied by *CYP2A6**1B polymorphisms. A1~A4 ($V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$), B1~B4 (K_m , $Content_M$, $Content_{LT}$, $Content_L$), C1~C4 (CL_P , CL_M , CL_{LT} , CL_L) were divided by *1A/*1A, *1A/*1B, and *1B/*1B genotypes (*1A/*1A, *n* = 11; *1A/*1B, *n* = 51; *1B/*1B, *n* = 16). D1~D4, E1~E4, G1~G4, correlation between K_m with $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$ in *1A/*1A, *1A/*1B and *1B/*1B genotypes, respectively. $V_{\max-P}$ (pmol/min per picomole), CL_P (μ l/min per picomole), the molecular phenotype or phenotype at the protein level. $V_{\max-M}$ (pmol/min per milligram), CL_M (μ l/min per milligram), $Content_M$ (pmol/mg), the subcellular phenotype or phenotype at microsomal level. $V_{\max-LT}$ (nmol/min per gram), CL_{LT} (ml/min per gram), $Content_{LT}$ (pmol/g), the tissue phenotype or phenotype at liver tissue level. $V_{\max-L}$ (μ mol/min per kilogram), CL_L (ml/min per kilogram), $Content_L$ (pmol/kg), the organ phenotype or phenotype at liver level. K_m (μ M), the Michaelis-Menten constant.

Fig. 4. Genotype-phenotype associations varied by *CYP2A6**4 polymorphisms. A1~A4 ($V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$), B1~B4 (K_m , Content_M , Content_{LT} , Content_L), C1~C4 (CL_P , CL_M , CL_{LT} , CL_L) were divided by *1/*1 and *1/*4 genotypes (*1/*1, $n = 78$; *1/*4, $n = 12$). D1~D4, E1~E4, correlation between K_m with $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$ in *1/*1 and *1/*4 genotypes, respectively. $V_{\max-P}$ (pmol/min per picomole), CL_P ($\mu\text{L}/\text{min}$ per picomole), the molecular phenotype or phenotype at the protein level. $V_{\max-M}$ (pmol/min per milligram), CL_M ($\mu\text{L}/\text{min}$ per milligram), Content_M (pmol/mg), the subcellular phenotype or phenotype at microsomal level. $V_{\max-LT}$ (nmol/min per gram), CL_{LT} (mL/min per gram), Content_{LT} (pmol/g), the tissue phenotype or phenotype at liver tissue level. $V_{\max-L}$ ($\mu\text{mol}/\text{min}$ per kilogram), CL_L (mL/min per kilogram), Content_L (pmol/kg), the organ phenotype or phenotype at liver level. K_m (μM), the Michaelis-Menten constant.

without considering *9/*9 homozygotes since only three individuals carried the *9/*9 genotype. No significant difference was found in $V_{\max-P}$ for *1/*1 carriers as compared with *1/*9 carriers, and there was a dramatically lower K_m for individuals with *1/*9 (2.07 μM) than for *1/*1 (3.03 μM) genotypes. The CL_P values, acquired by dividing individual $V_{\max-P}$ by each K_m as described above, of *1/*9 (12.72 $\mu\text{L}/\text{min}$ per picomole) individuals seemed to be higher than *1/*1 (8.71 $\mu\text{L}/\text{min}$ per picomole) subjects; however, this difference was not statistically significant. For both $V_{\max-M}$ and $V_{\max-LT}$, *1/*9 was associated with decreased activity as compared with wild type, which might be related to seemingly lower content_M and content_{LT} for *1/*9 individuals in spite of lack of statistical difference. Moreover, the CL_M for 2A6*1/*9 was significantly lower than that of *1/*1 individuals, which might be due to simultaneously lowered $V_{\max-M}$ and Content_M and a strongly significant association between K_m and $V_{\max-M}$. For $V_{\max-L}$, Content_{LT} , Content_L , CL_{LT} , and CL_L , no significant alteration was related with *9 polymorphisms, even with a lower tendency of $V_{\max-L}$, Content_{LT} , and Content_L for *1/*9 individuals. We note that K_m was strongly positively associated with $V_{\max}$ within different levels for both *CYP2A6**1/*1 and *CYP2A6**1/*9 genotypes except for $V_{\max-P}$ in *CYP2A6**9 wild-type carriers.

Intergenotype Variation

Intergenotype individual variations of *CYP2A6* phenotypes at different levels were charted, and significant differences in

variability among genetically different individuals are presented as seen in Fig. 6. Generally speaking, the *CYP2A6**4 polymorphism led to the most remarkable alteration, with decreased $V_{\max}$, Content , and CL at the majority of levels, whereas *CYP2A6**1B and *9 led to less obvious alteration either with increased or decreased phenotypes at some levels. Compared with wild type, various phenotypes including $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$, Content_M , Content_{LT} , CL_M , CL_{LT} , CL_L , and CL_H of *1/*4 showed an approximate decrease of 51%, 85%, 80%, 88%, 51%, 51%, 71%, 59%, 62%, and 57%, respectively, whereas the Content_L and CL_P were basically unchanged. For *CYP2A6**1B, Content_{LT} , Content_L , CL_{LT} , CL_L , and CL_H of mutant homozygotes were significantly higher than that of heterozygotes, whereas heterozygotes and mutant homozygotes were basically unchanged when compared with wild type. Most notably, *CYP2A6**1B led to distinctly increased Content_L with heterozygotes and mutant homozygotes showing 2.80-fold and 6.87-fold increases as compared with wild type, respectively. However, CL_L did not confirm this trend of change. With regard to *CYP2A6**9, lowered $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, Content_L , and CL_L of mutant heterozygotes were detected when compared with wild type, whereas Content_M , CL_P , and CL_M showed a reverse trend.

Moreover, with regard to K_m , in line with other phenotypes, the most remarkable alteration was with *1/*4 genotypes, which showed a 49% reduction, whereas *9 polymorphisms led

Fig. 5. Genotype-phenotype associations varied by *CYP2A6**9 polymorphisms. A1~A4 ($V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$), B1~B4 (K_m , Content_M , Content_{LT} , Content_L), C1~C4 (CL_P , CL_M , CL_{LT} , and CL_L) were divided by *1/*1, *1/*9, and *9/*9 genotypes (*1/*1, $n = 46$; *1/*9, $n = 29$; *9/*9, $n = 3$). D1~D4, E1~E4, correlation between K_m with $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$ in *1/*1 and *1/*9 genotypes, respectively. $V_{\max-P}$ (pmol/min per picomole), CL_P ($\mu\text{L}/\text{min}$ per picomole), the molecular phenotype or phenotype at the protein level. $V_{\max-M}$ (pmol/min per milligram), CL_M ($\mu\text{L}/\text{min}$ per milligram), Content_M (pmol/mg), the subcellular phenotype or phenotype at microsomal level. $V_{\max-LT}$ (nmol/min per gram), CL_{LT} (ml/min per gram), Content_{LT} (pmol/g), the tissue phenotype or phenotype at the liver tissue level. $V_{\max-L}$ ($\mu\text{mol}/\text{min}$ per kilogram), CL_L (ml/min per kilogram), Content_L (pmol/kg), the organ phenotype or phenotype at liver level. K_m (μM), the Michaelis-Menten constant.

to distinctly lower K_m , with heterozygotes and mutant homozygotes showing 32% and 47% decreases as compared with wild type, respectively. For *CYP2A6**1B, K_m of *1B carriers was basically unchanged for heterozygotes and homozygotes.

Discussion

In this present study of 90 human liver samples, the systematic delineation of a *CYP2A6* genotype-phenotype model including protein content and metabolic activity of *CYP2A6* at various levels such as molecular, cellular (microsomal, tissue, organ), and organismal levels was generated. We determined the median and range for the protein content, $V_{\max}$, and CL as well as relative phenotypes (R_{Content} , $R_{V_{\max}}$, and R_{CL}) at each level with every *CYP2A6* genotype to elucidate how different *CYP2A6* genotypes yield phenotypic traits (protein content and enzyme activity) at different levels. The high relative ICVs of phenotypes indicate large intra-individual variation within different levels, sometimes in a genotype-dependent manner. Intergenotype analysis indicated that *CYP2A6**4 had the most obvious effect on phenotypes, and protein content might be related to the *CYP2A6**4 allele. The correlation of phenotypes from the molecular level to the cellular level and then to the organismal level demonstrates how genotypes may affect phenotypes and

could be considered for better understanding of phenotypic variation and assessment of phenotypic outcome with specific genotypes.

We did find that the alteration of phenotypes from the molecular level to the cellular level and then to organismal level varied greatly, which agrees with our previous studies (Zhang et al., 2015b; Gao et al., 2017a). However, our previous study on the *CYP2D6* genotype-phenotype association (Gao et al., 2017a) indicated the effect of the *CYP2D6* genotype on the metabolic phenotype at each level but did not consider the alteration of phenotypes among different levels. In this study, the ICVs of $R_{V_{\max}}$, R_{CL} , and R_{Content} within different levels indicated noticeable intraindividual variation and varied with the *CYP2A6* genotype. Take the *CYP2A6**4 allele, for example; the ICVs of R_{CL} for *CYP2A6**1/*4, 41.6% (11.1%–133.5%), was nearly 1.56-fold of that for the *1/*1 genotype, 26.7% (2.39%–102.9%), whereas for $R_{V_{\max}}$, the difference was 1.28-fold. The reason for these differences might be the super-imposed effect of genotype on phenotype since the effect on the molecular level might influence the cellular level and then the organismal level. In line with this speculation, the effect on CL might be the combined effect on $V_{\max}$ and K_m since CL was obtained by dividing $V_{\max}$ by K_m . However, the precise mechanism underlying this needs further exploration.

The most impactful genotype-on-phenotype effect was with *CYP2A6**4, which is a gene-deletion genotype, in contrast to

Fig. 6. The diversity in CYP2A6 phenotypes at different levels. A1~A3 (fold changes of $V_{\max-P}$, $V_{\max-M}$, $V_{\max-LT}$, $V_{\max-L}$), A4 (fold changes of K_m), B1~B3 (fold changes of Content-M, Content-LT, Content-L), C1~C3 (fold changes of CL-P, CL-M, CL-LT, CL-L, CL-H) were sorted by different CYP2A6 genotypes. As compared with wild type, fold changes of other genotypes were calculated by dividing the absolute value of the median for the phenotype by the absolute value of median for the corresponding wild type. The fold change for all phenotypes with wild type were defined as one. CYP2A6*1B (*1A/*1A, $n = 11$; *1A/*1B, $n = 51$; *1B/*1B, $n = 16$); CYP2A6*4 (*1/*1, $n = 78$; *1*4, $n = 12$); CYP2A6*9 (*1/*1, $n = 46$; *1/*9, $n = 29$; *9/*9, $n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. wild type; # $P < 0.05$, ## $P < 0.01$ vs. mutant heterozygotes.

the *1B genotype (located in 3' untranslated region) and the *9 mutation (located in the "TATA box"), being the least impactful phenotype as measured herein. Results are in agreement with a series of previous studies (Ujii et al., 2002; Yoshida et al., 2003; Wang et al., 2006, 2011; Tanner et al., 2017), though those studies were conducted either in vitro by site-directed mutagenesis or in vivo by phenotyping using substrates. The location and type of mutation is clearly

important when evaluating the phenotypic impact of different genotypes.

It is noteworthy that protein content might play an important role for extrapolation from the genotype of CYP2A6 to the different levels of phenotypes, especially for CYP2A6*4. CYP2A6*4, a coding-deletion genotype, was associated with significant decreased protein and activity in our study and also with the same effect in previous reports (Peamkrasatam

et al., 2006; Wang et al., 2011). However, one such trend was detected in one study demonstrating protein content correlated well with activity (Tanner et al., 2017). The reason for the inconsistencies might be that liver samples conducted in that study were from two liver banks with different ethnicities, and only one *CYP2A6**1/*4 sample was identified, and so the effect of the *CYP2A6**4 genotype on phenotype could not be well evaluated. Our study focused on known but less common polymorphisms that were present at higher levels in the Chinese population. As depicted in Fig. 4, the markedly lower Content_M (10.62 pmol/mg) and Content_{LT} (3.70 ml/min per gram) in *1/*4 genotypes might help explain the much greater decrease in $V_{\max-M}$, $V_{\max-LT}$, and $V_{\max-L}$ compared with $V_{\max-P}$ correlated with *CYP2A6**4. This was consistent with the view that protein content and activity might play a role in determining metabolic phenotypes and susceptibility to disease (Tian et al., 2016; Gao et al., 2018a,b,c).

Most notably, one interesting finding was the highly significant positive correlation between K_m and $V_{\max}$ at each level in the *1/*1 wild type. Given that, we speculate that the significantly lowered CL_M, CL_{LT}, and CL_L in *4 carriers might be due to the more prominent decreased $V_{\max}$ when compared with K_m at each corresponding level, whereas for the phenotype at the molecular level, the almost uniform extent of change for $V_{\max-P}$ and K_m might be the reason for the insignificant alteration of CL_P related to the *4 variation. However, the explicit mechanism underlying this remains unknown and needs further investigation.

The interindividual variation of *CYP2A6* phenotypes in relation to different genotypes at various levels may play a role in interethnic or interindividual variability for *CYP2A6* phenotypes and the risk for *CYP2A6*-related disease. *CYP2A6**4, in particular, correlated with lower 2A6 phenotypes at a number of levels. For example, the median values of $V_{\max-M}$, Content_M, and CL_M were decreased to 15%, 49%, and 29%, respectively, in *4 carriers when compared with *1 wild-type carriers. This likely contributes to the known slower cotinine metabolism in Chinese-Americans than Caucasians (Benowitz et al., 2002) since the frequency of *CYP2A6**4 in Chinese was 6.6%~15.1%, much higher than the Caucasian level of 0.5%~4.9% (Xu et al., 2002).

The interethnic as well as interindividual variation in metabolic phenotypes might have several important clinical implications; for instance, *CYP2A6*-related metabolic activity (e.g., nicotine metabolism), behaviors (Malaiyandi et al., 2006; Gao et al., 2016a) (i.e., smoking), and disease (Fujieda et al., 2004) (i.e., lung cancer). Genetic studies on *CYP2A6* in Japanese indicated a decreased rate of lung cancer in inactive *CYP2A6* variants carriers in comparison with wild type (Miyamoto et al., 1999). In addition to the cigarette smoking-related lung cancer, *CYP2A6* is also involved in the metabolism of many other procarcinogens. However, a clearer understanding of the role for genotypes of *CYP2A6* in relation to disease is needed before conclusions can be reached.

Since liver samples of perfectly healthy persons with comprehensive background information are difficult to obtain, the liver samples used here were from patients undergoing hepatic surgery. However, the donors were with clear medical diagnoses mostly of cavernous hemangioma of liver and mitigated by techniques and confirmed by analysis (Supplemental Fig. 5) to minimize impact of tumors and other demographic factors (Supplemental Figs. 1–4). Moreover, the

total of 90 samples studied here with comprehensive background information is a large number of human samplings and is certainly adequate. Even so, high variability existed along with relatively low numbers per grouping and might still affect the ability to differentiate smaller differences. Many more samples are needed to rule out idiosyncratic variation as much as possible. Since it was not easy to simultaneously obtain in vivo and in vitro results for the same cohort of samples, no actual in vivo measurements were made in our study. We used in vitro measurements at different levels to predict hepatic clearance by in vitro–in vivo extrapolation to the in vivo level. After correction, our in vivo extrapolation by calculation yielded high accuracy, as indicated by the predicted CL of *CYP2A6* matching 82.8% of the cases within 2-fold error when compared with actual observed CL_{in vivo} determined in other studies (Ritschel et al., 1977)(Gao et al., 2016a). Even so, actual in vivo measurements are additional efforts needed to draw more clear and convincing conclusions.

In conclusion, we have developed a comprehensive *CYP2A6* genotype-to-phenotype model. In this model, a role for the *CYP2A6* genotypes in influencing phenotypic variation at various levels was evaluated, although clearly it may not be sufficient to explain variation in all phenotypic outcomes. It does provide the basal data for *CYP2A6* content and metabolic activities at different levels with each *CYP2A6* genotype as well as fundamental hierarchical relationships between *CYP2A6* genotypes and phenotypes. We identified a key role for decreased protein content for the *CYP2A6**4 genotype that is related to decreased metabolic phenotypes. These findings might help open new approaches for considering how genetic variation is translated to the phenotype of individuals, which might be a further step on the way to making useful predictions of outcomes for a particular individual as a part of personalized medicine and disease risk.

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Authorship Contributions

Participated in research design: Qiao.
Conducted experiments: Fang, Wang.
Contributed new reagents or analytic tools: Guo, Zhang, Wen.
Performed data analysis: Fang, Wang.
Wrote or contributed to the writing of the manuscript: Fang, Xing, Gao, Qiao.

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