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**Genetic variation in Aldo-keto reductase 1D1 (*AKR1D1*) affects the expression and activity
of multiple Cytochrome P450s (CYPs)**

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Running Title: *AKR1D1* Polymorphism affecting multiple CYPs

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Abbreviations: AKR1D1, Aldo-keto reductase 1D1 (aldo-keto reductase family 1, member D1 = delta 4-3-ketosteroid-5-beta-reductase = SRD5B1); CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; CYP, cytochrome P450; FXR, farnesoid X receptor; HAP, haplotype; LD, linkage disequilibrium; PXR, pregnane X receptor; SHP, small heterodimer partner; SNP, single nucleotide polymorphism; UTR, untranslated region; VAR, variant; WT, wild type.

Abstract

Human liver gene regulatory (Bayesian) network analysis (Yang et al., 2010) was previously used to identify a cytochrome P450 gene subnetwork with AKR1D1 as a key regulatory driver of this subnetwork. This study assessed the biological importance of AKR1D1 (a key enzyme in the synthesis of bile acids, ligand activators of FXR, PXR and CAR - known transcriptional regulators of CYPs) to hepatic CYP expression. Over-expression of AKR1D1 in primary human hepatocytes led to increased expression of *CYP3A4*, *2C8*, *2C9*, *2C19*, and *2B6*. Conversely, AKR1D1 knockdown decreased expression of these CYPs. We resequenced AKR1D1 from 98 donor livers and identified a 3'-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 the SNP increases AKR1D1 mRNA stability and/or translation efficiency. Consistent with AKR1D1's putative role as a driver of the CYP subnetwork, the AKR1D1 3'-UTR SNP was significantly associated with increased hepatic mRNA expression of multiple CYPs (*CYP3A4*, *2C8*, *2C9*, *2C19*, and *2B6*) 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 CYPs 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 CYP 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 CYP enzymes, a considerable percentage of variation in CYP activity cannot be explained by cis-genetic polymorphisms, and it is hypothesized that genetic variation in trans regulators influences CYP variation (Schuetz, 2004). Indeed, we previously showed that polymorphisms in hepatic transcription factors that regulate CYPs, such as *FoxA2*, *HNF4alpha*, *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 CYP trans regulators is to derive the CYP biological co-expression 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 CYP gene network. AKR1D1 (Δ^4 -3-oxosteroid, 5 β -reductase) was predicted as one master regulator of the CYP subnetwork or module (Fig 1). Expression of multiple CYPs, including *CYP2C9*, *CYP3A4* and *CYP2C19* were predicted to be influenced by AKR1D1.

AKR1D1 (aldo-keto reductase family 1, member D1 = delta 4-3-ketosteroid-5-beta-reductase = SRD5B1) is a biologically plausible candidate gene regulating CYP 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 CYPs 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 PXR and CAR (Congiu et al., 2009; Schuetz et al., 2001) – transcriptional regulators of *CYP3A4*, *CYP2C9* and *CYP2C19*. Bile acids also activate and regulate the nuclear hormone receptor FXR, a known transcriptional regulator of bile acid homeostasis (Modica et al., 2009; Urquhart et al., 2007) and *PXR* (Jung et al., 2006), and *CYP3A4* (Gnerre et al., 2004). *AKR1D1* could also regulate CYPs 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 CYP expression. In addition, we tested whether genetic variation in *AKR1D1* was associated with CYP 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 (Lemonde et al., 2003). In this study, we report that genetic variation in *AKR1D1* contributes to the observed variation in CYP expression and activity of the CYP 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 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 (NIH Contract #N01-DK-9-2310) and by the Cooperative Human Tissue Network. Liver tissue from 163 White donor livers (from the U. 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 CYP activities, respectively (Schadt et al., 2008; Yang et al., 2010). A subset of 145 White donor livers from the 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'-UTR-luciferase reporter construct was purchased from Switchgear Genomics, Menlo Park, CA and single mutants of rs1872929 and rs1872930 as well as different haplotypes (WT-HAP and 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 PCR amplified from the human AKR1D1 expression plasmid (Origene Cat no. SC116410) and subcloned into

pENTR/SD/D-TOPO (Invitrogen Part no. 45-0219) for entry into the Adv-BGFP-Gateway destination vector (from Dr. John Gray, St Jude) in order to make the Adv-BGFP-AKR1D1 overexpression vector. The pGIPz-AKR1D1-200099 lentiviral shRNAmir vector (Catalog no. RHS-4430-98709631), to knockdown human AKR1D1 and pGIPz non-silencing 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 SNP genotyping. The microarray design, RNA sample preparation, amplification, hybridization, and expression analysis as well as 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 ABI. The Snapshot primers for 1st round PCR were forward primer 5'-GCGCGATCATCCTGAATACCCATT-3' and reverse primer 5'-GACTGAGTCAAAGTCATTTACTGAC-3'. The extension primers used were forward extension primer 5'-TCCTGAACAGATTTTCACTCCC-3' and reverse extension primer – 5'-CATTGCACCGTCTTGGCACTC-3'.

CYP enzyme activity measurements in human liver microsomes. The CYP activities (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 Mem- α medium 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, 3×10^5 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 of 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 DMSO. 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 1500 g for 4 min, 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, Madison, WI). 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 Student's *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 number 454424). 4×10^5 cells per well in 24 well culture dishes were obtained from the company and cultured in hepatocytes culture media (BD Biosciences, Woburn, MA) 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 hrs. Cells were transfected with 20 MOI (multiplicity of infection) of Adv-BGFP-AKR1D1 or control virus for overexpression of AKR1D1. Similarly, for AKR1D1 knockdown experiments, hepatocytes were transfected with 5 MOI of either control (pGIPz-Lentiviral-RHS4346) or AKR1D1 specific shRNA 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% DMSO (vehicle). The CDCA treated cells were harvested 24 h after treatment; Adenoviral infected cells were harvested 48 h after infection; and Lentiviral infected cells were harvested 72 h after infection. Before harvesting, hepatocytes were washed once with PBS and were harvested by adding TRIzol reagent directly into the wells and passing a couple of time 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 phosphate buffer saline (PBS), harvested in 5 mL PBS and centrifuged at 1500 rpm for 10 min. 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 number 34076) from Thermo Scientific Waltham, MA.

mRNA quantification by Quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, NY). First-strand cDNA was prepared using oligo (dT) primers (ThermoScript RT-PCR system, Catalog number 11146-016, Invitrogen). Real-time PCR quantification of *AKR1D1*, *PXR*, *CAR*, *CYP3A4*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2B6*, *CYP7A1*, *SHP*, *FXR* and *GAPDH* mRNAs was carried out using the SYBR GreenER qPCR supermix (Invitrogen Catalog number 11760-100) 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 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 heterozygotes 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 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 LD with two intron 8 SNPs, the first round *AKR1D1* PCR product from subjects with rs1872930 TT, TC and CC genotypes was used as template to specifically amplify a region around exon 8/intron8 using primers AKR-FP1 and AKR-RP (Table 2). Again a semi-nested PCR was performed with AKR-FP1 and AKR-RPN primers to amplify a 416 bp normal or any low abundance transcripts that might have been associated with the rs1872930 allele. All 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 WT, 42 TC Heterozygotes and 2 CC variant homozygotes) 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 Mfold (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.

miRNA recognition sites using miRANDA. To define the potential for microRNAs to be differentially bound to *AKR1D1* wild-type or *AKR1D1* variant (rs1872930), miRanda (v3.0) 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. 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 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 CYP activity levels or mRNA expression, we consider $p < 0.0034$ as statistically significance after adjusting for multiple testing.

Results

The hepatic expression of multiple CYP mRNAs is significantly correlated. We first examined CYP 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 CYP's mRNA expression, and the results from the second analysis demonstrated virtually identical results. These CYPs 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 CYPs in the CYP 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 co-transfected 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, co-transfected AKR1D1 significantly stimulated *CYP3A4* expression ($p = 0.002$) in HepG2 cells to the same extent as the addition of 50 μ M 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 utilized 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 hrs with an AKR1D1 Adenovirus showed a ~443% increase in AKR1D1 immunoreactive protein (Fig 3B). Conversely, HepG2 cells transduced for 72 hr with the AKR1D1 ShRNA lentivirus exhibited ~90% cell transduction, and a ~64% decrease in AKR1D1 protein compared to cells transduced with the scrambled ShRNA (Fig 3C).

AKR1D1 overexpression induces CYP mRNA expression in primary human hepatocytes.

To test whether AKR1D1 is a key driver of the CYP 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 CYPs (*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 *CYP*, *PXR* and *CAR* induction profile

(Fig. 4B) similar to that observed with AKR1D1 overexpression. Although the fold induction of mRNA changes did not reach statistical significance for either CDCA treatment or AKR1D1 over expression, this is likely due to the large human variation in fold induction between the different hepatocyte preparations.

AKR1D1 silencing decreases multiple CYPs mRNA expression in primary human

hepatocytes. We next tested whether lentivirus shRNA mediated AKR1D1 knockdown would lead to down-regulation of CAR, PXR and CYP 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 CYPs (*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 CYPs, 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 ($p = 0.005$) (Table 3 and Fig. 5B). This 3'-UTR SNP (rs1872930) was in linkage disequilibrium (LD) with three other SNPs (an additional 3'-UTR SNP (rs1872929) and two SNPs in Intron 8 (rs2035647 and rs2306847)) in either a wild type haplotype (WT-HAP) or variant haplotype (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 WT, 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 if 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 that skip individual exons (exon 5 (125 bp), or exon 8 (83 bp)). The full length *AKR1D1* cDNA and UTR regions (1535 bp) were amplified from livers WT, heterozygote 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 (a) alternative *AKR1D1* transcripts differing from the WT mRNA by only a few nucleotides, and (b) alternative transcripts with premature termination codons that undergo accelerated degradation by nonsense mediated decay. Hence, the 1535 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 to 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. 5C,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. 5C,D) corresponded well with the increased expression of *AKR1D1* in livers with the

VAR-HAP compared to the WT-HAP (Fig. 5A,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 CYP subnetwork. Having established that AKR1D1 may act as a master regulator of mRNA expression of multiple CYPs 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 CYP subnetwork (Fig. 6). The *AKR1D1* functional SNP (rs1872930) was most significantly associated with expression of the nuclear hormone receptor *CAR* (*NR1I3*) 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 CYPs 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 CYPs 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 CYP 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, CYP2B6 could not escape the stringent p value threshold ($p < 0.0034$) of multiple testing.

To determine if association of the *AKR1D1* rs1872930 genotype with expression of other members of the CYP subnetwork was specific, and not simply because of the higher degree of correlation in mRNA expression between subnetwork members, we examined the relationship between several *CYP* polymorphisms with *AKR1D1* and *CYP* mRNA expression. Livers heterozygous for either the *CYP2C9**2 or *2C9**3 allele had significantly lower CYP2C9 activity compared to 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* WT 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 nt) 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 for the variant allele was -80.01 and for the WT allele was -78.1. The more negative, thermodynamically favorable, free energy of the variant vs. wild-type allele suggests 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* WT and variant 3'-UTR sequences. Both the full length WT and VAR *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 WT and VAR 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 CYP gene regulatory (Bayesian) subnetwork that showed striking correlation between expression of many drug metabolizing CYPs (Yang et al., 2010) (Fig 2). This network modeling approach identified AKR1D1 as one master regulatory node for the human liver CYP subnetwork (Yang et al., 2010). In this report we validated that AKR1D1 is a key regulator of the CYP subnetwork. Overexpression of AKR1D1 induced expression of CYPs, while lentiviral mediated AKR1D1 knockdown caused a decrease in CYP 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 CYPs. This result makes mechanistic sense since, presumably, alterations in AKR1D1 expression, an enzyme critical in both the classical 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 CYPs. The fact 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, as

human AKR1D1 deficiency is rare, 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 non-conservative 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 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 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 *CYP* 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 (MAF) of rs1872930 varies among populations i.e., 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 is further substantiated by the DNA sequencing data (Caucasians) from 1000 genome project which also revealed the absence of any *AKR1D1* coding region SNP in LD to rs1872930.

The CYP co-expression subnetwork (Yang et al., 2010) included a significant number of drug metabolizing CYPs and nuclear hormone (PXR, CAR) regulators of these CYPs.

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

The finding that *AKR1D1* sequence variation was predictive of mRNA expression for *AKR1D1* and multiple *CYPs* 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 *CYP* cis-variation does not explain the majority of human variation in *CYP* activity. Moreover, genetic variation in *CYP* (e.g., *CYP3A4*) trans-regulatory factors such as *PXR*, *FoxA2* and *HNF4a*, can only explain another 10% of *CYP* phenotypic variation (Lamba et al., 2010).

This study demonstrates that genetic variation in genes (*AKR1D1*) that control the amount of *ligands* that activate *CYP* transcriptional regulators such as *PXR*, *CAR* and *FXR*, can be another important source of phenotypic variation in drug metabolizing *CYPs*. 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 UV sunlight and reported seasonal levels of vitamin D (Thirumaran et al., 2012).

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Authorship Contributions

Participated in research design: Schuetz, Chaudhry, Thirumaran, Yasuda

Conducted experiments: Chaudhry, Yasuda

Contributed new reagents or analytic tools: Strom, Thirumaran

Performed data analysis: Schuetz, Chaudhry, Thirumaran, Yasuda, Fan

Wrote or contributed to the writing of the manuscript: Schuetz, Chaudhry, Thirumaran, Yasuda, Yang, Fan, Strom

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Legends for Figures

Figure 1. Model for regulation of expression/activity of multiple CYPs by AKR1D1.

Cholesterol is converted to cholic acid (CA) and chenodeoxycholic acid (CDCA) via a classical pathway (starting with CYP7A1) and an alternative pathway (starting with CYP27A1). Both bile acid synthesis pathways include aldo-keto reductase family 1, member 1 (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 chenodeoxycholic acid and cholic acid which can serve as endogenous ligands of nuclear receptors PXR and CAR which upon activation can regulate the transcription of multiple CYPs including CYP3A, CYP2C9 and CYP2C19.

Figure 2. Significant human liver CYP-CYP mRNA correlation indicates these CYPs share trans-regulation. Correlation between *CYP3A4* vs. *CYP2C9* mRNA (A), *CYP3A4* vs.

CYP2C19 mRNA (B), and *CYP3A4* vs. *CYP2B6* mRNA (C). CYP mRNA expression data from 163 human liver samples was taken from an earlier study (Schadt et al., 2008; Yang et al., 2010) and was employed 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 in the figures.

Figure 3. AKR1D1 over-expression 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. Co-transfected pSV40-LacZ served as a transfection efficiency

control. Next day, empty vector transfected cells were treated with either 50 μ M CDCA or DMSO. Twenty-four hr 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 hr were infected with AKR1D1 Adenovirus (B), or with AKR1D1 ShRNA Lentiviral particles (C). Cell lysates were prepared and 100 μ g protein was subjected to SDS-PAGE and immunoblotted with anti-AKR1D1 or anti- β -Actin antibody.

Figure 4. AKR1D1 over-expression increased and AKR1D1 silencing decreased expression of multiple CYPs in primary Human Hepatocytes.

Primary human hepatocytes were transduced with Adv-BGFP-AKR1D1 or control virus, cells were harvested after 48 hr and mRNA quantification was performed using RT-PCR. Results from four donors are presented as mean \pm SEM (A). As a positive control hepatocytes were treated with either DMSO or 50 μ M chenodeoxycholic acid (CDCA). Results from three donors are presented as mean \pm SD (B). For AKR1D1 knockdown experiments hepatocytes were transfected with either control or AKR1D1 lenti-viruses. Cells were harvested 72 hr after infection for RT-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.

Figure 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 Q-PCR analysis (B) was 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, D) and 24 hr later luciferase assays were performed. Results are expressed as fold change with respect to vector control. Statistical difference between groups was determined using Student's *t* test (D). VAR-HAP- variant haplotype, WT-HAP- wild type haplotype.

Figure 6. The *AKR1D1* 3'-UTR (rs1872930) polymorphism is associated with increased liver mRNA expression of downstream targets including *PXR*, *CAR* and multiple *CYPs*. A pruned CYP subnetwork showing the master regulator *AKR1D1* (dark grey oval) and downstream target *CYPs* (light grey ovals) and other transcription factors including the nuclear receptors *PXR* & *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 is 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 vs. livers with one ($n=57$) or two ($n=5$) variant allele (TC+CC genotypes). To account for multiple testing, we adjusted the statistical significance threshold using permutation method in consideration of the correlation among phenotypes. For the association between genotype and mRNA expression, we consider $p < 0.0034$ as statistically significance after adjusting for multiple testing. The statistically significant values after multiple testing are marked with an asterisk.

Figure 7. The *AKR1D1* 3'-UTR variant (rs1872930) is associated with increased activity of multiple CYPs in human liver microsomes. CYP activities were compared between the 83 livers with the *AKR1D1* rs1872930 wild type TT genotype vs. livers with one (n=57) or two (n=5) variant alleles (indicated as TC genotype). The activities of CYPs 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 CYP activities are shown on the top of each panel. For the association between genotype and CYP activity, we consider $p < 0.0034$ as statistically significance after adjusting for multiple testing. The statistical significant value after multiple testing is marked with an asterisk.

Table 1. Primers used for real-time Q-PCR.

	mRNA	Sequence (5' → 3')
1	<i>GAPDH-FP</i>	GGACCACCAGCCCCAGCAAGAG
2	<i>GAPDH-RP</i>	GAGGAGGGGAGATTCAGTGTGGTG
3	<i>AKR1D1-FP</i>	TCAGAACCTAAATCGACCCCT
4	<i>AKR1D1-RP</i>	CCCTGTGTCAATAGCAACCTTC
5	<i>CYP3A4-FP</i>	CCTTACACATACACACCCTTTGGAAGT
6	<i>CYP3A4-RP</i>	AGCTCAATGCATGTACAGAATCCCCGGTTA
7	<i>CYP2C8-FP</i>	GCAGGAAAAGGACAACCAAA
8	<i>CYP2C8-RP</i>	GTGTAAGGCATGTGGCTCCT
9	<i>CYP2C9-FP</i>	GATCTGCAATAATTTTTCTC
10	<i>CYP2C9-RP</i>	TCTCAGGGTTGTGCTTGTC
11	<i>CYP2C19-FP</i>	GAGGAGTTTTCTGGAAGAGGCC
12	<i>CYP2C19-RP</i>	CATTGCTGAAAACGATTCCAAA
13	<i>CYP2B6-FP</i>	CACCCTAACACCCATGACCG
14	<i>CYP2B6-RP</i>	GATCACACCATATCCCCGGA
15	<i>FXR-FP</i>	TATCTAGCCCAATATTTACAGTT
16	<i>FXR-RP</i>	GTATAACTTTCTTTATTTAC
17	<i>CYP7A1-FP</i>	AAACTGGATGGAAATAGCCATTTG
18	<i>CYP7A1-RP</i>	TTTCATCTGCAAGTCATTTAGCG
19	<i>SHP-FP</i>	GCTGTCTGGAGTCCTTCTGG
20	<i>SHP-RP</i>	CCAATGATAGGGCGAAAGAA
21	<i>CAR-FP</i>	CCAGCTCATCTGTTTATCCA
22	<i>CAR-RP</i>	GGTAACTCCAGGTCGGTCAG
23	<i>PXR-FP</i>	CAAGCGGAAGAAAAGTGAAC
24	<i>PXR-RP</i>	CACAGATCTTTCCGGACCTG

FP, forward primer; RP, reverse primer

Table 2. Primers used to test for alternative *AKR1D1* mRNA transcripts.

Primer	Sequence (5' → 3')	Location in mRNA	Length
AKR-FP AKR-RP	CCCTAGGACACCTTTCTA TCATATGTTGCCTTTGATG	5' UTR 3' UTR	1353 bp
AKR-FPN AKR-RPN	ATGGATCTCAGTGCTGCAAGT CCCTGCAGTCAGTATTCATCA	Start of Ex 1 Ex 9/3' UTR	989 bp
AKR-FP1 AKR-RP	AACCAGGTTGAGTGCCAT TCATATGTTGCCTTTGATG	Ex5/Ex 6 3' UTR	711 bp
AKR-FP1 AKR-RPN	AACCAGGTTGAGTGCCAT CCCTGCAGTCAGTATTCATCA	Ex 5/Ex 6 Ex 9/3' UTR	416 bp

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

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

MAF: minor allele frequency; (-): SNP not genotyped. The r^2 threshold for tag SNPs analysis was 0.8.

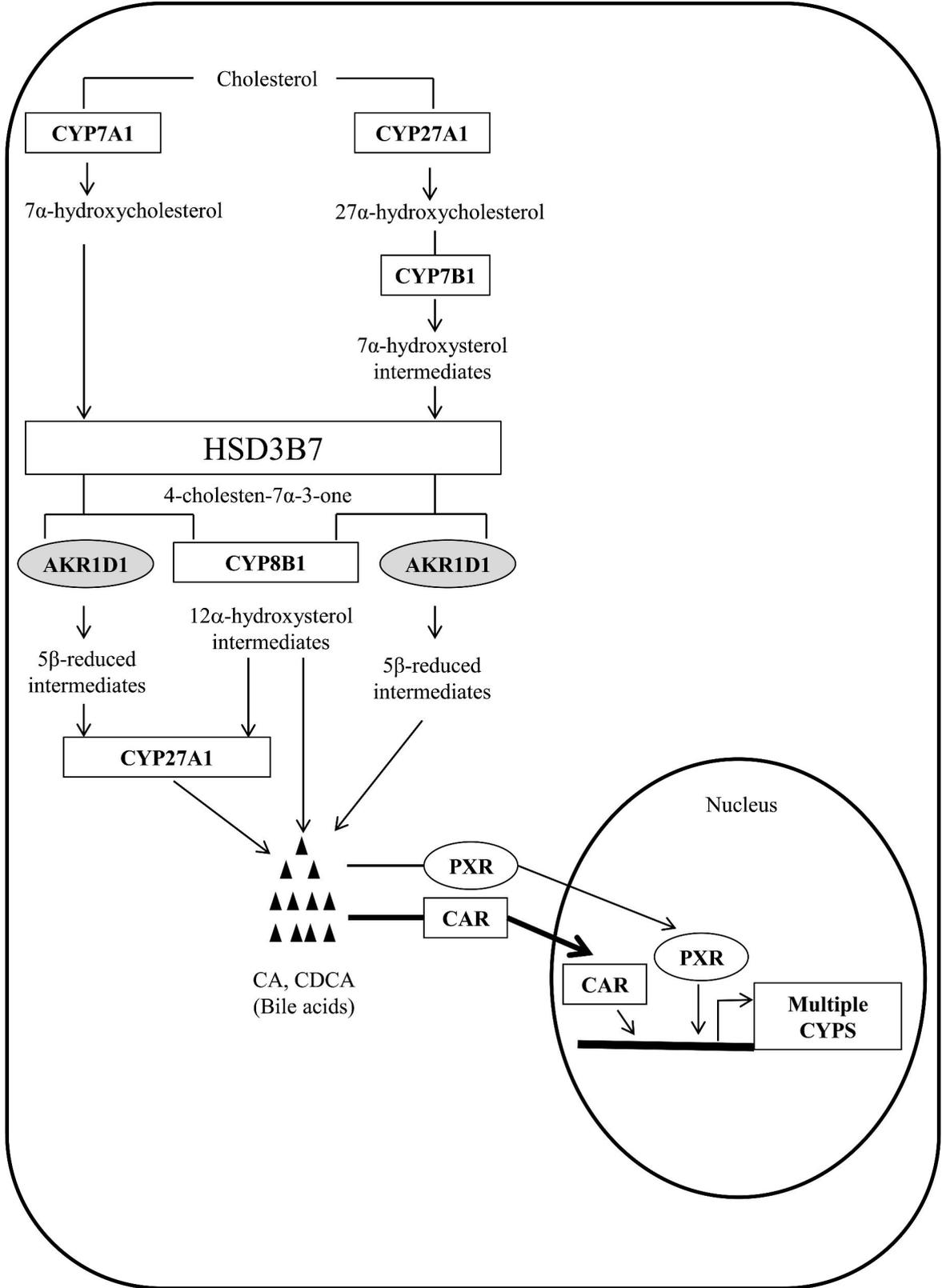
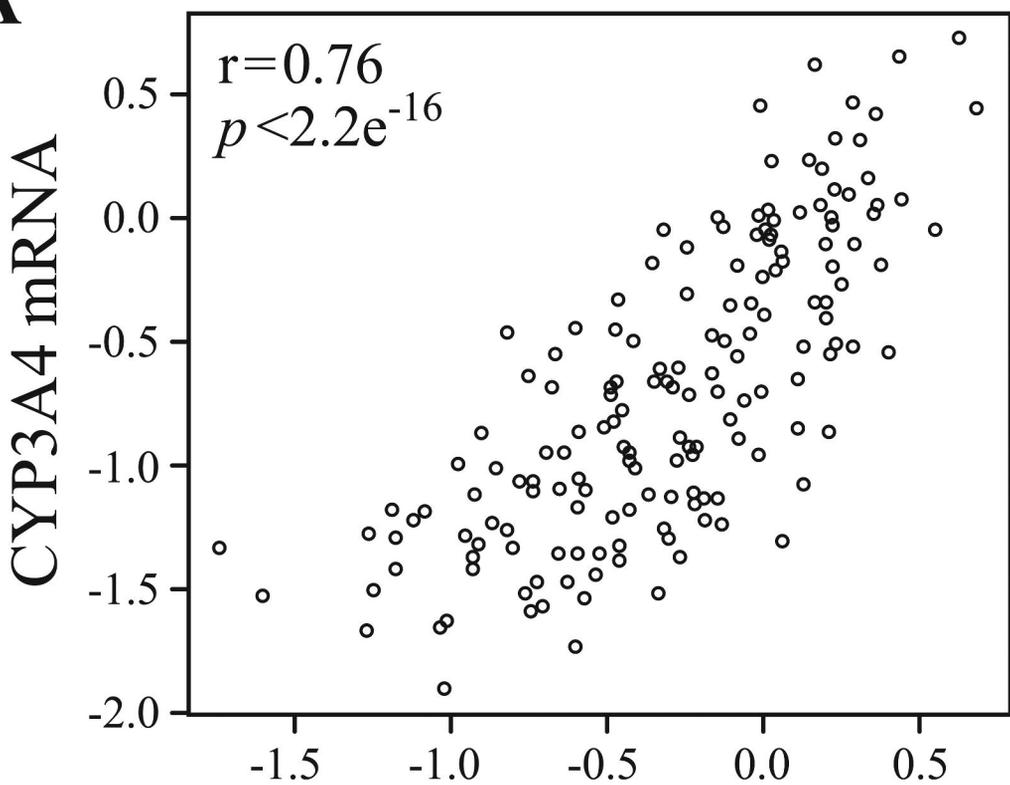


Figure 1

A

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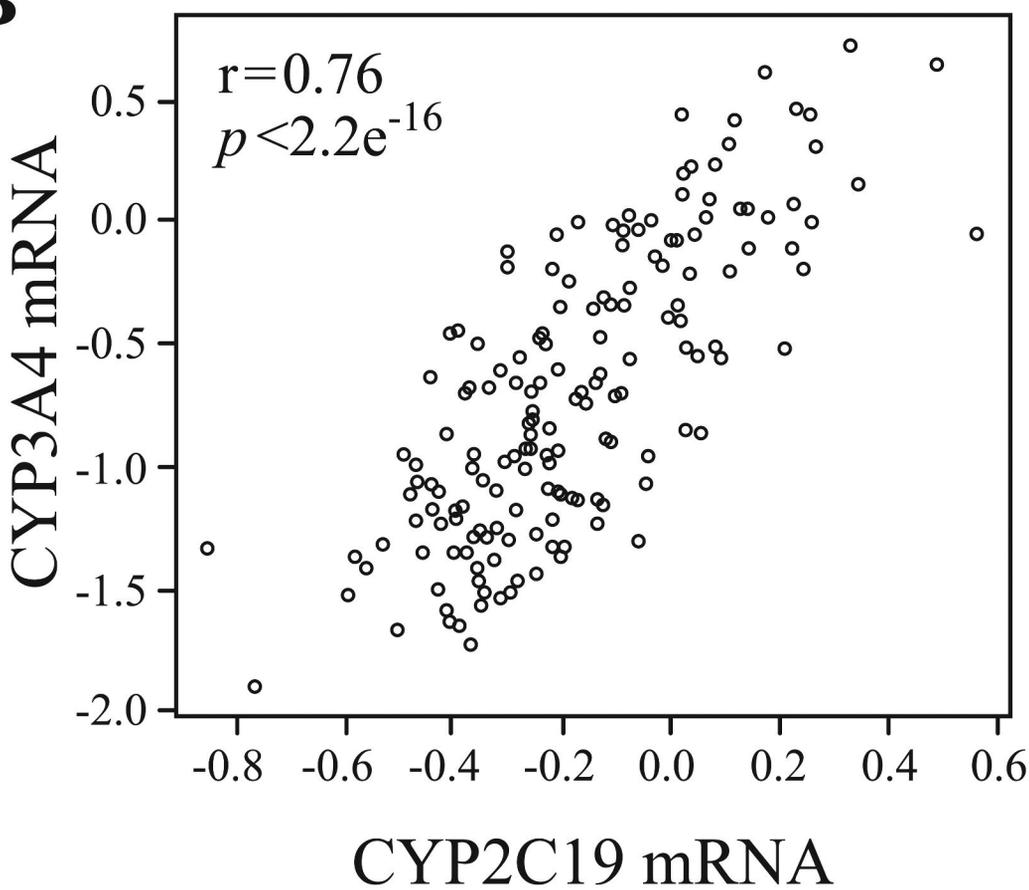
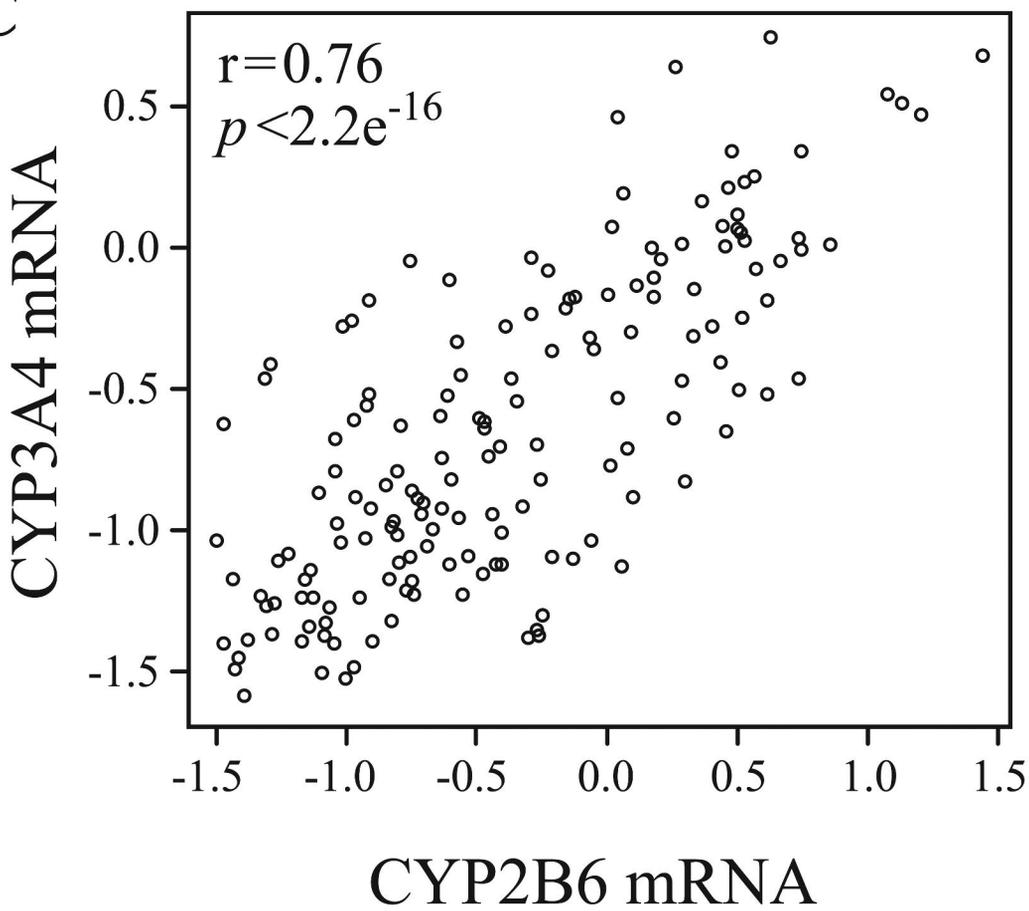
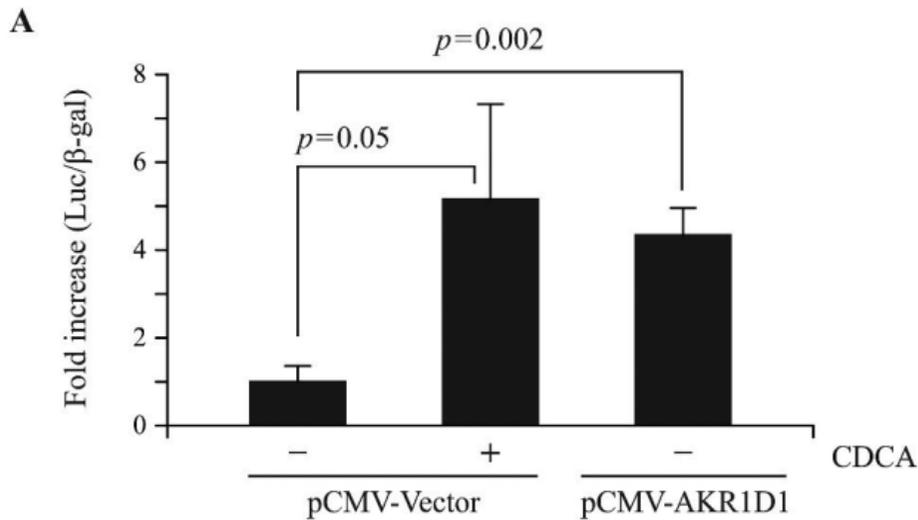
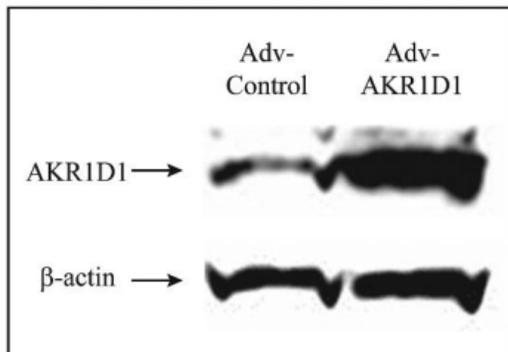
B**C**

Figure 2



B



C

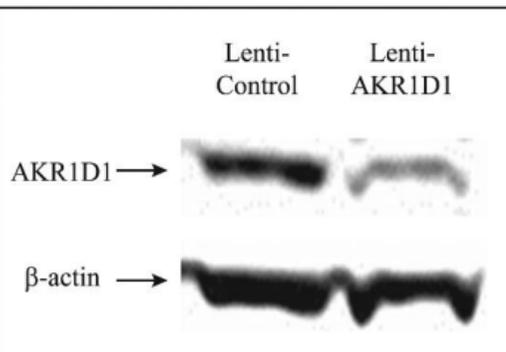


Figure 3

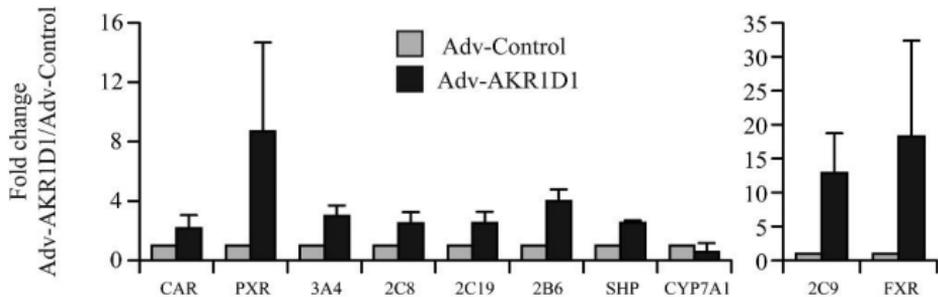
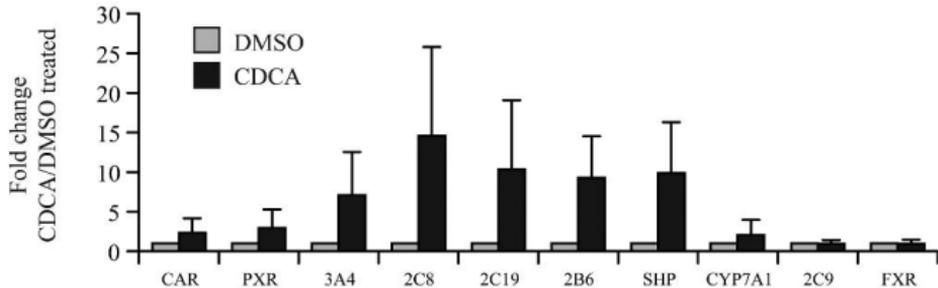
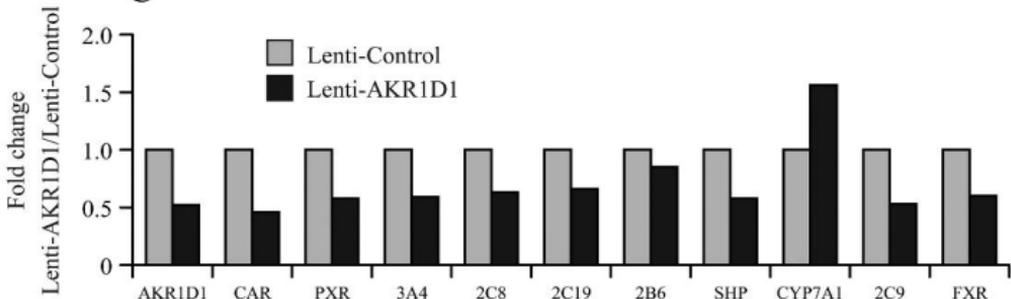
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Figure 4

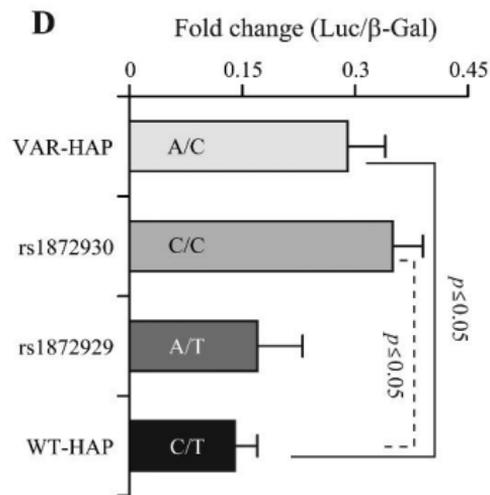
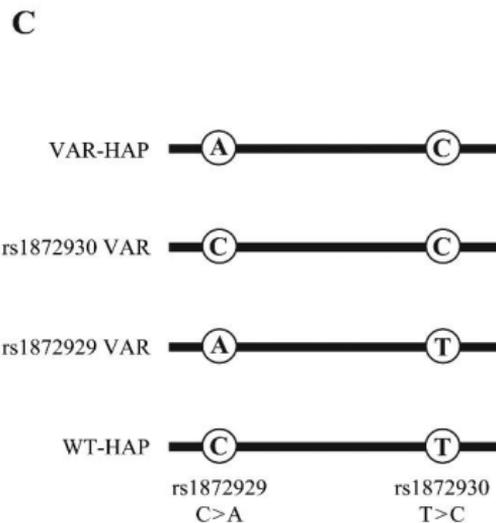
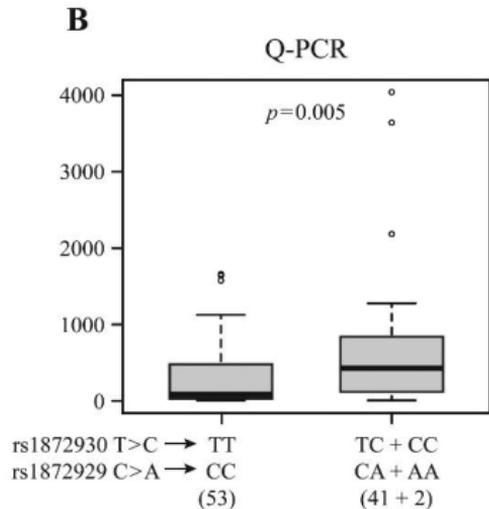
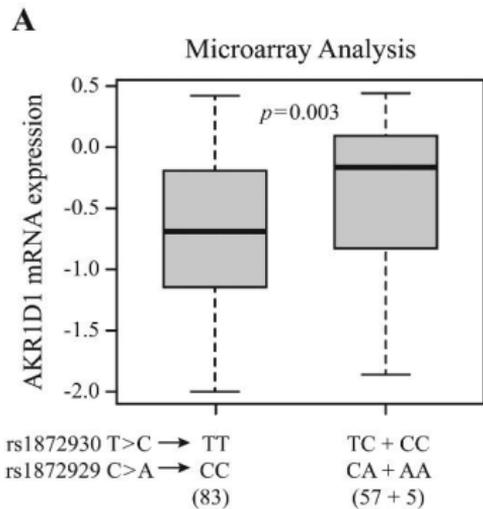


Figure 5

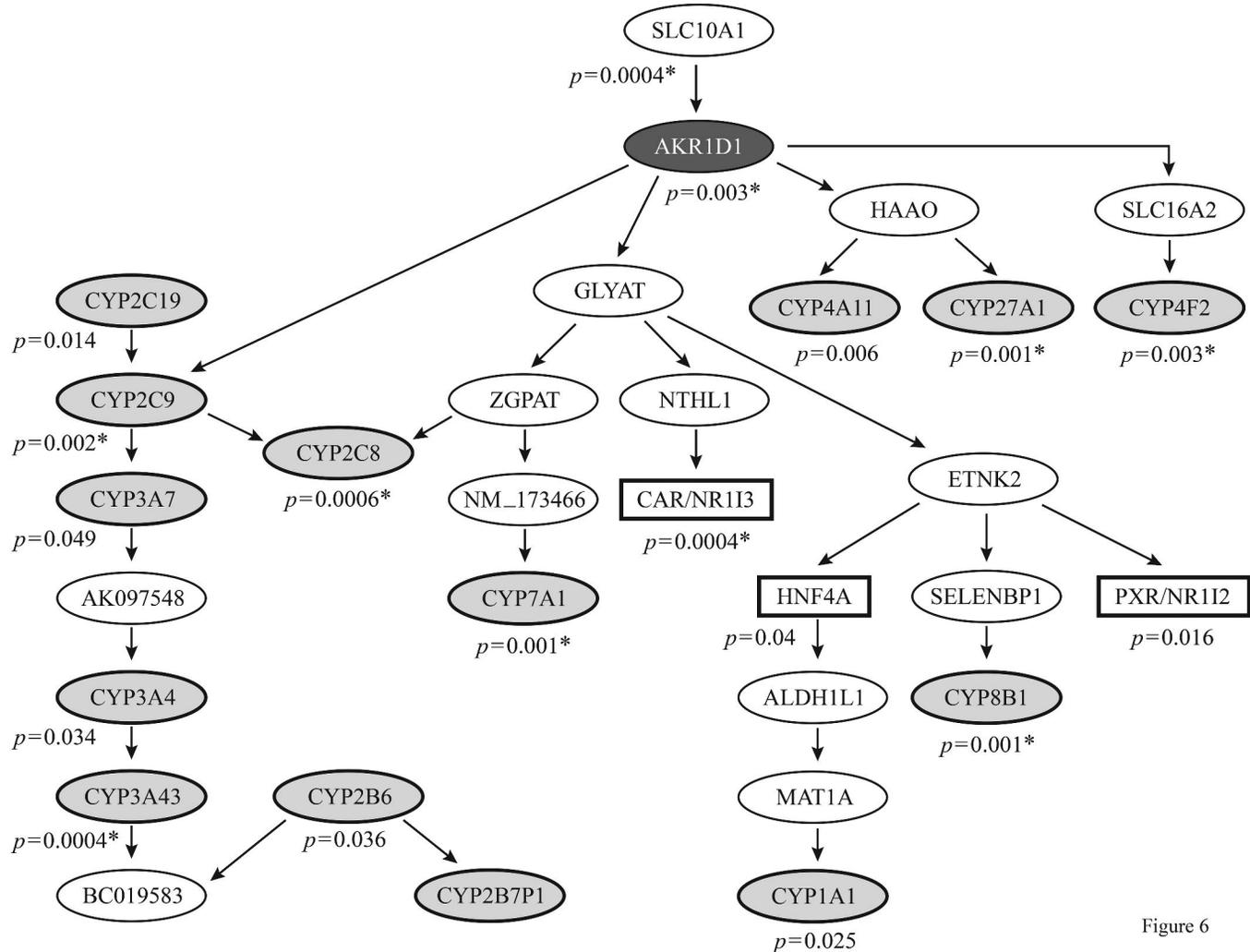


Figure 6

CYP activity (nmol/min/mg protein)

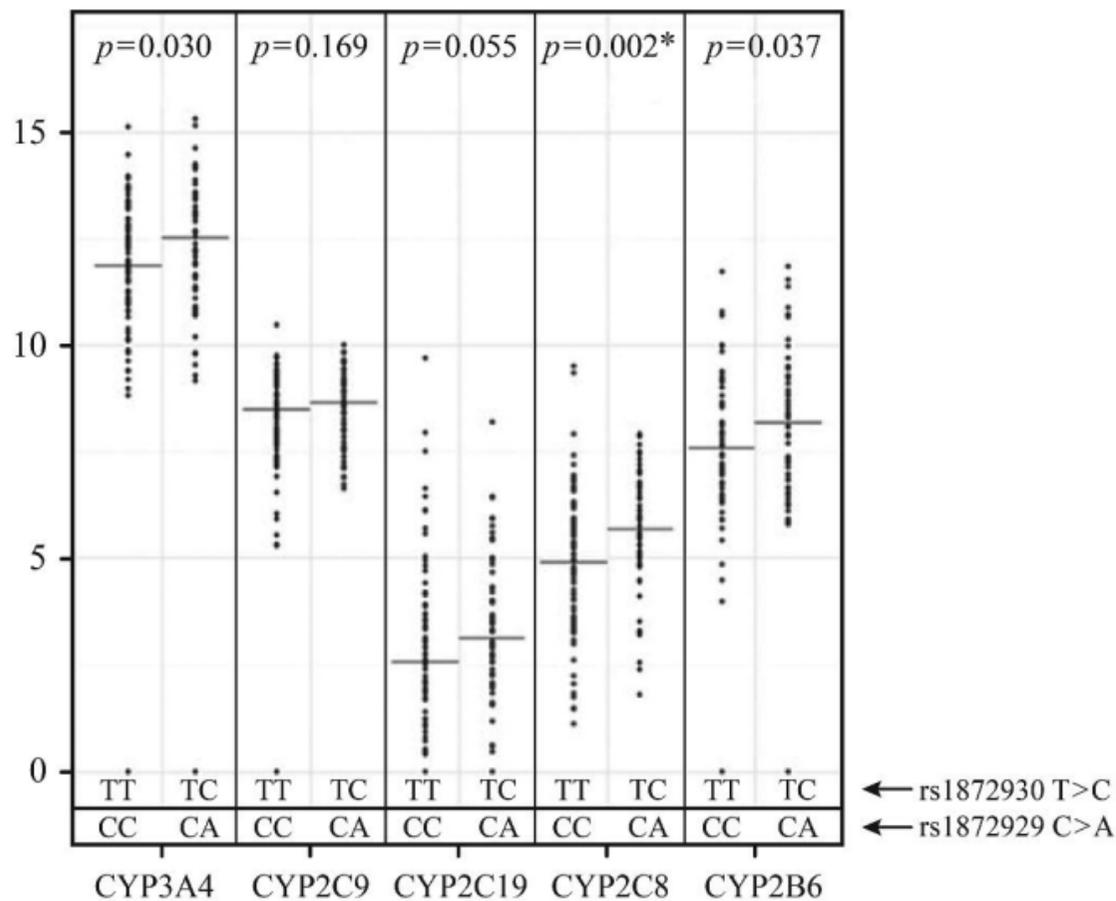


Figure 7

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

In the above article [Chaudhry AS, Thirumaran RK, Yasuda K, Yang X, Fan Y, Strom SC, and Schuetz EG (2013) *Drug Metab Dispos* **41**:1538–1547; doi:10.1124/dmd.113.051672], the funding information is missing.

The correct funding information is shown below:

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The online versions of this article have been corrected.

The author regrets this error and any inconvenience it may have caused.