

Genetic Variation in Aldo-Keto Reductase 1D1 (*AKR1D1*) Affects the Expression and Activity of Multiple Cytochrome P450s

Amarjit S. Chaudhry, Ranjit K. Thirumaran, Kazuto Yasuda, Xia Yang, Yiping Fan, Stephen C. Strom, and Erin G. Schuetz

Departments of Pharmaceutical Sciences (A.S.C., R.K.T., K.Y., E.G.S.) and Computational Biology (Y.F.), St. Jude Children's Research Hospital, Memphis, Tennessee; Department of Integrative Biology and Physiology, University of California, Los Angeles, California (X.Y.); and Division of Pathology, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden (S.C.S.)

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ABSTRACT

Human liver gene regulatory (Bayesian) network analysis was previously used to identify a cytochrome P450 (P450) gene subnetwork with Aldo-keto reductase 1D1 (*AKR1D1*) as a key regulatory driver of this subnetwork. This study assessed the biologic importance of *AKR1D1* [a key enzyme in the synthesis of bile acids, ligand activators of farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR), known transcriptional regulators of P450s] to hepatic P450 expression. Overexpression of *AKR1D1* in primary human hepatocytes led to increased expression of *CYP3A4*, *CYP2C8*, *CYP2C9*, *CYP2C19*, and *CYP2B6*. Conversely, *AKR1D1* knockdown decreased expression of these P450s. We resequenced *AKR1D1* from 98 donor livers and identified a 3'-untranslated region (UTR) (rs1872930) single nucleotide polymorphism (SNP) significantly associated with higher *AKR1D1* mRNA expression. *AKR1D1* 3'-UTR-luciferase reporter

studies showed that the variant allele resulted in higher luciferase activity, suggesting that the SNP increases *AKR1D1* mRNA stability and/or translation efficiency. Consistent with *AKR1D1*'s putative role as a driver of the P450 subnetwork, the *AKR1D1* 3'-UTR SNP was significantly associated with increased hepatic mRNA expression of multiple P450s (*CYP3A4*, *CYP2C8*, *CYP2C9*, *CYP2C19*, and *CYP2B6*) and *CYP3A4*, *CYP2C8*, *CYP2C19*, and *CYP2B6* activities. After adjusting for multiple testing, the association remained significant for *AKR1D1*, *CYP2C9*, and *CYP2C8* mRNA expression and *CYP2C8* activity. These results provide new insights into the variation in expression and activity of P450s that can account for interindividual differences in drug metabolism/efficacy and adverse drug events. In conclusion, we provide the first experimental evidence supporting a role for *AKR1D1* as a key genetic regulator of the P450 network.

Introduction

Pharmacogenetic efforts to date have largely been focused on identifying *cis*-coding and *cis*-regulatory polymorphisms affecting candidate gene expression and activity. This approach has been successful in identifying functional polymorphic alleles for *CYP2C9*, *CYP2C19*, *CYP2B6*, *CYP2C8*, and *CYP3A4* (<http://www.cypalleles.ki.se/>). However, for many of these cytochrome P450 (P450) enzymes, a considerable percentage of variation in P450 activity cannot be explained by *cis*-genetic polymorphisms, and it is hypothesized that genetic variation in *trans*-regulators influences P450 variation (Schuetz, 2004). Indeed, we previously showed that polymorphisms in hepatic transcription factors that regulate P450s such as *FoxA2*, *HNF4B*, *FoxA3*, and *PXR*, together explained as much as 10% of the variation in hepatic *CYP3A4* expression (Lamba et al., 2010).

One additional approach to identify P450 *trans*-regulators is to derive the P450 biologic coexpression networks using both a weighted gene coexpression network approach and a Bayesian network reconstruction approach. We previously (Yang et al., 2010) applied these integrative genomic approaches using mRNA expression from

466 human livers, to construct a probabilistic, causal P450 gene network. *AKR1D1* (Δ^4 -3-oxosteroid, 5β -reductase) was predicted as one master regulator of the P450 subnetwork or module (Fig. 1). Expression of multiple P450s including *CYP2C9*, *CYP3A4*, and *CYP2C19*, was predicted to be influenced by Aldo-keto reductase 1D1 (*AKR1D1*).

AKR1D1 is a biologically plausible candidate gene regulating P450 expression because it participates in regulating bile acid concentrations in liver (Fig. 1). *AKR1D1* catalyzes the NADPH-dependent reduction of the double bond in the steroid A-ring in bile acids and steroid hormones, and is a central enzyme in the synthesis of both chenodeoxycholic acid and cholic acid (Lee et al., 2009). Bile acids are now recognized not just as fat solubilizers, but as signaling hormones with endocrine effects that affect regulation of P450s as well as lipid and glucose metabolism (Houten and Auwerx, 2004; Thomas et al., 2008). Indeed, bile acids are known ligands/activators of the nuclear hormone receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Schuetz et al., 2001; Congiu et al., 2009), which are transcriptional regulators of *CYP3A4*, *CYP2C9*, and *CYP2C19*. Bile acids also activate and regulate the nuclear hormone farnesoid X receptor (FXR), a known transcriptional regulator of bile acid homeostasis (Urquhart et al., 2007; Modica et al., 2009) and *PXR* (Jung et al., 2006), and *CYP3A4* (Gnerre et al., 2004). *AKR1D1* could

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ABBREVIATIONS: *AKR1D1*, Aldo-keto reductase 1D1; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; FP, forward primer; FXR, farnesoid X receptor; HAP, haplotype; LD, linkage disequilibrium; miRNA, microRNA; P450, cytochrome P450; PCR, polymerase chain reaction; PXR, pregnane X receptor; SHP, small heterodimer partner; shRNA, short hairpin RNA; shRNAmir, microRNA-adapted short hairpin RNA; RP, reverse primer; SNP, single nucleotide polymorphism; UTR, untranslated region; VAR-HAP, variant haplotype; WT-HAP, wild-type haplotype.

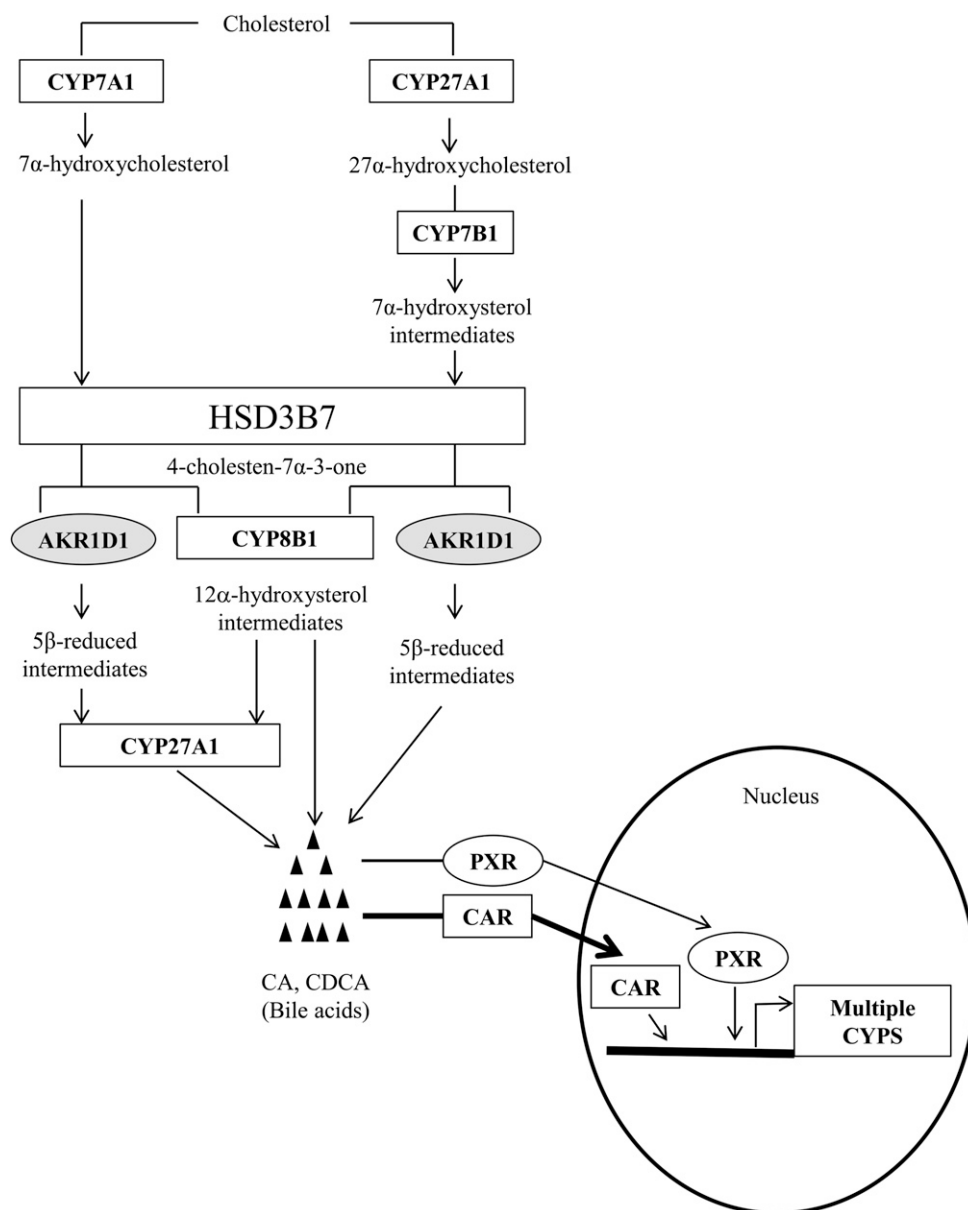


Fig. 1. Model for regulation of expression/activity of multiple P450s by AKR1D1. Cholesterol is converted to CA and CDCA via a classic pathway (starting with CYP7A1) and an alternative pathway (starting with CYP27A1). Both bile acid synthesis pathways include AKR1D1-mediated catalysis of the 5 β -reduction of bile acid intermediates. These 5 β -reduced intermediates are finally converted to the primary bile acids CDCA and CA, which can serve as endogenous ligands of nuclear receptors PXR and CAR that upon activation can regulate the transcription of multiple P450s including CYP3A, CYP2C9, and CYP2C19. CA, cholic acid.

also regulate P450s through reduction of steroid hormones. For example, AKR1D1-mediated 5 β -reduction of progesterone to 5 β -pregnane-3, 20-dione results in activation of PXR (Bertilsson et al., 1998).

This study was undertaken to validate whether AKR1D1 was indeed a regulator of hepatic P450 expression. In addition, we tested whether genetic variation in *AKR1D1* was associated with P450 mRNA expression and activity. To date, genetic variation in *AKR1D1* has not been explored in normal individuals, and rare genetic variants have been reported only in infants that lack cholic acid and chenodeoxycholic acid (Lemond et al., 2003). In this study, we report that genetic variation in *AKR1D1* contributes to the observed variation in P450 expression and activity of the P450 liver network.

Materials and Methods

Procurement of Human Liver Tissue. The St. Jude Children's Research Hospital and University of Pittsburgh Institutional Review Boards approved the human liver studies. Liver tissue from white donors was processed through St. Jude Liver Resource at St. Jude Children's Research Hospital and was provided

by the Liver Tissue Procurement and Distribution System (National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases Contract N01-DK92310) and by the Cooperative Human Tissue Network. Liver tissue from 163 white donor livers (from the University of Pittsburgh and St. Jude Liver Resource) was previously used, together with livers from other resources, to isolate DNA for genotyping and RNA and microsomes for phenotyping mRNA expression and P450 activities, respectively (Schadt et al., 2008; Yang et al., 2010). A subset of 145 white donor livers from the University of Pittsburgh and St. Jude Liver Resource had sufficient tissue available for follow-up analysis in this study.

Plasmids. The PXR expression plasmid (pSG5-hPXR) was generously provided by Dr. Steve Kliewer (Lehmann et al., 1998). The reporter plasmid [CYP3A4 +53 to 362(7836/7208)-Luciferase], hereafter called CYP3A4-PXRE2-LUC, was generously provided by Dr. Richard Kim (Zhang et al., 2001). The AKR1D1 3'-untranslated region (UTR)-luciferase reporter construct was purchased from Switchgear Genomics (Menlo Park, CA) and single mutants of rs1872929 and rs1872930 as well as different haplotypes [wild-type haplotype (WT-HAP) and variant haplotype (VAR-HAP)] were constructed by site directed mutagenesis. All nucleotide substitutions were confirmed by direct DNA sequencing.

Generation of Vectors, Adenoviruses, and Lentiviruses. *AKR1D1* was polymerase chain reaction (PCR) amplified from the human *AKR1D1* expression plasmid (catalog no. SC116410; OriGene, Rockville, MD) and subcloned into pENTR/SD/D-TOPO (part no. 45-0219; Invitrogen, Grand Island, NY) for entry into the Adv-BGFP-Gateway destination vector (from Dr. John Gray, St. Jude) to make the Adv-BGFP-*AKR1D1* overexpression vector. The pGIPz-*AKR1D1*-200099 lentiviral microRNA-adapted short hairpin RNA (shRNAmir) vector (catalog no. RHS-4430-98709631) to knockdown human *AKR1D1* and pGIPz nonsilencing lentiviral shRNA control vector (catalog no. RHS4346) were purchased from Thermo Scientific Open Biosystems (Lafayette, CO). These vectors were used to make adenoviral or lentiviral particles by the Vector Development & Production shared resource at St. Jude Children's Research Hospital.

RNA Profiling and Single Nucleotide Polymorphism Genotyping. The microarray design, RNA sample preparation, amplification, hybridization, and expression analysis as well as single nucleotide polymorphism (SNP) genotyping were previously described in detail (Schadt et al., 2008; Yang et al., 2010). The *AKR1D1* SNP rs1872930 was also genotyped with a Snapshot genotyping kit from Applied Biosystems (Grand Island, NY). The Snapshot primers for first-round PCR were as follows: forward primer, 5'-GCGCGAT-CATCTGAATACCCATT-3'; and reverse primer, 5'-GACTGAGTCAAA-GTCATTTACTGAC-3'. The extension primers used were as follows: forward, 5'-TCCTGAACAGATTTTCTACTCCC-3'; and reverse, 5'-CATTG-CACCGTCTGGCACTC-3'.

P450 Enzyme Activity Measurements in Human Liver Microsomes. The P450 activities of CYP2B6 (bupropion to 1'-hydroxybupropion), CYP2C8 (paclitaxel to 6 α -hydroxypaclitaxel), CYP2C9 (tolbutamide to hydroxytolbutamide), CYP2C19 ((S)-mephenytoin to 4'-hydroxymephenytoin), and CYP3A4 (midazolam to 1'-hydroxymidazolam and testosterone to 6 β -hydroxytestosterone) were previously determined in microsomes isolated from human liver samples using probe substrate metabolism assays (Yang et al., 2010).

CYP3A4-PXRE2-LUC Reporter Assays and *AKR1D1* 3'-UTR-Luciferase Reporter Assays in HepG2 Cells. HepG2 cells were cultured in minimum Eagle's medium- α supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin, and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂. For transfection studies, 3 \times 10⁵ cells per well were seeded into 24-well culture dishes. Twenty-four hours later, cells were transfected overnight by calcium phosphate precipitation with 300 ng PXRE2-CYP3A4-LUC reporter plasmid, 100 ng pSG5-hPXR, and 50 ng SV40- β -galactosidase along with either 100 ng pCMV-*AKR1D1* expression plasmid or pCMV-XL5 empty vector plasmid as a control. The next day, cells were washed once with medium and incubated with fresh medium containing 10% charcoal dextran-treated fetal bovine serum (Hyclone Laboratories, Logan, UT) with either 50 μ M chenodeoxycholic acid (CDCA) (Sigma-Aldrich, St. Louis, MO) or dimethylsulfoxide. For *AKR1D1* 3'-UTR-Luciferase reporter assays, HepG2 cells were transfected with 250 ng of different *AKR1D1* 3'-UTR-Luc reporter plasmid constructs along with 50 ng SV40- β -galactosidase. Twenty-four hours later, cells were harvested, lysed, and centrifuged at 1500g for 4 minutes, and luciferase activities were determined in an aliquot of supernatant according to the manufacturer's instructions (Luciferase Assay System; Promega, Madison, WI) using Clarity luminescence microplate reader (BIO-TEK, Winooski, VT). β -galactosidase activities were determined in an aliquot of supernatant according to the manufacturer's instructions (β -Galactosidase Enzyme Assay System; Promega). Luciferase activity was normalized to β -galactosidase activity to correct for transfection efficiency and expressed as fold change with respect to vector control. Data are reported as mean \pm S.D. of three determinations and were representative of multiple experiments. Statistical difference between groups was determined by use of the *t* test. All experiments were performed in triplicate.

Adv-*AKR1D1* and Lentiviral shRNAmir *AKR1D1* Transduction of Primary Human Hepatocytes. Freshly plated primary human hepatocytes isolated from four different male donor livers were purchased from BD Biosciences (catalog no. 454424; Woburn, MA). 4 \times 10⁵ cells per well in 24-well culture dishes were obtained from BD Biosciences and cultured in hepatocyte culture media supplemented with G418, amphotericin B, and L-glutamine at a final concentration of 50 mg/l, 750 μ g/l, and 292 mg/l in the media, respectively. Hepatocytes were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Transfections were done after acclimatizing the cells for 24 hours. Cells were transfected with 20 m.o.i. (multiplicity of

infection) of Adv-BGFP-*AKR1D1* or control virus for overexpression of *AKR1D1*. Similarly, for *AKR1D1* knockdown experiments, hepatocytes were transfected with 5 m.o.i. of either control (pGIPz-Lentiviral-RHS4346) or *AKR1D1*-specific shRNAmir lentiviruses (pGIPz-*AKR1D1*-200099). For bile acid treatment, cells were washed once with medium and incubated with fresh medium containing either 50 μ M CDCA or 0.1% dimethylsulfoxide (vehicle). The CDCA-treated cells were harvested 24 hours after treatment, adenoviral infected cells were harvested 48 hours after infection, and lentiviral infected cells were harvested 72 hours after infection. Before harvesting, hepatocytes were washed once with phosphate-buffered saline (PBS) and were harvested by adding TRIzol reagent (Invitrogen) directly into the wells and passing a couple of times through the pipette tip.

Western Blotting for *AKR1D1* in HepG2 Total Cell Lysate. HepG2 cells were cultured as described above. Cells were infected with *AKR1D1* lentiviral or adenoviral particles as described for human hepatocytes. After treatment cells were washed once with ice-cold PBS, harvested in 5 ml PBS, and centrifuged at 1500g for 10 minutes. The cell pellets were reconstituted in 50 μ l microsome storage buffer (100 mM potassium phosphate, pH 7.4, 1.0 mM EDTA 20% glycerol with protease inhibitor cocktail) and sonicated to prepare total cell lysate. Cell lysates (100 μ g protein) were subjected to SDS-PAGE and immunoblotted with anti-*AKR1D1* (SC-67710; Santa Cruz Biotechnology, Dallas, TX) or anti- β -actin antibody, followed by appropriate secondary antibodies, and developed with SuperSignal West Dura Extended Duration Substrate (catalog no. 34076; Thermo Scientific, Waltham, MA).

mRNA Quantification by Quantitative Real-Time PCR. Total RNA was isolated using TRIzol reagent. First-strand cDNA was prepared using oligo (dT) primers (catalog no. 11146-016, ThermoScript RT-PCR system; Invitrogen). Real-time PCR quantification of *AKR1D1*, *PXR*, *CAR*, *CYP3A4*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2B6*, *CYP7A1*, small heterodimer partner (SHP), *FXR*, and *GAPDH* mRNAs was carried out using the SYBR GreenER quantitative PCR supermix (catalog no. 11760-100; Invitrogen) according to the manufacturer's instructions. cDNA was analyzed in duplicate by quantitative real-time PCR on an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primers used for real-time quantification are provided in Table 1. Specificity of amplification was confirmed in each case by performing melt curve analysis and agarose gel electrophoresis. The averaged Ct values were analyzed by the comparative Ct method to obtain relative mRNA expression levels.

***AKR1D1* cDNA Amplification for Sequencing and To Screen for Alternative *AKR1D1* mRNAs Associated with Polymorphisms.** RNA was extracted from 98 donor livers with tissue available using TRIzol and was used

TABLE 1
Primers used for real-time quantitative PCR

| S. No. | mRNA | Sequence (5' \rightarrow 3') |
|--------|-------------------|--------------------------------|
| 1 | <i>GAPDH-FP</i> | GGACCACCAGCCCCAGCAAGAG |
| 2 | <i>GAPDH-RP</i> | GAGGAGGGGAGATTTCAGTGTGGTG |
| 3 | <i>AKR1D1-FP</i> | TCAGAACCTAAATCGACCCT |
| 4 | <i>AKR1D1-RP</i> | CCCTGTGTCAATAGCAACCTTC |
| 5 | <i>CYP3A4-FP</i> | CCTTACACATACACACCTTTGGAAGT |
| 6 | <i>CYP3A4-RP</i> | AGCTCAATGCATGTACAGAATCCCCGGTTA |
| 7 | <i>CYP2C8-FP</i> | GCAGGAAAAGGACAAACAAA |
| 8 | <i>CYP2C8-RP</i> | GTGTAAGCATGTGGCTCCT |
| 9 | <i>CYP2C9-FP</i> | GATCTGCAATAATTTTCTC |
| 10 | <i>CYP2C9-RP</i> | TCTCAGGGTGTGCTTGTG |
| 11 | <i>CYP2C19-FP</i> | GAGGAGTTTTCTGGAAGAGGCC |
| 12 | <i>CYP2C19-RP</i> | CATTCTGAAAACGATTCAAA |
| 13 | <i>CYP2B6-FP</i> | CACCCTAACACCCATGACCG |
| 14 | <i>CYP2B6-RP</i> | GATCACACCATATCCCCGGA |
| 15 | <i>FXR-FP</i> | TATCTAGCCCAATATTTACAGTT |
| 16 | <i>FXR-RP</i> | GTATAACTTTCCTTTATTTTAC |
| 17 | <i>CYP7A1-FP</i> | AAACTGGATGGAATAGCCATTG |
| 18 | <i>CYP7A1-RP</i> | TTTTCATCTGCAAGTCATTAGCG |
| 19 | <i>SHP-FP</i> | GCTGTCTGGAGTCTTCTGG |
| 20 | <i>SHP-RP</i> | CCAATGATAGGGCGAAAGAA |
| 21 | <i>CAR-FP</i> | CCAGCTCATCTGTTTCATCCA |
| 22 | <i>CAR-RP</i> | GGTAATCCAGGTCGGTCAG |
| 23 | <i>PXR-FP</i> | CAAGCGGAAGAAAAGTGAAC |
| 24 | <i>PXR-RP</i> | CACAGATCTTCCGGACCTG |

to prepare cDNA. In the first PCR, a forward primer (AKR-FP) (Table 2) that annealed in the 5'-UTR and a reverse primer (AKR-RP) (Table 2) that annealed in 3'-UTR were used to amplify all 9 exons of *AKR1D1* from 12 samples heterozygous for rs1872930. To detect any low abundance alternatively spliced transcripts that might undergo nonsense-mediated decay, the first-round PCR product was used as template for PCR with a second primer pair (AKR-FPN and AKR-RPN) (Table 2). This PCR amplified a 989 bp fragment that still included all of the 9 exons of *AKR1D1*. Because amplification of a long PCR product might fail to detect alternatively spliced products with small insertions or deletions, and because rs1872930 was in linkage disequilibrium (LD) with two intron 8 SNPs, DNA from subjects with rs1872930 TT, TC, and CC genotypes was used as a template to specifically amplify a region around exon 8/intron8 using primers AKR-FP1 and AKR-RP (Table 2). Again to detect any low abundance alternatively spliced transcripts that might undergo nonsense-mediated decay, the first round PCR product was used as template for PCR with a second primer pair (AKR-FP1 and AKR-RPN) to amplify a 416 bp normal or any low abundance transcripts that might have been associated with the rs1872930 allele. All of the amplified PCR products were analyzed by agarose gel electrophoresis to look for differential banding patterns among genotypes caused by polymorphic splicing events. The first PCR products from 98 subjects (54 TT wild type, 42 TC heterozygous, and 2 CC variant homozygous) were sequenced directly to look for any coding region SNPs.

RNA Secondary Structure Analysis. The RNA secondary structure of *AKR1D1* wild-type and variant haplotypes was determined using the Mfold web server (Zuker, 2003). For this analysis, a 370 nucleotide region surrounding the SNPs (from 170 nucleotides upstream of rs1872929 to 170 nucleotides downstream of rs1872930) was used to create wild-type and variant haplotypes and these sequences were used to predict the effect on secondary structure of RNA.

microRNA (miRNA) Recognition Sites Using miRanda. To define the potential for microRNAs to be differentially bound to *AKR1D1* wild-type or *AKR1D1* variant (rs1872930), miRanda software (version 3.0; <http://www.microrna.org/microrna/getDownloads.do>) was used with the default settings and with a decreased energy reporting threshold (-en -10). Human microRNA sequences were obtained from the miRBase database (version 18; <http://www.mirbase.org/index.shtml>). The full-length mRNA coding sequence of *AKR1D1* with and without the rs1872930 variant was used as the reference sequence. The best energy and heuristic score for each pairing was compared between wild-type and rs1872930 variant *AKR1D1* sequences to find the maximum difference in either of these parameters.

Statistical Analysis. The chi-squared test for deviation from Hardy-Weinberg equilibrium was used to calculate the observed versus expected distribution of genotypes. Because the phenotypic markers were not normally distributed, group differences were analyzed nonparametrically using the Wilcoxon rank sum test, which is more robust against outliers, to compare binary groups (e.g., TT versus TC+CC). The Kruskal-Wallis test was used to compare three groups of genotype for each polymorphism (e.g., TT versus TC versus CC) with the phenotype. To account for multiple testing, we adjusted the statistical significance threshold using the permutation method in consideration of the correlation among phenotypes. For the association between genotype and P450 activity levels or mRNA expression, we considered $P < 0.0034$ as statistically significant after adjusting for multiple testing.

TABLE 2

Primers used to test for alternative *AKR1D1* mRNA transcripts

| Primer | Sequence (5' → 3') | Location in mRNA | Length |
|---------|-----------------------|------------------|--------|
| AKR-FP | CCCTAGGACACCTTTCTA | 5' UTR | 1353 |
| AKR-RP | TCATATGTTGCCTTTGATG | 3' UTR | |
| AKR-FPN | ATGGATCTCAGTGCTGCAAGT | Start of Exon 1 | 989 |
| AKR-RPN | CCCTGCAGTCAGTATTCATCA | Exon 9/3' UTR | |
| AKR-FP1 | ACCAGGTTGAGTGCCAT | Exon 5/Exon 6 | 711 |
| AKR-RP | TCATATGTTGCCTTTGATG | 3' UTR | |
| AKR-FP1 | AACCAGGTTGAGTGCCAT | Exon 5/Exon 6 | 416 |
| AKR-RPN | CCCTGCAGTCAGTATTCATCA | Exon 9/3' UTR | |

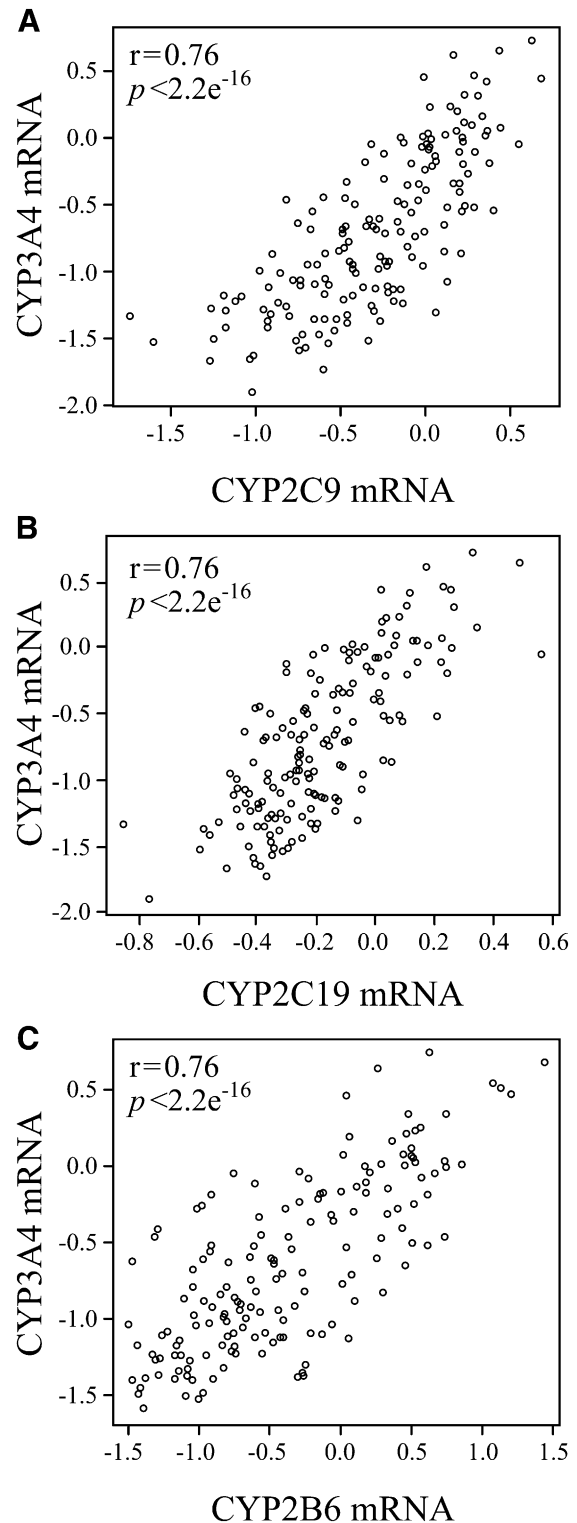


Fig. 2. Significant human liver P450-P450 mRNA correlation indicates that these P450s share *trans*-regulation. Correlation between *CYP3A4* versus *CYP2C9* mRNA (A), *CYP3A4* versus *CYP2C19* mRNA (B), and *CYP3A4* versus *CYP2B6* mRNA (C). P450 mRNA expression data from 163 human liver samples were taken from an earlier study (Schadt et al., 2008; Yang et al., 2010) and were utilized to study this correlation. The microarray design, RNA sample preparation, amplification, hybridization, and expression analysis were previously described in detail (Schadt et al., 2008; Yang et al., 2010). The Pearson's product-moment correlation r values are indicated.

Results

The Hepatic Expression of Multiple P450 mRNAs Is Significantly Correlated. We first examined P450 expression (previously profiled by Agilent custom expression arrays) in a cohort of 163 human livers from white donors (Schadt et al., 2008) and found a significant degree of correlation between expression of mRNAs for *CYP3A4* and *CYP2C9* ($r = 0.76$, $P < 2.2e^{-16}$), *CYP3A4* and *CYP2C19* ($r = 0.76$, $P < 2.2e^{-16}$), and *CYP3A4* and *CYP2B6* ($r = 0.76$, $P < 2.2e^{-16}$) (Fig. 2). A second cohort of 206 human liver tissues that had been separately profiled with a different Agilent custom expression array (Innocenti et al., 2011) was used to confirm correlation between P450s mRNA expression, and the results from the second analysis demonstrated virtually identical results. These P450s are located on different chromosomes (*CYP3A4*, *CYP2C19*, and *CYP2B6* on chromosomes 7, 10, and 19, respectively). These results are consistent with the membership of these P450s in the P450 co-expression module (Yang et al., 2010).

AKR1D1 Overexpression in HepG2 Cells Induces CYP3A4 Transcription. In our initial experiment, we used a mechanistic gain-of-expression study for experimental validation that AKR1D1 is a regulator of CYP3A transcription. Human hepatoblastoma HepG2 cells were transiently cotransfected with a CYP3A4-luc reporter plasmid (containing multiple PXR binding elements) and a PXR expression plasmid, with and without an AKR1D1 expression plasmid. As expected, CDCA treatment induced CYP3A4 transcription (Fig. 3A). In the presence of transfected PXR, cotransfected AKR1D1 significantly stimulated CYP3A4 expression ($P = 0.002$) in HepG2 cells to the same extent as the addition of 50 μM CDCA alone ($P = 0.05$). We speculate that transfected AKR1D1 increases endogenous bile acids that ligand activate PXR to induce CYP3A4 transcription.

Successful Lentiviral shRNA Mediated AKR1D1 Knockdown and Adenoviral Mediated Overexpression of AKR1D1 in Hepatic Cells. Since transient transfection is inefficient in introducing plasmid DNA into 100% of cells, we used adenovirus-mediated overexpression and lentiviral-mediated shRNA knockdown approaches to demonstrate that we could experimentally modulate AKR1D1 expression in human hepatic cells. HepG2 cells transduced for 48 hours with an AKR1D1 adenovirus showed an approximately 443% increase in AKR1D1 immunoreactive protein (Fig. 3B). Conversely, HepG2 cells transduced for 72 hours with the AKR1D1 shRNA lentivirus exhibited approximately 90% cell transduction, and an approximately 64% decrease in AKR1D1 protein compared with cells transduced with the scrambled shRNA (Fig. 3C).

AKR1D1 Overexpression Induces P450 mRNA Expression in Primary Human Hepatocytes. To test whether AKR1D1 is a key driver of the P450 network, presumably through elevation in bile acid levels, we used adenovirus-mediated overexpression of AKR1D1 in primary human hepatocytes and quantified mRNA expression of bile acid regulated target genes. Adenoviral-mediated AKR1D1 overexpression increased the mRNA expression of a variety of bile acid regulated genes including the nuclear receptors *CAR* and *PXR* and multiple P450s (*CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2B6*, and *CYP3A4*) (Fig. 4A). AKR1D1 overexpression, and presumably increased bile acids, engaged the FXR feedback loop to maintain bile acid homeostasis. Specifically, AKR1D1 overexpression induced expression of the bile acid sensor farnesoid X receptor (*FXR*), resulting in feedback inhibition of *CYP7A1* (the rate limiting enzyme in bile acid synthesis) through induction of small heterodimer partner (*SHP*) (Fig. 4A). Treatment of hepatocytes with the primary bile acid CDCA elicited a *P450*, *PXR*, and *CAR* induction profile (Fig. 4B) similar to that observed with AKR1D1 overexpression. Although the fold

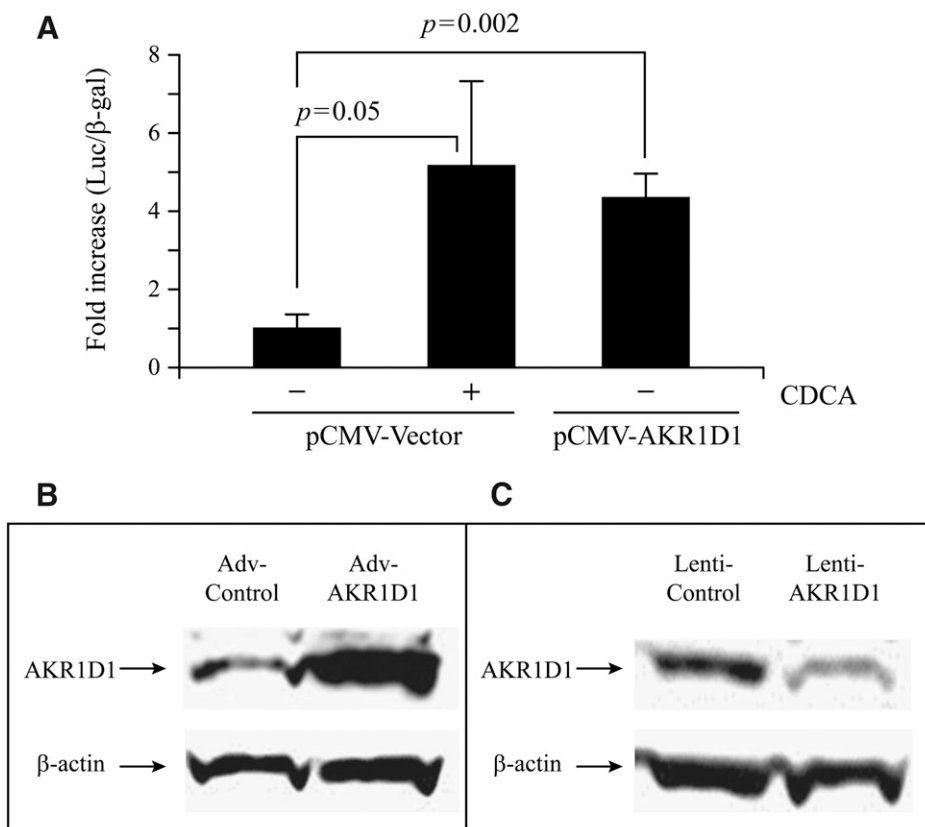


Fig. 3. AKR1D1 overexpression increases CYP3A4 promoter activity, and modulating AKR1D1 expression in HepG2 cells. HepG2 cells were transfected with PXRE2-CYP3A4-LUC and pSG5-hPXR along with either the pCMV-AKR1D1 expression plasmid or pCMV-XL5 empty vector control plasmid. Cotransfected pSV40-LacZ served as a transfection efficiency control. The next day, empty vector transfected cells were treated with either 50 μM CDCA or dimethylsulfoxide. Twenty-four hours later, luciferase and β -galactosidase activities were determined. Results are presented as fold change with respect to vector control (A). HepG2 cells cultured for 24 hours were infected with AKR1D1 adenovirus (B) or with AKR1D1 shRNA lentiviral particles (C). Cell lysates were prepared and 100 μg protein was subjected to SDS-PAGE and immunoblotted with anti-AKR1D1 or anti- β -actin antibody.

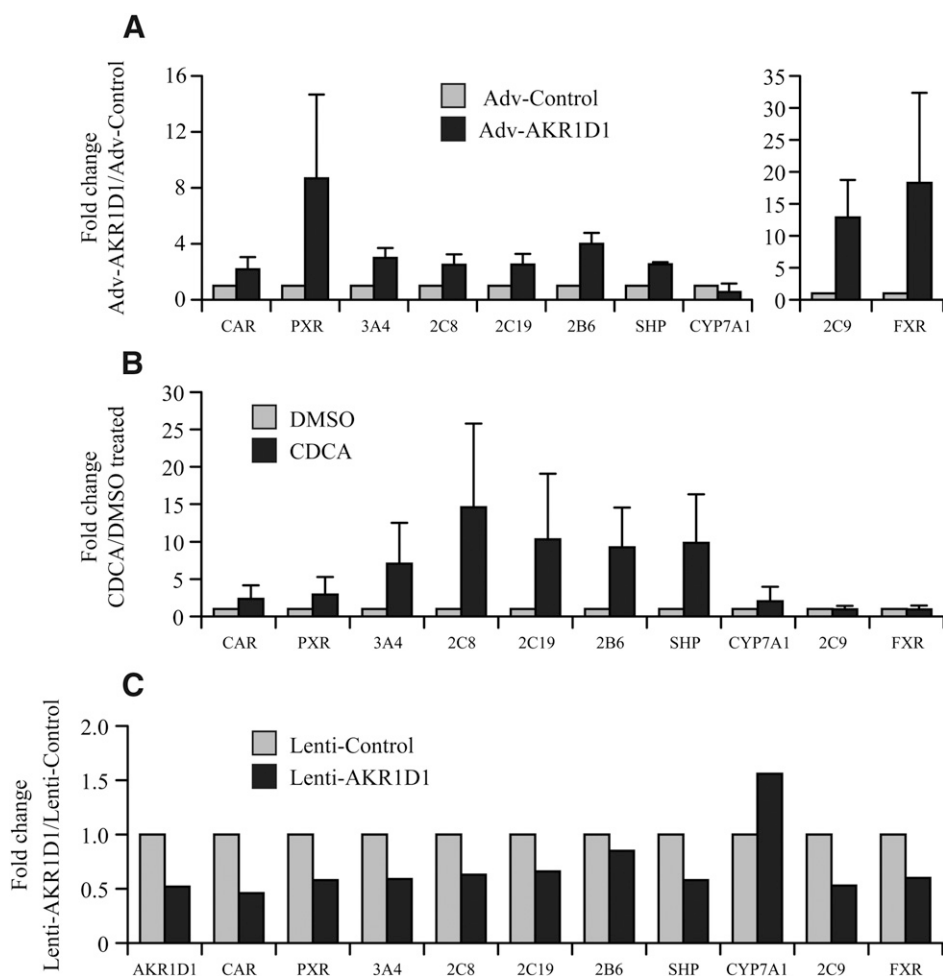


Fig. 4. AKR1D1 overexpression increased and AKR1D1 silencing decreased expression of multiple P450s in primary human hepatocytes. Primary human hepatocytes were transduced with Adv-BGFP-AKR1D1 or control virus, cells were harvested after 48 hours, and mRNA quantification was performed using real-time PCR. Results from four donors are presented as mean \pm S.E.M. (A). As a positive control, hepatocytes were treated with either dimethylsulfoxide or 50 μ M CDCA. Results from three donors are presented as mean \pm S.D. (B). For AKR1D1 knockdown experiments, hepatocytes were transfected with either control or AKR1D1 lentiviruses. Cells were harvested 72 hours after infection for real-time PCR experiments. Results are presented as the average of two livers (C). The expression of genes involved in bile acid homeostasis FXR, SHP, and CYP7A1 was also studied and served as positive controls. The results did not reach statistical significance, likely due to the small number of donor hepatocytes tested and the wide human interindividual variability in the observations.

induction of mRNA changes did not reach statistical significance for either CDCA treatment or AKR1D1 overexpression, this is likely due to the large human variation in fold induction between the different hepatocyte preparations.

AKR1D1 Silencing Decreases Multiple P450s mRNA Expression in Primary Human Hepatocytes. We next tested whether lentivirus shRNA-mediated AKR1D1 knockdown would lead to downregulation of CAR, PXR, and P450 mRNA expression. Primary hepatocytes transduced with the AKR1D1 shRNA lentiviruses (but not the control shRNA scrambled lentivirus) had decreased expression of *AKR1D1* (50%) and a similar decrease in expression of *CAR*, *PXR*, and multiple P450s (*CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2B6* and *CYP3A4*) (Fig. 4C). ShRNA-mediated AKR1D1 knockdown led to a decrease in *FXR*, a decrease in *SHP* (the *CYP7A1* repressor), and a corresponding induction of *CYP7A1* (Fig. 4C), although these changes did not reach statistical significance.

The AKR1D1 3'-UTR rs1872930 Variant Allele Is Associated with Increased Hepatic AKR1D1 Expression. Having established that AKR1D1 can act as a *trans*-regulator of multiple P450s, we hypothesized that functional SNPs in *AKR1D1* could affect its expression. To identify *AKR1D1* functional polymorphisms, we first surveyed *AKR1D1* [Chromosome 7: 137761178 - 137803050 (+)] genotype information from 90 unrelated HapMap (<http://gvs.gs.washington.edu/GVS137/index.jsp>) white subjects. There were 22 *AKR1D1* SNPs reported and the Genome Variation server binned the *AKR1D1* SNPs into 12 linkage groups with the tag SNPs for each

group (Table 3). Tag SNP genotypes for nine of the groups were already available in our St. Jude human liver cohort ($n = 145$ livers) (Schadt et al., 2008). The *AKR1D1* 3'-UTR SNP rs1872930 was most significantly associated with higher hepatic *AKR1D1* mRNA expression quantified by both microarray analysis ($P = 0.003$) (Fig. 5A) and by quantitative PCR ($P = 0.005$) (Fig. 5B; Table 3). This 3'-UTR SNP (rs1872930) was in LD with three other SNPs [an additional 3'-UTR SNP (rs1872929) and two SNPs in intron 8 (rs2035647 and rs2306847)] in either a WT-HAP or VAR-HAP.

AKR1D1 cDNA Resequencing. The tag SNPs may not represent the functional SNPs but could be in LD with other variants with functional significance. Hence, the *AKR1D1* cDNA was resequenced in 196 alleles from 98 human livers for which tissue was available and with the following known *AKR1D1* rs1872930 genotypes: 54 TT wild type, 42 TC heterozygous, and 2 CC variant homozygous. *AKR1D1* resequencing identified no coding region polymorphism.

AKR1D1 Polymorphisms Did Not Influence Expression of Individual Alternatively Spliced mRNA Transcripts. Since four SNPs in LD [two in the 3'-UTR (rs1872929 and rs1872930) and two in intron 8 (rs2035647 and rs2306847)] were significantly associated with *AKR1D1* mRNA expression, we first determined whether the intron 8 SNPs (or any other intronic SNPs in partial LD) were affecting *AKR1D1* mRNA splicing, particularly since there are two alternative *AKR1D1* mRNAs in the UCSC Genome Browser (<http://genome.ucsc.edu>) that skip individual exons [exon 5 (125 bp), or exon 8 (83 bp)]. The full-length *AKR1D1* cDNA and UTR regions (1353 bp) were

TABLE 3
Tag SNPs from Caucasian HapMap genotype data and the association of SNP genotypes with liver *AKR1D1* mRNA expression

The r^2 threshold for tag SNPs analysis was 0.8. A dash indicates that the SNP was not genotyped.

| Group | Minor Allele Frequency | TAG SNPs | Location in Gene | Hepatic <i>AKR1D1</i> mRNA Association |
|-------|------------------------|------------|------------------|--|
| | % | | | <i>P</i> |
| 1 | 23 | rs2120846 | Intron 1 | 0.045 |
| | | rs6977075 | Intron 2 | — |
| | | rs6980334 | Intron 4 | — |
| | | rs7795946 | Intron 4 | — |
| 2 | 43 | rs8180809 | Intron 3 | 0.14 |
| 3 | 21 | rs1872929 | 3' UTR | 0.003 |
| | | rs1872930 | 3' UTR | 0.003 |
| 4 | 18 | rs2035647 | Intron 8 | — |
| | | rs2306847 | Intron 8 | — |
| | | rs2306846 | Intron 6 | — |
| | | rs3805362 | Intron 7 | 0.17 |
| 5 | 7 | rs12668157 | Intron 1 | — |
| | | rs6943935 | Intron 2 | — |
| | | rs17169518 | Intron 7 | 0.53 |
| | | rs17169521 | Intron 8 | 0.22 |
| 6 | 37 | rs2035648 | Intron 8 | 0.1 |
| 7 | 3 | rs2306845 | Intron 4 | 0.9 |
| 8 | 3 | rs2882947 | Intron 7 | — |
| 9 | 17 | rs3735023 | 3' UTR | 0.87 |
| 10 | 13 | rs6467735 | Intron 4 | 0.26 |
| 11 | 1 | rs17169523 | 3' UTR | — |
| 12 | 11 | rs17543126 | Intron 4 | — |

amplified from livers wild type, heterozygous, and homozygous for the 3'-UTR SNPs. No alternative mRNAs were observed in any of the livers. However, this amplification strategy would fail to detect the consequences of SNPs that lead to the following: 1) alternative *AKR1D1* transcripts differing from the wild-type mRNA by only a few nucleotides, and 2) alternative transcripts with premature termination codons that undergo accelerated degradation by nonsense-mediated decay. Hence, the 1353 bp *AKR1D1* first-round PCR product was used as template for a second round of nested PCR amplification of various portions of *AKR1D1* (Table 2) including amplification that would have detected any skipping of exon 8. *AKR1D1* alternative mRNAs were detected on second-round PCR amplification, but the pattern of amplified bands was the same for all samples regardless of intron 8 (rs2035647 and rs2306847), or any other *AKR1D1* genotypes. Hence, we unequivocally determined by PCR analysis of *AKR1D1* mRNA transcripts in human livers that none of the *AKR1D1* alternative mRNAs was related to a polymorphism.

The *AKR1D1* VAR-HAP Has Increased Transcript Stability and/or Translation Efficiency Compared with the WT-HAP. We conducted *AKR1D1* 3'-UTR-luciferase reporter assays (the entire 3'UTR is cloned downstream of a luciferase cDNA) to address whether either of the 3'-UTR SNPs (rs1872929 and rs1872930) was affecting mRNA abundance (by affecting mRNA translational efficiency or mRNA stability). Reporter plasmids containing the WT-HAP, VAR-HAP (rs1872929 and rs1872930), and single 3'-UTR variants were constructed by site directed mutagenesis, and the plasmids transiently transfected into HepG2 cells (Fig. 5, C and D). The *AKR1D1* VAR-HAP and the rs1872930 single 3'-UTR variant construct had similar reporter activity that was significantly higher compared with the WT-HAP and the rs1872929 single 3'UTR variant. These *AKR1D1* 3'-UTR-luciferase reporter assay results (VAR-HAP > WT-HAP) (Fig. 5, C and D) corresponded well with the increased expression of *AKR1D1* in livers with the VAR-HAP compared with the WT-HAP (Fig. 5, A and B). These results suggest that the rs1872930 3'-UTR SNP is increasing *AKR1D1* mRNA transcript stability and/or translational efficiency.

The *AKR1D1* 3'-UTR rs1872930 Polymorphism Is Associated with Increased Expression of Many mRNAs in the P450 Subnetwork.

Having established that *AKR1D1* may act as a master regulator of mRNA expression of multiple P450s in primary human hepatocytes (Fig. 4), and that the rs1872930 is a functional SNP affecting hepatic *AKR1D1* mRNA expression, our next goal was to study the association of this polymorphism with the mRNA expression of other members of the P450 subnetwork (Fig. 6). The *AKR1D1* functional SNP (rs1872930) was most significantly associated with expression of the nuclear hormone receptor *CAR* (*NRII3*) and the bile acid uptake carrier Na⁺-taurocholate cotransporting polypeptide (*NTCP*) or solute carrier family 10 (sodium/bile acid cotransporter family) member (*SLC10A1*), but was also significantly associated with expression of multiple P450s involved in bile acid synthesis including *CYP27A1*, *CYP7A1*, and *CYP8B1* (Fig. 6). The *AKR1D1* polymorphism was also significantly associated with mRNA expression of multiple drug-metabolizing P450s including *CYP2C19*, *CYP2C9*, *CYP2C8*, and *CYP3A4* (Fig. 6). To account for multiple testing, we adjusted the statistical significance threshold using the permutation method in consideration of the correlation among phenotypes. After adjusting for multiple testing, the association remained statistically significant for *AKR1D1*, *SLC10A1*, *CYP2C9*, *CYP2C8*, *CYP27A1*, *CYP7A1*, *CYP8B1*, and *CAR* mRNA expression (Fig. 6). Similarly, the *AKR1D1* genotype was significantly associated with P450 enzyme activities measured in liver microsomes from the same livers. Livers with the *AKR1D1* variant allele had significantly higher activity of *CYP2C8* ($P = 0.002$) using paclitaxel as substrate, *CYP3A4* ($P = 0.03$) using testosterone as substrate, *CYP2B6* ($P = 0.037$) using bupropion as substrate, and *CYP2C19* ($P = 0.055$) using *S*-mephenytoin as substrate (Fig. 7). The association for *CYP2C8* remained statistically significant even after adjusting for multiple testing, whereas the association for *CYP3A4*, *CYP2C19*, and *CYP2B6* could not escape the stringent P value threshold ($P < 0.0034$) of multiple testing.

To determine whether association of the *AKR1D1* rs1872930 genotype with expression of other members of the P450 subnetwork was specific, and not simply because of the higher degree of

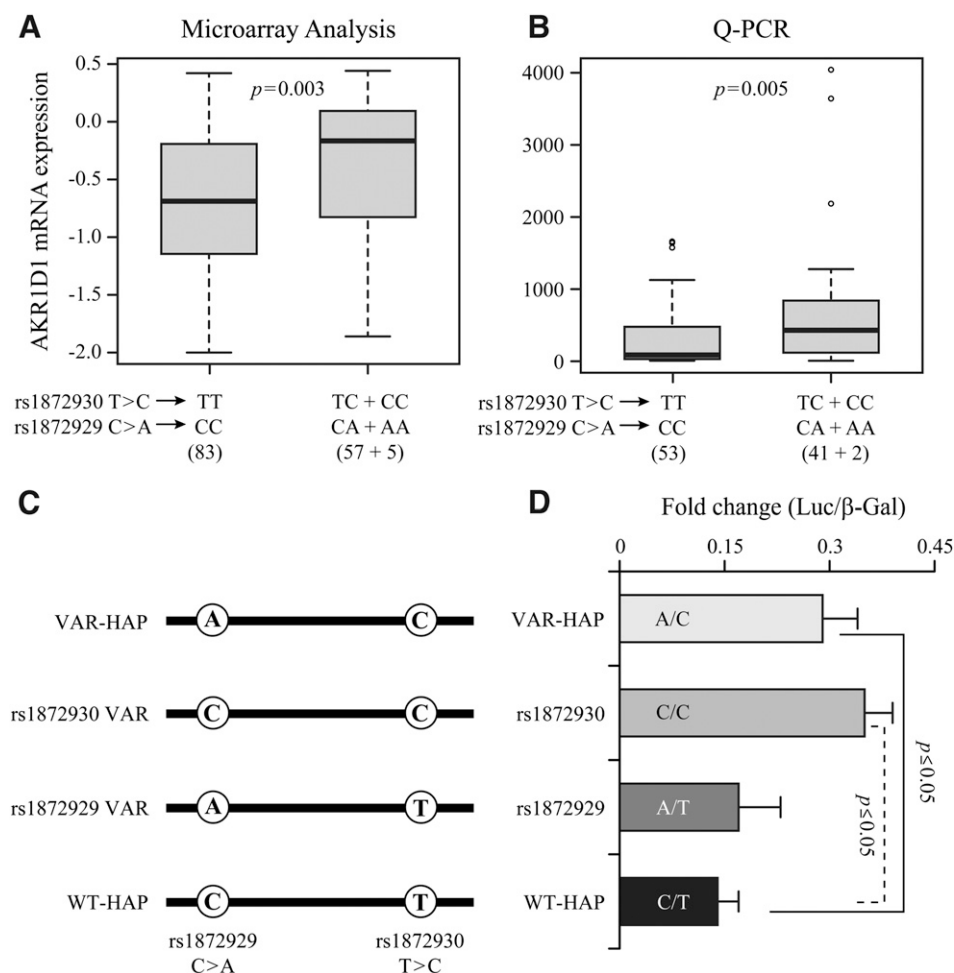


Fig. 5. *AKR1D1* SNP (rs1872930) variant is associated with increased human liver *AKR1D1* mRNA expression as well as increased transcript stability and/or translation efficiency. *AKR1D1* microarray expression data taken from an earlier study (Schadt et al., 2008) (A) or analyzed in this study by quantitative PCR analysis (B) were significantly higher in livers with the *AKR1D1* 3'-UTR SNPs ($P = 0.003$ and $P = 0.005$, respectively). HepG2 cells were transfected with wild-type or variant *AKR1D1* 3'-UTR-Luc reporter plasmid constructs (C and D) and luciferase assays were performed 24 hours later. Results are expressed as fold change with respect to vector control. Statistical difference between groups was determined using the *t* test (D).

correlation in mRNA expression between subnetwork members, we examined the relationship between several *P450* polymorphisms with *AKR1D1* and *P450* mRNA expression. Livers heterozygous for either the *CYP2C9**2 or *2C9**3 allele had significantly lower *CYP2C9* activity compared with livers with the *CYP2C9**1 genotype ($P = 0.004$), but there was no effect of the same genotypes on mRNA expression of *CYP3A4* ($P = 0.43$) or *AKR1D1* ($P = 0.75$). Similarly, the *CYP2C19* diplotypes (*2C19**1/*1, *1/*17, *1/*2, *17/*17, *2/*17, or *2/*2) were not associated with mRNA expression of either *CYP3A4* ($P = 0.3229$) or *CYP2C9* ($P = 0.9716$).

Mfold Analysis of *AKR1D1* Wild-Type and Variant mRNA Structure. To test whether the two 3'-UTR SNPs change *AKR1D1*'s mRNA secondary structure, we performed Mfold analysis of *AKR1D1* mRNA. The local sequences of *AKR1D1* mRNA (370 nucleotide) surrounding the two 3'-UTR SNPs were used to predict mRNA secondary structure. Since RNAs can potentially adopt multiple conformations, Mfold calculated 13 alternative structures for the variant allele and 12 for the wild type. The average minimum free energy was -80.01 for the variant allele and -78.1 for the wild-type allele. The more negative, thermodynamically favorable, free energy of the variant versus wild-type allele suggests that this is one reason the variant allele has a higher level of *AKR1D1* mRNA. The 3'-UTR polymorphism has altered the thermodynamic folding landscape of *AKR1D1*'s mRNA to a more thermodynamically favorable conformation, which may be affecting its mRNA expression (Fig. 5).

In Silico Analysis of *AKR1D1* Wild-Type and Variant 3'-UTR Sequences. Both the full-length wild-type and variant *AKR1D1*

mRNA (3'-UTR) were investigated for any differential miRNA recognition/binding sites (using miRanda) that might affect the expression of *AKR1D1* mRNA. There was no significant difference ($P < 0.05$) in the binding scores or energy of any known miRNAs between the wild-type and variant alleles demonstrating the absence of miRNA-mediated regulation of *AKR1D1* via the rs1872930 SNP.

Discussion

This study was prompted by a recent report that expression profiled 466 human liver samples and constructed a *P450* gene regulatory (Bayesian) subnetwork that showed striking correlation between expression of many drug-metabolizing *P450*s (Yang et al., 2010) (Fig. 2). This network modeling approach identified *AKR1D1* as one master regulatory node for the human liver *P450* subnetwork (Yang et al., 2010). In this report, we validated that *AKR1D1* is a key regulator of the *P450* subnetwork. Overexpression of *AKR1D1* induced expression of *P450*s, whereas lentiviral-mediated *AKR1D1* knockdown caused a decrease in *P450* mRNA expression in hepatic cells. Our results show that an *AKR1D1* 3'-UTR polymorphism causes a perturbation in *AKR1D1* mRNA expression that ripples through the network, ultimately affecting mRNA expression of multiple *P450*s. This result makes mechanistic sense since, presumably, alterations in *AKR1D1* expression (an enzyme critical in both the classic and alternative bile acid synthesis pathways) would be expected to result in altered production of cholic acid and chenodeoxycholic acid, known activators of nuclear hormone receptors, such as PXR and CAR, that transcriptionally regulate a number of drug-metabolizing *P450*s. The fact

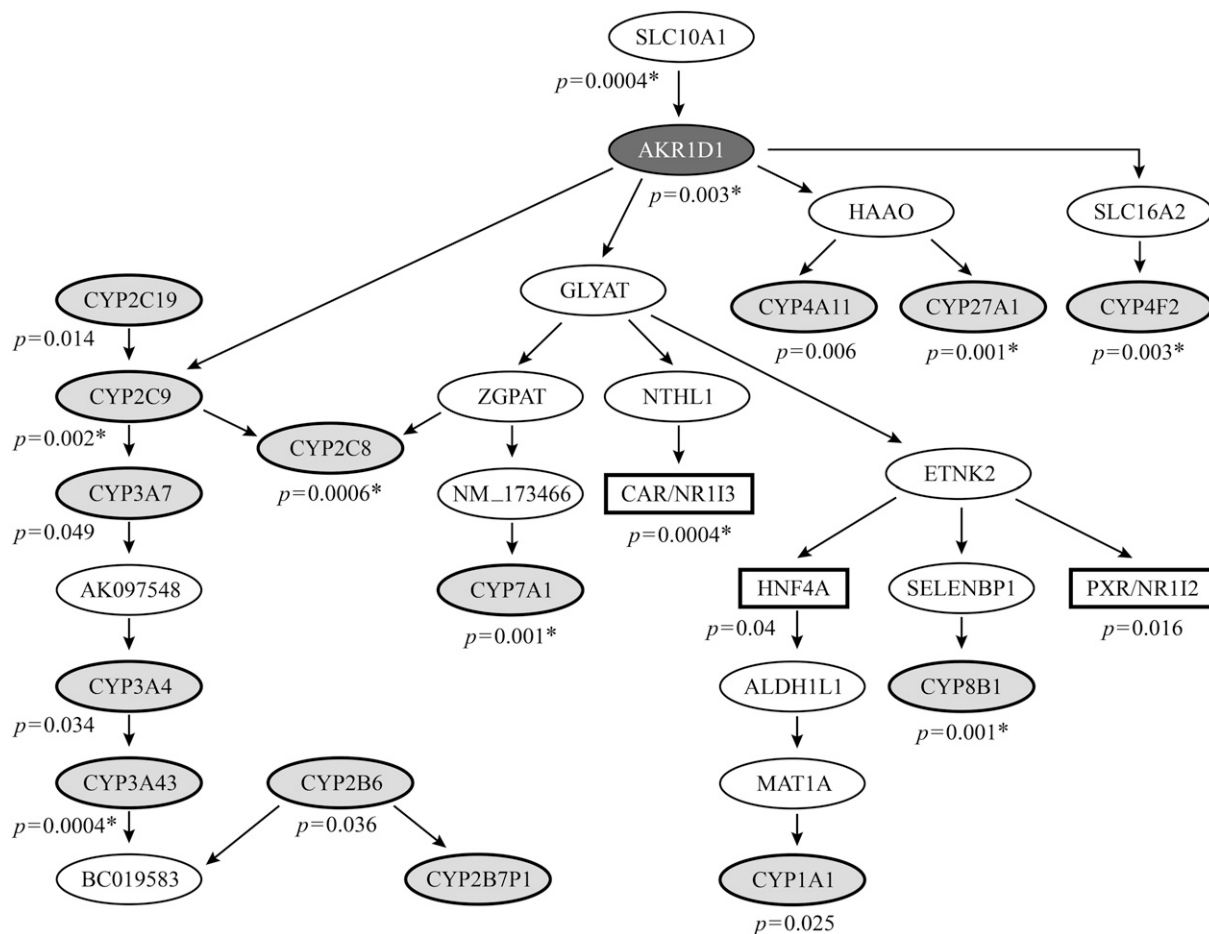


Fig. 6. The *AKR1D1* 3'-UTR (rs1872930) polymorphism is associated with increased liver mRNA expression of downstream targets including *PXR*, *CAR*, and multiple *P450s*. A pruned *P450* subnetwork showing the master regulator *AKR1D1* (dark gray oval) and downstream target *P450s* (light gray ovals) and other transcription factors including the nuclear receptors *PXR* and *CAR* (boxes) is shown along with the *P* values of association of the variant allele with increased liver mRNA expression. The mRNA expression data used for calculating associations are from previous microarray studies (Schadt et al., 2008; Yang et al., 2010). mRNA expression was compared between the 83 livers available with the *AKR1D1* rs1872930 wild-type TT genotype versus livers with one ($n = 57$) or two ($n = 5$) variant alleles (TC+CC genotypes). To account for multiple testing, we adjusted the statistical significance threshold using the permutation method in consideration of the correlation among phenotypes. For the association between genotype and mRNA expression, we consider $P < 0.0034$ as statistically significant after adjusting for multiple testing. The statistically significant values after multiple testing are marked with an asterisk.

that the association of the *AKR1D1* SNP is greater with the bile acid pathway genes than with the genes involved in drug metabolism is further support for the notion that the functional consequence of the *AKR1D1* polymorphism is on hepatic bile acid homeostasis. The fact that *AKR1D1* overexpression also resulted in induction of the FXR target gene *SHP* and feedback repression of the *CYP7A1* gene further suggests that *AKR1D1* was modifying primary bile acid synthesis in hepatocytes.

Our *AKR1D1* resequencing study failed to find any *AKR1D1* coding variation. The absence of *AKR1D1* coding variation likely reflects the important function of this gene in regulating bile acid levels. Human *AKR1D1* deficiency is rare and patients with *AKR1D1* deficiency accumulate bile acid intermediates and lack downstream functional bile acids, and leads to hepatotoxicity (Drury et al., 2010). For example, *AKR1D1* sequence variation was reported in infant patients presenting with primary bile acid deficiency (Gonzales et al., 2004) who had a compound heterozygous status represented by two nonconservative missense mutations, P133R (C467G) in exon 4 and R261C (C850T) in exon 7. However, these variations were not detected in 100 chromosomes of control individuals of the same ethnicity. *AKR1D1* genetic variations were also reported in three patients with neonatal onset cholestatic liver disease. These patients had rare disease causing *AKR1D1* genetic variation that was not

detected in 100 chromosomes of control individuals indicating that these are disease-specific mutations (Lemondé et al., 2003). Our study did identify a common 3'-UTR SNP (rs1872930) genotype that correlated significantly with total hepatic *AKR1D1* and *P450* mRNA expression. To our knowledge, this is the first study to report the occurrence of a functional genetic variation (rs1872930) in *AKR1D1* in healthy livers. The minor allele frequency of rs1872930 varies among populations (e.g., Caucasians, 21%; Chinese, 39%; Japanese, 23%; African Americans, 12%; and Mexicans, 39%) and one could imagine the population-specific pharmacogenetic implications associated with this SNP. To rule out the possibility of another linked coding SNP responsible for the higher activity, we directly sequenced the cDNA from these samples and found no coding region polymorphism in *AKR1D1*. This data are further substantiated by the DNA sequencing data (Caucasians) from the 1000 Genome Project, which also revealed the absence of any *AKR1D1* coding region SNP in LD to rs1872930.

The *P450* coexpression subnetwork (Yang et al., 2010) included a significant number of drug-metabolizing *P450s* and nuclear hormone (*PXR*, *CAR*) regulators of these *P450s*. Importantly, the interconnected genes in the *P450* subnetwork can sense perturbations from genetic loci (Wang et al., 2012) and identify the key intermediates that contribute to the perturbations. It was thus informative that the

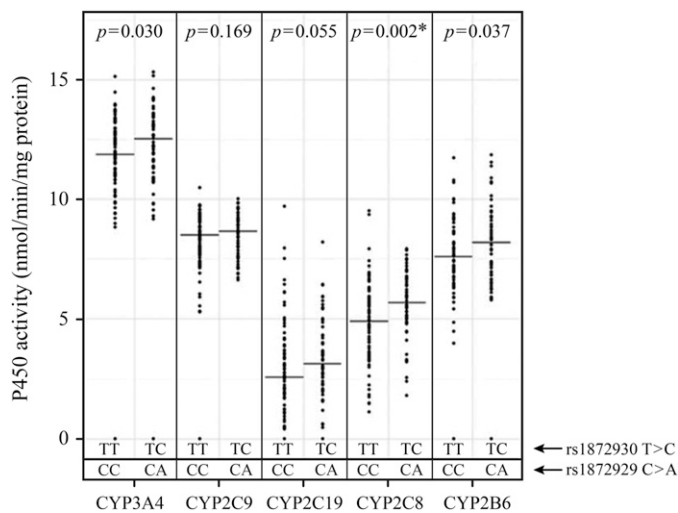


Fig. 7. The *AKR1D1* 3'-UTR variant (rs1872930) is associated with increased activity of multiple P450s in human liver microsomes. P450 activities were compared between the 83 livers with the *AKR1D1* rs1872930 wild-type TT genotype versus livers with one ($n = 57$) or two ($n = 5$) variant alleles (indicated as TC genotype). The activities of P450s were measured using probe substrate metabolism assays for CYP3A4 (testosterone), CYP2C8 (paclitaxel), CYP2C9 (tolbutamide), CYP2C19 (*S*-mephenytoin), and CYP2B6 (bupropion). *P* values of the association between *AKR1D1* genotypes and P450 activities are shown on the top of each panel. For the association between genotype and P450 activity, we considered $P < 0.0034$ statistically significant after adjusting for multiple testing. The statistically significant value after multiple testing is marked with an asterisk.

subnetwork was also enriched for a number of genes important for the bile acid biologic pathway, including *AKR1D1* which was identified as a key regulator of the network. Indeed, the confirmation that the P450 subnetwork senses perturbations in *AKR1D1* (driven by its genetic variation) supports the regulatory role for *AKR1D1* in the P450 network.

The finding that *AKR1D1* sequence variation was predictive of mRNA expression for *AKR1D1* and multiple P450s also validates the notion that identification of regulatory nodes in transcription networks is a reasonable approach to identify new candidate genes whose genetic variation is predictive of expression of other genes in regulatory networks. This approach represents a radical departure from the traditional pharmacogenetic reductionist candidate gene strategy, and it represents an elegant approach that harnesses the ability of systems biology to identify the nodes that, when individually perturbed, regulate the entire network. This is particularly useful since the common *P450 cis*-variation does not explain the majority of human variation in P450 activity. Moreover, genetic variation in *P450* (e.g., *CYP3A4*) trans-regulatory factors such as *PXR*, *FoxA2* and *HNF4a*, can only explain another 10% of P450 phenotypic variation (Lamba et al., 2010).

This study demonstrates that genetic variation in genes (*AKR1D1*) that control the amount of ligands that activate P450 transcriptional regulators such as *PXR*, *CAR*, and *FXR* can be another important source of phenotypic variation in drug-metabolizing P450s. This is an interesting finding since we also recently found that variation in intestinal *CYP3A4* expression was directly related to genetic variation in both its transcriptional regulator *VDR*, and probably in the level of its ligand 1,25-dihydroxyvitamin D3 because *CYP3A4* intestinal expression varied seasonally, correlating with the documented levels of ultraviolet sunlight and reported seasonal levels of vitamin D (Thirumaran et al., 2012).

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Authorship Contributions

Participated in research design: Chaudhry, Thirumaran, Yasuda, Schuetz.
Conducted experiments: Chaudhry, Yasuda.
Contributed new reagents or analytic tools: Thirumaran, Strom.
Performed data analysis: Chaudhry, Thirumaran, Yasuda, Fan, Schuetz.
Wrote or contributed to the writing of the manuscript: Chaudhry, Thirumaran, Yasuda, Yang, Fan, Strom, Schuetz.

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Address correspondence to: Dr. Erin G. Schuetz, Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105. E-mail: erin.schuetz@stjude.org