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Determinants of Cytochrome P450 2D6 mRNA Levels in Healthy Human Liver Tissue

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Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme that exhibits large interindividual variability. Recent studies suggest that differential transcriptional regulation of *CYP2D6* in part may be responsible for the variability. In this study, we characterized potential determinants of CYP2D6 transcript levels in healthy human liver tissue samples (n = 115), including genetic polymorphisms in *CYP2D6* and the genes encoding transcription regulators for CYP2D6 expression; mRNA expression of the transcription factors and their known target genes; and hepatic levels of bile acids and retinoids, agents that modulate the expression/activity of the transcription factors. Their associations with CYP2D6 mRNA levels in the tissues were examined. Results from multivariable linear regression analysis revealed *CYP8B1* mRNA level and rs3892097, the single- nucleotide polymorphism defining the nonfunctional *CYP2D6*4* allele, as the two most significant predictors of *CYP2D6* mRNA levels in the liver tissue samples, explaining 30% of the variability.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Differential transcriptional regulation of *CYP2D6* may in part be responsible for interindividual variability in cytochrome P450 2D6 (CYP2D6)-mediated drug metabolism.
WHAT QUESTION DID THIS STUDY ADDRESS?
☑ We investigated relative contributions of multiple genetic and nongenetic factors to hepatic CYP2D6 expression by examining their correlation with basal CYP2D6 mRNA levels in 115 human liver tissue samples. They include genetic polymorphisms in *CYP2D6* as well as those in *NR0B2* and *HNF4A*, the genes encoding transcription factors that regulate CYP2D6 transcription. The mRNA levels of CYP8B1, a target gene of small heterodimer partner (SHP) and hepatocyte nuclear factor (HNF)4α, were also examined as an activity marker of SHP/HNF4α regulatory pathway.
WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ CYP8B1 expression was found to be the best predictor of hepatic CYP2D6 transcript levels.
HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Future studies using a larger sample size are needed to elucidate the contribution of rare *CYP2D6* polymorphisms to the variability in CYP2D6 transcript levels. Transcriptional regulation via SHP and HNF4α seems important in determining hepatic CYP2D6 transcript levels.

Cytochrome P450 (CYP2D6) is a major drug-metabolizing enzyme, responsible for eliminating over 20% of clinically used drugs.[1] CYP2D6-mediated drug metabolism exhibits large interindividual variability, and CYP2D6 phenotype of an individual can fall into one of the four categories of poor metabolizer, intermediate metabolizer, normal metabolizer, and ultrarapid metabolizer.[2] Genetic polymorphisms of *CYP2D6* linked to no enzyme activity are known to contribute to the poor metabolizer, whereas increased copy number of normal function *CYP2D6* alleles is associated with ultrarapid metabolizer phenotypes.[2,3] Of interest, recent studies indicate that differential transcriptional regulation of CYP2D6 may in

part explain the variability in CYP2D6-mediated drug metabolism.[4–9] Significant association between CYP2D6 mRNA and activity levels was reported in human liver tissue.[4–6] In addition, studies using *CYP2D6*-humanized mice showed that transcriptional regulation of *CYP2D6* can lead to serial changes in CYP2D6 protein and activity levels.[7–9]

CYP2D6 transcription can be regulated by actions of *cis*-elements (e.g., genetic polymorphisms affecting transcription factor binding) or *trans*-elements (e.g., differential expression/activity of transcription factors). Hepatocyte nuclear factor (HNF) 4α is a transcription factor known to regulate the expression of multiple hepatic

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genes, including *CYP2D6*.[10,11] HNF4α binds to a response element (-53/-41) in the *CYP2D6* promoter and enhances CYP2D6 transcription.[11] Of note, HNF4α activity is modulated by its interaction with other transcription factors, including small heterodimer partner (small heterodimer partner (SHP); encoded by *NR0B2*). SHP is a representative target gene of farnesoid X receptor (FXR), the bile acid sensor.[12] Bile acids induce *NR0B2* promoter activity via FXR activation, as well as enhancing SHP protein stability by post translational modifications.[13–15] SHP (as a transcriptional repressor), in turn, decreases the expression of genes encoding bile acid synthesis enzymes (e.g., CYP8B1), thus maintaining bile acid homeostasis.[13,16] In addition to bile acids, retinoids induce SHP expression.[7,17,18] Pharmacological or pathological induction of SHP (e.g., retinoid administration or cholestasis) was shown to trigger CYP2D6 downregulation in *CYP2D6*-humanized mice by inhibiting HNF4α transactivation of the *CYP2D6* promoter.[7,8]

Despite accumulating knowledge about multiple factors that modulate CYP2D6 transcription, a comprehensive study examining their relative contribution to CYP2D6 transcript levels has been lacking. The purpose of this study was to explore genetic and nongenetic factors that may govern CYP2D6 mRNA levels. To this end, we examined the relationship between the following factors and CYP2D6 mRNA levels in 115 healthy human liver tissue samples: (i) *CYP2D6* gene copy number, (ii) genetic variations in *CYP2D6* (including the regulatory region that may alter transcription factor binding), *HNF4A*, and *NR0B2*, (iii) differential expression/activity of HNF4α and SHP, and (iv) hepatic levels of endogenous compounds that modulate them (i.e., bile acids and retinoid). Based on the results, the best predictors of hepatic CYP2D6 mRNA expression levels have been identified using a panel of human liver tissue samples.

MATERIALS AND METHODS
Reagents
Cholic acid (CA), glycocholic acid (GCA) hydrate, taurocholic acid sodium salt hydrate, chenodeoxycholic acid (CDCA), sodium glycochenodeoxycholate (GCDCA), sodium taurochenodeoxycholate, deoxycholic acid (DCA), glycodeoxycholic acid monohydrate, sodium taurodeoxycholate hydrate, ursodeoxycholic acid, lithocholic acid, sodium taurolithocholate, sodium tauroursodeoxycholate, and all-*trans* retinoic acid (*at*RA), were purchased from Sigma Aldrich (St. Louis, MO). D5-*at*RA was purchased from Toronto Research Chemicals (Toronto, Ontario). The α-muricholic acid salt, tauro-α-muricholic acid, β-muricholic acid, tauro-β-muricholic acid, ω-muricholic acid, taurohyodeoxycholic acid, hyocholic acid, glycohyocholic acid, taurohyocholic acid, glycoursodeoxycholic acid, glycolithocholic acid, hyodeoxycholic acid, murocholic acid, taurodehydrocholic acid sodium salt, glycodehydrocholic acid, deoxycholic acid-2,2,4,4-D4 (DCA-D4), lithocholic acid-2,2,4,4-D4 (LCA-D4), glycochenodeoxyxholic acid-2,2,4,4-D4 (GCDCA-D4), cholic acid-2,2,4,4-D4 (CA-D4), and glycocholic acid-2,2,4,4-D4 (GCA-D4) were purchased from Steraloiods (Newport, RI).

Human liver tissue
Frozen human liver tissue samples without gross pathology were obtained from liver tissue and cell distribution system (funded by NIH Contract #HHSN276201200017C) and Corning (Corning, NY). Mean ages of donors were 49.4 ± 16.1 for liver tissue and cell distribution system tissue samples (*n* = 65; 32 men and 33 women) and 49.4 ± 14.7 for Corning (*n* = 50; 38 men and 12 women).

DNA sequencing
Single-nucleotide polymorphisms (SNPs) in *CYP2D6*, *NR0B2*, and *HNF4A* were examined using a custom-designed Ampliseq panel (ThermoFisher Scientific, Waltham, MA), which is a highly specific, multiplex polymerase chain reaction (PCR)-based sequence enrichment method using a customizable pool of oligonucleotide primer pairs. The custom Ampliseq panel was designed to target the *CYP2D6* gene region (4-kb upstream of the transcriptional start site to 1-kb downstream; hg19 chr22:42521501-42530883), the *NR0B2* gene region (2-kb upstream to 1-kb downstream; hg19 chr1:27236975-27242567), and exons, including adjacent intron/putative regulatory regions, of the *HNF4A* gene. The exon regions that could not be covered by the Ampliseq panel (hg19 chr22:42525025-42525235 and hg19 chr1:27240340-27240390) were sequenced using Sanger sequencing. Sequencing was completed using the Ion Torrent next-generation sequencing platform (ThermoFisher Scientific). Raw data from Ion Torrent sequencing were filtered for quality control and aligned to hg19 genome assembly. SNP calling was completed as previously described.[19] Sanger sequencing results for *CYP2D6* and *NR0B2* were aligned to hg19 chr22:42525025-42525235 and hg19 chr1:27240340-27240390 regions, respectively, using novoSNP software.[20]

CYP2D6 genotyping
Genotyping was performed as previously described.[19]

RNA isolation and quantitative real time-PCR
RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR) for CYP2D6 mRNA levels was previously described.[19] TaqMan assay IDs for other genes are listed in **Table S1**. Of note, additional CYP2D6 assay (i.e., Hs00164385_m1 assay) was used (i) to ensure accurate measurement of CYP2D6 mRNA levels and (ii) to explore potential interference of amplification by *CYP2D6*17*. The results from two assays showed an excellent correlation,[19] and data obtained using Hs.PT.56a.45336286.g assay were used in the final analysis. The relative mRNA expression level was determined after normalizing the expression levels to those of *HPRT1* and relative to an arbitrarily chosen sample (liver ID 1530) using $2^{-\Delta\Delta Ct}$ method.[21]

Measurement of hepatic bile acid levels
Hepatic bile acid levels were measured as previously reported,[22] with minor modifications. Briefly, human liver tissue (~ 100 mg) was weighed and homogenized in ice-cold methanol (0.6 mL) and internal standard solution (0.2 mL, 1 μM CA-D4, DCA-D4, GCA-D4, GCDCA-D4, and 2 μM

LCA-D4 in acetonitrile) using Precellys soft tissue homogenizing CK14 kit (Bertin, Rockville, MD) and a bead beater. Tissue was homogenized at 4°C at maximum speed in 30-second on-and-off cycles twice or until complete homogenization was achieved. The homogenate was then centrifuged at 3,000 *g* for 5 minutes. The supernatant was collected, and the pellet was extracted again using 0.4 mL cold methanol via the same procedure. The supernatant from the second extraction was combined with that from the first. The pooled supernatant was aliquoted (150 μL) and stored at −80°C until analyzed. The aliquots were evaporated to dryness in a Savant Speedvac concentrator (ThermoFisher Scientific) at medium temperature for 1 hour and reconstituted in methanol:water (50:50 v/v; 50 μL), vortexed vigorously for 30 seconds, and centrifuged at 16,000 *g* for 10 minutes at 4°C. The supernatant was used for liquid chromatography tandem mass spectrometer (LC-MS/MS) analysis. Two aliquots were analyzed for each liver sample.

For standard curves, individual bile acid stock solutions (5 or 10 mg/mL) were used to make working solution in methanol:water (50:50), with a final concentration of 100 μM for each bile acid, except for lithocholic acid (200 μM). Each working solution was diluted serially using methanol:water (50:50) to concentrations ranging from 5 nM to 50 μM. Internal standard solutions of DCA-D4, GCDCA-D4, CA-D4, GCA-D4, and LCA-D4 were prepared in methanol:water (50:50) at 1 μM concentrations for each compound and except for LCA-D4 (2 μM). Equal volumes (25 μL) of standard solution and internal standard solution were added to 100 μL of cold methanol. The mixture was vacuum-dried and reconstituted as described above. Both standard curve samples and human liver samples were prepared in duplicate.

The liquid chromatography separation was performed using 1290 infinity ultra-performance liquid chromatography (UPLC) system (Agilent, Danbury, CT) equipped with Acquity UPLC BEH C18 (1.7 μm, 2.1 × 100 mm; Waters) column, using an injection volume of 4 μL. Column and autosampler temperature was set at 50°C and 4°C, respectively. The following mobile phase was used: 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) in gradient elution (0 minute, 5% B; 0.5 minutes, 5% B, 7 minutes 25% B, 16 minutes, 40% B; 19.5 minutes, 95% B; 19.9 minutes, 95% B; 20 minutes, 5% B; and 22 minutes, 5% B) at a flow rate of 0.5 mL/minute. Eluents from 5.5–21.5 minutes were directed to the mass spectrometer system for analysis.

A QTRAP 6500 MS/MS system (Sciex) with electrospray ionization (ESI) source in negative-ion mode was used for analysis. Medium collisionally activated dissociation (CAD), ion source of −4,500 V, and a source temperature of 600°C were used. Curtain gas, TurbolonSpray nebulizer, and heater gas flow were set at 30, 60, and 60 psi, respectively. Multiple reaction monitoring (MRM) transitions for individual bile acids are listed in **Table S2**. The dwell time for each MRM was 50 ms. Data acquisition and analysis were performed using Analyst 1.6.2 software. Samples that show concentrations of single or multiple bile acid species at levels higher than the upper limit of quantification were diluted 10 times and analyzed again. The accuracy and quantification limits of the bile acid assays are shown in **Table S3**. Typical chromatograms are shown in **Figure S1**.

Measurement of hepatic *at*RA levels

Hepatic *at*RA levels were measured by LC-MS/MS, as previously described.[23] Briefly, liver tissues (~ 100 mg) were homogenized in a 1:1 volume of 0.9% NaCl, and the sample was transferred into a 15-mL glass culture tube. Two volumes of acetonitrile containing 0.1% formic acid were added along with D5-*at*RA (internal standard). Retinoic acid was extracted using 10 mL of hexanes, and the organic layer was dried under nitrogen at 37°C. The samples were reconstituted in 65 μL of acetonitrile/H_2O (60:40) for LC-MS/MS analysis. Standard curve and quality control samples were generated using ultraviolet (UV) light–exposed mouse liver homogenate spiked with *at*RA at concentrations of 0, 2, 5, 10, 15, and 20 nM for the standards and 3, 7.5, and 17.5 nM for the quality control samples. The retinoids were separated using an Agilent 1290 UPLC (Santa Clara, CA) equipped with a Sigma (St. Louis, MO) Ascentis Express RP Amide column (2.7 μm; 150 mm × 2.1 mm). Analytes were detected using an AB Sciex 5500 QTRAP MS/MS (Foster City, CA) operated in positive ion atmospheric pressure chemical ionization mode. MRM transitions for *at*RA and internal standard were 301/205 and 306/116, respectively.

Statistical analysis

Statistical analysis was performed using SAS version 9.4 (Cary, NC). All variables of interest (predictors and outcomes) were assessed for normality; nonnormal distributions were normalized using log transformations. For normality assessment, graphical inspections (e.g., histograms and boxplots) as well as the Shapiro-Wilk tests of normality ($P < 0.05$) were used. Following log transformation of variables, normality, including graphical inspection, skewness, and kurtosis, were re-examined. To determine basic predictor–outcome associations, a series of unadjusted linear regression analyses were first conducted. SNPs with complete linkage disequilibrium ($r^2 = 1$) were trimmed to reduce the redundancy in data analysis. Based on these analyses, a series of multivariable linear regressions was then conducted whereby each predictor was examined in relation to the outcome after adjusting for age, sex, and source of liver. Predictors that remained significant across models were included in a stepwise linear regression analyses. Predictors were retained in the models if $P < 0.15$.

RESULTS
CYP2D6 copy number

A positive association was observed between *CYP2D6* copy number and mRNA levels in 115 healthy human liver tissue samples (**Figure 1**). Seven liver tissue samples with copy number 3 had *CYP2D6*4x2/*17*, **2x2/*1*, **2x2/*2*, **2x2/*1*, **4x2/*1*, **10x2/*1*, and **1x2/*41* alleles. Two samples with copy number 4 carried *CYP2D6*2x3/*1* and **10x2/*36x2* alleles.
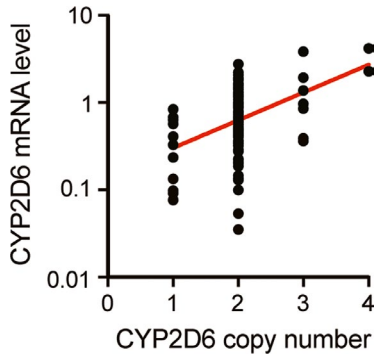
Figure 1 Relationship between cytochrome P450 (CYP)2D6 mRNA levels and *CYP2D6* copy number. The line represents unadjusted linear regression line for *CYP2D6* copy number vs. mRNA levels (*n* = 114, after excluding one liver tissue sample that is homozygous for *CYP2D6*5*, and thus, the gene is deleted). *N* = 11, 94, 7, and 2 for copy number 1, 2, 3, and 4 groups, respectively.

Genetic polymorphisms in *CYP2D6, HNF4A,* and *NR0B2*

A total of 86, 25, and 76 SNPs were identified in *CYP2D6* (4-kb upstream to 1-kb downstream), *NR0B2* (2-kb upstream to 1-kb downstream), and *HNF4A* (exon and adjunctive intron/regulatory regions), respectively (**Table S4**), most of which were previously annotated. None of the eight unannotated *CYP2D6* SNPs was nonsynonymous. Considering the small sample size (*n* = 115), only common SNPs (i.e.,

observed frequency of homozygous or heterozygous carriers ≥ 15%) were screened for their association with CYP2D6 mRNA levels. Among 58 common SNPs (**Table S5**), the following SNPs were found to be associated with CYP2D6 mRNA levels: rs16947 (NG_008376.3:g.2851C>T in *CYP2D6* gene; nonsynonymous, found in numerous haplotypes), rs3892097 (NG_008376.3:g.1847G>A in *CYP2D6*4* allele; intronic), and rs28371703 (NG_008376.3:g.973C>A in *CYP2D6*4* allele; nonsynonymous) (**Table S6**). No SNPs in *NR0B2* or *HNF4A* were found significantly associated with CYP2D6 mRNA levels.

HNF4α and SHP expression/activity

CYP2D6 mRNA was positively associated with HNF4α mRNA expression levels in samples having two *CYP2D6* gene copies (**Figure 2a**), consistent with a previous report.[4] Interestingly, mRNA expression of SHP (a transcriptional repressor of CYP2D6) was also positively associated with CYP2D6 mRNA levels in the liver tissue (**Figure 2b**). A positive association between HNF4α and SHP mRNA levels was observed (**Figure S2**), likely reflecting HNF4α transactivation of the *NR0B2* promoter.[24]

SHP protein expression and activity are functionally modulated by post translational modification,[13–15] and, thus, SHP mRNA levels may not be associated with SHP activity levels. In consideration of these findings, the relationship between mRNA levels of CYP2D6 and a target gene of SHP-HNF4α regulatory pathway, namely CYP8B1,[25,26] was examined. A prominent positive association was observed between
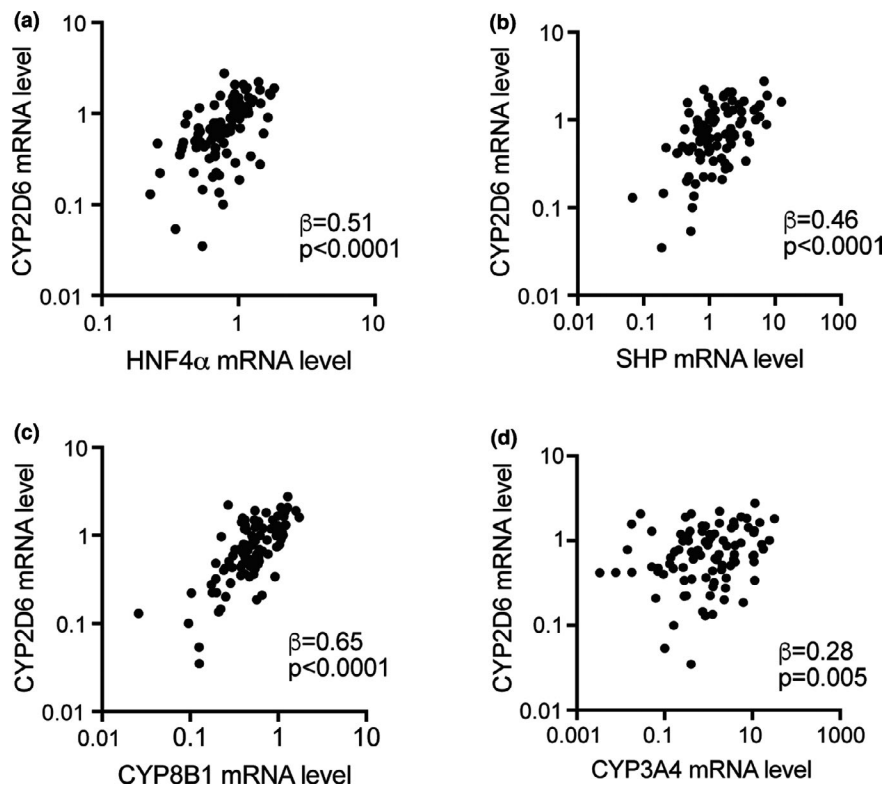

Figure 2 Associations between mRNA levels of cytochrome P450 (CYP)2D6 and other hepatic genes. (**a–d**) Associations in liver samples with *CYP2D6* copy number of 2 (*n* = 94) are shown. β and *P* values are standardized regression coefficients and corresponding *P* values after adjusting for age, sex, and liver source. HNF4α, hepatic nuclear factor 4α; SHP, small heterodimer partner.

CYP2D6 and CYP8B1 mRNA levels in the liver tissue samples with two *CYP2D6* gene copies (**Figure 2c**), suggesting significant roles of SHP-HNF4α regulatory pathway explaining at least a portion of the variability in CYP2D6 mRNA levels. The relationship between CYP8B1 and SHP mRNA levels was similar to that between CYP2D6 and SHP (**Figure S3**).

To ensure that the observed association was not driven by a technical artifact (e.g., sample quality), the association between mRNA levels of CYP2D6 and CYP3A4 (a major drug-metabolizing enzyme) was examined in the samples. The association between CYP2D6 and CYP3A4 was much weaker (**Figure 2d**), suggesting that differences in sample quality (e.g., RNA quality) has little contribution, if any, to the association between CYP2D6 and CYP8B1.

Modulators of SHP expression/activity

SHP expression and/or activity is known to be induced by bile acids and retinoic acid.[27,28] To determine whether SHP modulators contribute to interindividual variability in CYP2D6 mRNA levels, hepatic concentrations of bile acids (28 primary and secondary bile acids) and *at*RA were measured, and their association with CYP2D6 mRNA levels was examined. Median total bile acid quantity was 60 pmol/mg liver (**Figure 3a**). Over 99% of the bile acids in the liver were conjugated with glycine or taurine (**Figure 3b**). A positive and marginal association was observed between CYP2D6 mRNA level and total bile acid levels (**Figure 3c**). The direction and extent of association between hepatic CYP8B1 expression and bile acid pool size were similar to those of
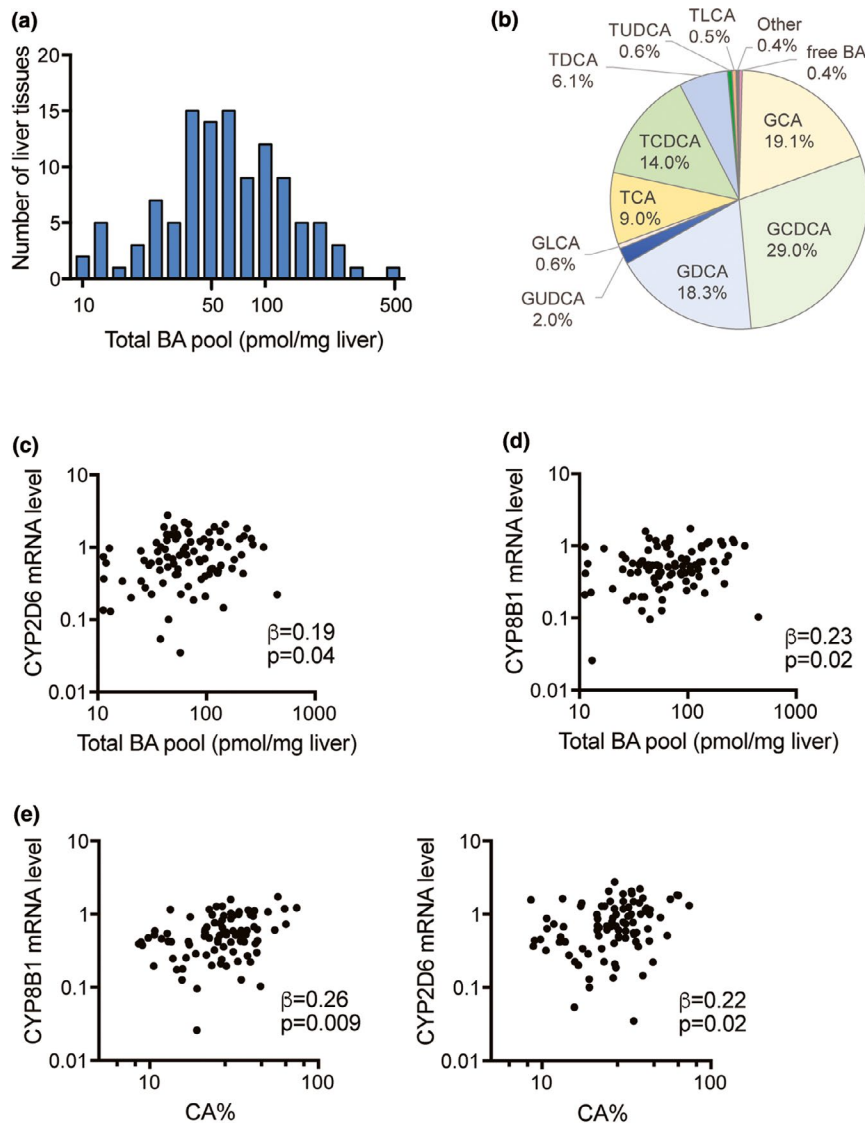

Figure 3 Associations between hepatic bile acid (BA) levels and cytochrome P450 (CYP)2D6 mRNA levels. (**a**) Distribution of total BA pool in human liver tissues is shown. (**b**) Average composition of BA species in human livers. (**c–e**) Association between CYP2D6 (or CYP8B1) mRNA levels and total hepatic bile acid level (pmol/mg liver) or cholic acid (free and conjugated) percentage (CA%) in the samples with *CYP2D6* copy number of 2 (*n* = 94). β and *P* values are standardized regression coefficients and corresponding *P* values after adjusting for age, sex, and liver source. GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholate; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA tauroursodeoxycholic acid.
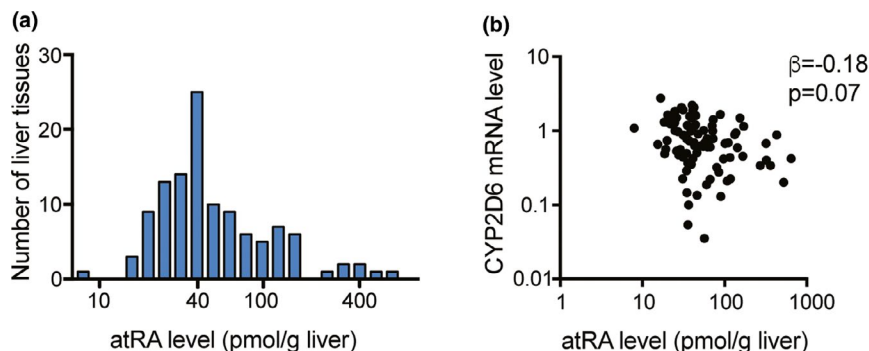
Figure 4 Association between hepatic retinoic acid levels and cytochrome P450 (CYP)2D6 mRNA levels. (**a**) Distribution of all-*trans* retinoic acid (*at*RA) levels in human liver tissue samples is shown. (**b**) Association between CYP2D6 mRNA and hepatic *at*RA levels in the samples with *CYP2D6* copy number of 2. β and *P* values are standardized regression coefficients and corresponding *P* values after adjusting for age, sex, and liver source.

CYP2D6 (**Figure 3d**). Total CA percentage (CA% hereafter) was positively associated with both CYP8B1 and CYP2D6 mRNA expression but to a small extent (**Figure 3e**).

The hepatic level of *at*RA, the bioactive form of retinoids,[29] ranged from 8–644 pmol/g liver, the median being 41 pmol/g liver (**Figure 4a**). No association was found between hepatic *at*RA and CYP2D6 mRNA levels (**Figure 4b**).

Stepwise regression analysis

To identify the best predictors of hepatic CYP2D6 mRNA levels, multivariable linear regression analysis was performed, including the variables described above. After adjustment of covariates (i.e., age, sex, and tissue source), only the following remained significantly associated with CYP2D6 mRNA levels and were included in the stepwise regression analysis: CYP8B1 mRNA, HNF4α mRNA, total bile acids, CA%, rs16947A, rs3892097T, and rs28371703T (linked to rs3892097T) (**Table S6**). Log CYP8B1 mRNA level and rs3892097 were found to be two best predictors of CYP2D6 mRNA expression (*P* < 0.05), each explaining 25% and 5% of the variability **Table 1**). When the stepwise regression analysis was limited only to the liver tissue samples with two *CYP2D6* gene copies, a higher portion (i.e., 36%) of CYP2D6 variability was explained by log CYP8B1 mRNA level (data not shown).

DISCUSSION

In this study, stepwise multiple regression analysis in 115 healthy human liver tissue samples revealed that CYP8B1 mRNA levels and the presence of rs3892097 are the significant predictors of CYP2D6 mRNA levels (after covariate adjustment). CYP8B1 mRNA level was the most significant predictor of CYP2D6 expression, explaining 25% of the overall variability in CYP2D6 mRNA expression. This may be attributable to the common regulatory mechanisms of their transcription, involving both SHP and HNF4α; SHP represses HNF4α transactivation of *CYP2D6* and *CYP8B1* promoters.[7,25,26] Supporting this idea, HNF4α expression alone was an insignificant predictor of CYP2D6 mRNA levels in the final model. The biological significance of the finding that *CYP8B1* and *CYP2D6* transcription is correlated

Table 1 Final regression model of CYP2D6 mRNA expression (*n* = 115)

Variable	β	SE	*P* value	Adjusted *r*²
Male	−0.0730	0.0726	0.32	0.122
Age	0.0702	0.0702	0.30	
Tissue source (i.e., Corning)	−0.1321	0.0784	0.095	
Log (CYP8B1 mRNA expression)	0.3744	0.0984	0.0002	0.372
rs3892097T	−0.2453	0.0712	0.0008	0.426
Log (HNF4α mRNA expression)	0.1707	0.0966	0.080	0.435
Log (total bile acids)	0.1197	0.0732	0.105	0.443

CYP, cytochrome P450.

is unclear. Most substrates of CYP2D6 are hydrophobic bases containing a planar hydrophobic aromatic ring and a nitrogen atom that can be protonated at the physiological pH. Considering that bile acids are without a nitrogen atom, it seems unlikely that CYP2D6 metabolizes bile acids. Of note, hepatic CYP8B1 activity determines the fraction of CA in total bile acids excreted into the bile, governing bile composition in the intestine and subsequent nutrient absorption.[30] Speculatively, the intestinal absorption of certain diet-derived CYP2D6 substrates[31] may be affected by bile composition, potentially contributing to coregulation of CYP2D6 and CYP8B1 expression.

The SHP-HNF4α pathway is regulated by multiple factors, including compounds that modulate SHP expression/activity.[13,14,28,32] For example, bile acids and *at*RA induce SHP expression and, thus, repress expression of HNF4α target genes, such as CYP2D6 and CYP8B1.[7,33] To explore their roles in explaining interindividual variability in CYP2D6 mRNA levels, we measured hepatic levels of bile acids and *at*RA and examined their relationships with CYP2D6 mRNA levels. Total bile acid levels were used in examining the association between bile acid levels and CYP2D6 mRNA levels, based on the findings that (i) CA, CDCA, and DCA have similar potency in activating FXR and inducing SHP expression[34]; (ii) conjugated bile acids bind to FXR and

stimulate target gene expression[35,36]; and (iii) CA, CDCA, and DCA, and their conjugates were the major bile acid species in human liver tissues (> 95% hepatic bile acid pool). Unexpectedly, a positive association was observed between hepatic total bile acid levels and CYP2D6 mRNA levels. A similar positive association was observed between bile acid levels and CYP8B1 expression. Our snapshot view of hepatic gene regulation in the healthy liver tissue (i.e., without pathological conditions, such as cholestasis) failed to capture bile acids behaving as a negative feedback regulator of CYP8B1 expression. Instead, we observed the apparent role of CYP8B1 as the enzyme mediating bile acid synthesis. Considering that (i) CYP2D6 mRNA levels exhibited the strongest association with CYP8B1, (ii) CYP8B1 catalyzes 12-hydroxylation reaction in bile acid synthesis that leads to CA production, and (iii) the percentage of total CA in the bile acid pool may serve as a potential marker of CYP8B1 activity,[30] CA% was estimated in the samples with two *CYP2D6* gene copies, and its association with CYP8B1 or CYP2D6 mRNA level was examined. Although CA% was also positively associated with both CYP8B1 and CYP2D6 mRNA expression, it was not included in the final model for the best predictors for CYP2D6 mRNA levels.

Limitations of this study include (i) the lack of correction for multiple comparisons in the exploratory steps (for heuristic purposes to examine the potential predictors of CYP2D6 mRNA levels, before stepwise regression analysis), (ii) a relatively small sample size, and (iii) the focus on transcriptional regulators of *CYP2D6* in investigating the determinants of CYP2D6 mRNA levels. Factors involved in post transcriptional regulation of CYP2D6 were not examined in this study, although microRNA hsa-miR-370-3p was recently shown to bind to a coding region of CYP2D6 transcript and regulate CYP2D6 expression in liver cells.[37] Due to the small sample size, the contribution of *CYP2D6* SNPs of low allele frequency (accounting for ~ 70% of *CYP2D6* SNPs identified in this study) to variable CYP2D6 mRNA levels was not examined in our liver tissue cohort. The rs3892097 (CYP2D6 NG_008376.3:g.1847G>A) was the only SNP significantly associated with CYP2D6 mRNA levels. The rs3892097 (minor allele frequency of 0.154; located at the junction of intron 3 and exon 4) is the determinant SNP for *CYP2D6*4* allele and known to cause aberrant splicing.[38] The geometric mean of CYP2D6 mRNA level in samples carrying *CYP2D6*4/wt* (*wt* as *CYP2D6*1* or **2* allele) carriers was at 68% of that in *CYP2D6 wt/wt* carriers (data not shown). Contribution of most other CYP2D6 SNPs to differential mRNA levels remains to be determined in the liver tissue of larger sample sizes.

In conclusion, we explored the potential determinants of interindividual variability in CYP2D6 mRNA levels in healthy human liver tissue. We found that among different parameters tested in this study, CYP8B1 expression level is the best predictor of hepatic CYP2D6 transcript levels. Although the biological implication remains to be defined, this finding suggests critical roles of the SHP-HNF4α pathway in the regulation of CYP2D6 expression. Potential contributions of numerous *CYP2D6* SNPs to the variability CYP2D6 transcript levels are yet to be determined using a larger sample size.

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