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CYP2C9 promoter variable number tandem repeat polymorphism (pVNTR) regulates mRNA expression in human livers

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Running title: CYP2C9 promoter pVNTR polymorphism regulates gene expression

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Abbreviations: pVNTR, promoter variable tandem repeat; AEI, allelic expression imbalance; LD, linkage disequilibrium

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Abstract:

Cytochrome P450 2C9 (CYP2C9) is involved in metabolism of nearly 25% of clinically used drugs. Coding region polymorphisms *CYP2C9*2* and **3* contribute to inter-person variability in drug dosage and clinical outcomes, while the role of regulatory polymorphism remains uncertain. Measuring allelic RNA expression in 87 human liver samples, combined with genotyping, sequencing and reporter gene assays, we identified a promoter variable number tandem repeat polymorphism (pVNTR) that fully accounted for allelic CYP2C9 mRNA expression differences. Present in three different variant forms (short (pVNTR-S), medium (pVNTR-M) and long (pVNTR-L)), only the pVNTR-S allele reduced CYP2C9 mRNA level compared to the pVNTR-M (reference) allele. pVNTR-S is in linkage disequilibrium with *3, with LD R^2 of 0.53 to 0.75 in different populations. In patients who were taking maintenance dose of warfarin, the mean warfarin dose was associated with the copies of pVNTR-S (p=0.0001). However, in multivariate regression models that included the *CYP2C9**3, pVNTR-S was no longer a significant predictor of the warfarin dose (p=0.60). These results indicate that although pVNTR-S reduced CYP2C9 mRNA expression, the *in vivo* effects of pVNTR-S on warfarin metabolism cannot be separated from the effects of *3. Therefore, it is not necessary to consider pVNTR-S as an additional biomarker for warfarin dosing. Larger clinical studies are needed to define whether the pVNTR-S has minimal effect *in vivo*, or the effect attributed to *3 is really a combination of effects on expression by the pVNTR-S along with effects on catalytic activity from the non-synonymous *3 variant.

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Introduction

Cytochrome P450 2C9 (CYP2C9) metabolizes nearly 25% of clinically used drugs. Genetic variability in *CYP2C9* can exert robust effects on treatment outcomes with drugs displaying a narrow therapeutic index, including the commonly prescribed anticonvulsant phenytoin, anticoagulant warfarin, antidiabetic tolbutamide and glipizide, antihypertensive losartan, antidepressant fluoxetine, and the nonsteroidal anti-inflammatory drugs ibuprofen, diclofenac, and celecoxib (Davies *et al.*, 2000; Klose *et al.*, 1998; Miners *et al.*, 1998). The human gene encoding the CYP2C9 protein was mapped to chromosome 10q24.2 and spans over 55kb. Coding region polymorphisms in *CYP2C9* have been extensively studied, with more than 30 alleles identified (http://www.cypalleles.ki.se). The two clinically most important alleles, *CYP2C9*2* and *CYP2C9*3*, occur at minor allele frequency of ~10% and 6%, respectively, in Caucasian populations, but are less abundant or undetectable in individuals of African and Asian descent (Lee *et al.*, 2002). In contrast, other potentially important variants, *e.g.*, *CYP2C9*8, *9,* and **11*, occur in African population with frequencies of 3.6, 13 and 5.6%, respectively, while these alleles in turn are low or undetectable in other racial groups (Allabi *et al.*, 2004; Blaisdell *et al.*, 2004). Convincing evidence suggests that *CYP2C9*2* and *CYP2C9*3* alleles convey reduced enzyme activity and have been associated with drug dosage requirements and treatment outcomes (Aithal *et al.*, 1999; Higashi *et al.*, 2002; Lee *et al.*, 2002). Consequently, *CYP2C9* variants are listed as a candidate biomarker test in FDA's "Table of Pharmacogenomics Biomarkers in Drug Labels" for celecoxib and warfarin.

Genetic variability in CYP2C9 is not fully accounted for by the known coding region polymorphisms (Shintani *et al.*, 2001; Takahashi *et al.*, 2004). The clearance of warfarin varies

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twelve-fold (Scordo *et al.*, 2002), and the level of CYP2C9 protein expression six-fold in human liver microsomes in homozygous *CYP2C9*1/*1* carriers (Yasar *et al.*, 2001). Moreover, warfarin metabolism varied among individuals carrying different promoter haplotypes that do not contain *2 and *3 (Veenstra *et al.*, 2005). For example, haplotype *1D carriers required a significantly lower warfarin dose than reference *1/*1 allele carriers in a small subject group (Veenstra *et al.*, 2005), suggesting that regulatory polymorphisms exist in *CYP2C9*. To take full advantage of CYP2C9 as a genetic biomarker for drug therapy, it is important to consider the full complement of relevant polymorphisms.

Studies on regulatory polymorphisms in the *CYP2C9* promoter region affecting transcription (Kramer *et al.*, 2008; Shintani *et al.*, 2001; Takahashi *et al.*, 2004) have yielded inconsistent results (King *et al.*, 2004; Veenstra *et al.*, 2005). Specifically, reporter gene assays showed promoter SNP -4302C>T and haplotypes H3A and H3B (or pattern 6, containing -981G>A, -1537C>T, -1885C>G and -1911T>A) reduced constitutive promoter activity (Kramer *et al.*, 2008; Takahashi *et al.*, 2004), while -2663delTG and/or -3089G>A reduced PXR or phenytoin-mediated induction of *CYP2C9* promoter activity (Chaudhry *et al.*, 2010; Kramer *et al.*, 2008). However, it is unclear whether these polymorphisms or haplotypes affect CYP2C9 mRNA expression in human livers, since conclusions derived from reporter gene assays in transfected cells do not always consistent with *in vivo* gene expression and regulation. The purpose of this study was to determine the presence of any regulatory *CYP2C9* polymorphisms that would change the constitutive mRNA expression in human livers. Since the total mRNA level is strongly influenced by *trans*-acting factors confounding the effect of *cis*-acting polymorphisms, we measured allelic mRNA expression, that is, the relative amount of mRNA derived from each of the two alleles in the same individual. This cancels out *trans*-acting factors that would have

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the same effects on both alleles. Allelic RNA ratios significantly deviating from 1 (normalized by measured DNA ratios) demonstrates allelic expression imbalance (AEI), a strong indicator of *cis*-acting regulatory polymorphism(s). Then we used AEI as phenotypic trait to scan for functional polymorphism(s) that are responsible for observed AEI, as demonstrated previously (Wang *et al.*, 2010; Wang *et al.*, 2005). Using this approach, we re-evaluated the effects of previously identified polymorphisms on constitutive liver CYP2C9 expression, and searched for novel regulatory variants. We identified a variable number tandem repeat polymorphism (pVNTR) located 4kb upstream of the translation start site to be functional that affects constitutive CYP2C9 mRNA expression in human livers. We then assessed the clinical effect of this pVNTR polymorphism by testing the association of the pVNTR genotype with warfarin dose requirements in patients undergoing warfarin therapy.

Materials and Methods:

Tissue and DNA samples: One hundred and sixty eight human liver biopsy or autopsy samples were obtained from the Cooperative Human Network Midwestern and Western Division, under the approval by The Ohio State University Institutional Review Board (IRB). Since *CYP2C9* promoter polymorphisms can affect CYP2C9 inducibility (Chaudhry *et al.*, 2010; Kramer *et al.*, 2008), we excluded livers from individuals with known usage of CYP2C9 inducers (phenytoin, phenobarbital, ethanol, and carbamazepine, etc.). Four hundred and thirty DNA samples from patients who were taking sulfomethoxazole (SMX cohort), collected for another study (Wang *et al.*, 2011b), were also used in this study to determine the distribution of *CYP2C9* *2, *3 and the newly identified pVNTR-S. The study protocol was approved by The Ohio State University IRB.

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DNA and RNA preparation: Preparation of genomic DNA and RNA was performed as described (Pinsonneault *et al.*, 2004). cDNA was prepared using gene specific primers and oligo-dT as described (Wang *et al.*, 2010; Wang *et al.*, 2005).

Genotyping: Twenty eight SNPs were genotyped in liver genomic DNA using multiplex SNap-Shot assays or gene specific real-time PCR. The *CYP2C9* promoter pVNTR polymorphism was genotyped using PCR with fluorescently labeled primer, yielding three main amplicons of different lengths: long (pVNTR-L), medium (pVNTR-M reference sequence) and short (pVNTR-S). PCR conditions and sequence of primers are provided in Supplemental Table 1.

Promoter region sequencing: *CYP2C9* promoter region (6343 bp's upstream of translation start site, reference sequence NT_030059) was PCR amplified from two samples with allelic RNA ratios deviating from 1, showing significant AEI (L012 and L052), and two samples without AEI (L50 and L71). PCR products were purified and sequenced using primers shown in Supplemental Table 1. To sequence the promoter pVNTR polymorphism region, a fragment of ~1000bp surrounding the pVNTR was PCR amplified from 10 samples having different genotypes for *pVNTR* polymorphisms and cloned into pCR 2.1-TOPO vector, using TOPO cloning kit (Invitrogen, Carlsbad, CA). Clones were sequenced using PCR primers shown in Supplemental Table 1.

Quantitative analysis of allelic ratios in genomic DNA and mRNA using SNapShot: SNapShot assays were performed as described (Wang *et al.*, 2005). We used three marker SNPs, two in the coding region (rs1057911, A>T and rs9332242, C>G) and one in intron region (rs2298037, C>T), to detect allelic RNA expression of mRNA or heteronuclear RNA (hnRNA) as described for CYP3A4 (Wang *et al.*, 2010). All 168 livers were first genotyped for these marker SNPs, and

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then 87 samples heterozygous for at least one marker SNP were selected for measuring allelic RNA expression. The allelic RNA ratios were normalized to DNA ratios set to 1. Copy number variants (e.g., gene duplication) were not detectable in gDNA.

Reporter gene constructs: Luciferase reporter gene constructs containing a 4465bp promoter region (from 388355 to 392820 in NT_030059) were generated from two PCR-amplified fragments. Overlapping fragments of 1487bp (from 388355 to 389842 in NT_030059, 5P construct) and 3598bp (from 389221 to 392819 in NT_030059, 3P construct) were PCR amplified and cloned into pCR 2.1-TOPO vector, respectively. Both forward primers were tagged with X*hoI* site and reverse primers were tagged with N*coI* sites. Then, the 3P construct was cut with K*pnI* (located at pCR 2.1-TOPO vector) and N*deI* (located in an overlapping segment of 3P and 5P) and joined with the 5P construct cut with the same enzymes. The resulting 4465 bp promoter fragment was cut with X*hoI* and *NcoI* and cloned into pGL3 vector. Constructs containing different alleles were generated by PCR amplification from different individuals with certain haplotypes and exchanging the fragments representing the 5P or 3P portions. The inserted SNPs/haplotypes were confirmed by DNA sequencing and shown to be free of spurious *in vitro* mutations.

Reporter gene assay: Reporter gene constructs (1 µg) were transfected into HepG2 cells together with a transcription factor expression cocktail (HNF1α 50 ng + pCDB1 25 ng + HNF4α 50ng, co-transfected in all experiments) known to be essential for *CYP2C9* promoter activity (Kramer *et al.*, 2008). As an internal transfection control, a TK promoter-driven Renilla luciferase construct (TK-pRL, 50 ng) was co-transfected. Cells were harvested after 48 hours and luciferase activity measured with Dual-Glo luciferase assay (Promega) on a luminescence plate

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reader (PerkinElmer Life and Analytical Science, Waltham, MA). In some experiments, in addition to the transcription factor expression cocktail, we co-transfected 75 ng of other transcription factors (CAR, PXR, CEBPA or GATA4), or as a control, pcDNA3 empty vector, together with pGL3 fused reporter plasmids to test the effects of transcription factors on promoter activity. To test the effects of inducers, 50 µM phenytoin or 10 µM rifampicin were added to cells 24 hr post transfection and luciferase activity measured another 24hr after treatment. For each construct, we selected 3 clones for plasmid DNA preparation. Each experiment was repeated three times, each with triplicates.

Cell culture and transfection: HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were plated into 24-well plates a day before transfection. Transfection was performed by using LipoJet reagent (SignaGen, Ijamsville, MD) according to the manufacturer's protocol.

Transcription factor constructs: Full length cDNA of human hepatocyte nuclear factor 1α (HNF1α), HNF4α, constitutive androstane receptor (CAR) splice variant 1, CCAAT/enhancer binding protein alpha (CEBPA), pregnane X receptor (PXR) splice variant 1, and GATA4 were PCR amplified from human liver cDNA using primers in Supplemental Table 1. PCR products were cloned into pCR 2.1-TOPO vector using TOPO cloning kit, and confirmed by DNA sequencing. Then the cDNA fragments were subcloned into pCDNA3 vector for transfection. HNF1α cofactor PCBD1 (DCoH) was purchased from ThermoFisher Scientific (Huntsville, AL) and subcloned into pcDNA3 vector.

Clinical warfarin dosing study: To test the clinical effect of the promoter variable tandem repeat (pVNTR-S) polymorphism, we assessed the association of the pVNTR genotype with the

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maintenance warfarin dose required in patients undergoing stable warfarin anticoagulation therapy from University of Florida (n=348) (Aquilante *et al.*, 2006) and Cairo, Egypt (n = 207) (Shahin *et al.*, 2011). The relevant protocols were approved by the local institutional review boards.

Data analysis: Data are expressed as mean ± SD. Statistical analysis of allelic DNA and RNA ratios was performed using Prism (GraphPad Software, San Diego, CA). Correlations between AEI status (displaying AEI, allelic RNA ratio ≥1.25 or ≤0.8, or no AEI, allelic RNA ratio <1.25 and >0.8) and SNP heterozygosity were determined by calculating Kappa-coefficients using SPSS (SPSS Inc, Chicago, IL, USA). A Kappa coefficient of 1.0 indicates a perfect agreement between heterozygosity and displaying AEI (all heterozygotes for the SNP show AEI and all homozygotes show no AEI), which would indicate that the SNP itself or other polymorphism in completely LD with it, is responsible for the AEI. The significance of Kappa coefficients was calculated using Z-score with one-sided P value (kappa coefficient >0).

For analysis of the clinical study, the weekly warfarin maintenance dose was transformed using square root transformation to improve model fit. Different pVNTR genotypes (L/L, M/L, M/M, S/L, S/M, S/S) were combined into 0, 1 or 2 copies of the short allele (pVNTR-S). The contribution of pVNTR-S to the model predicting the weekly warfarin dose was assessed using linear regression. Stepwise selection method was used in multiple linear regression to evaluate the contribution of pVNTR-S, *CYP2C9**2 and *CYP2C9**3 to the warfarin dose requirement, using $p < 0.2$ and $p < 0.05$ as criteria for entering and staying in the model. Independent studies in Caucasians (Aquilante *et al.*, 2006), African Americans (Gage *et al.*, 2004), Asians (Zhao *et al.*, 2004) and Egyptians (Shahin *et al.*, 2011) have confirmed that both *CYP2C9**2 and *3 are functional variants that influence warfarin dose required to maintain the therapeutic INR.

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Therefore we performed combined analysis in all the warfarin patients. This is the approach that the International Warfarin Pharmacogenomics Consortium (IWPC) (Klein *et al.*, 2009) used to create the warfarin dosing algorithm. The linkage disequilibrium between pVNTR-S and *CYP2C9* *2 and *3 alleles was analyzed in Haploview software (Barrett *et al.*, 2005). All statistical analyses for the clinical association were performed in SAS (version 9.2 Cary, NC).

Results:

1. Allelic expression imbalance of CYP2C9 in human livers:

Allelic RNA expression of CYP2C9 was measured in 87 livers heterozygous for at least one of the three SNP markers including two exonic (SNP26 rs1057911 and SNP28 rs9332242) to measure mature mRNA, and one intronic (SNP25 rs2298037) to measure heteronuclear RNA as reported previously for CYP3A4 (Wang *et al.*, 2010). Thirteen samples showed allelic RNA ratios significantly different from 1 and were considered displaying allelic expression imbalance (AEI), with the major allele consistently associated with higher levels of mRNA than the minor allele (allelic RNA ratio = 1.38 ± 0.12 (mean ± SD), ranging from 25 to 60% in different samples). This result suggests the presence of *cis*-acting regulatory polymorphism(s) that affect mRNA level by influencing either RNA transcription or RNA processing.

Searching for polymorphisms responsible for observed AEI:

We genotyped 28 SNPs in samples with allelic RNA expression data, including promoter SNPs that have been reported to affect promoter activity, and coding region SNPs (Table 1), which could also affect mRNA stability and change RNA level as reported for MDR1 (Wang *et al.*, 2005) and OPRM1 (Zhang *et al.*, 2005), or modulate early hnRNA processing. Six SNPs

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were monomorphic in this population, due to low allele frequency in Caucasians (83 of 87 liver samples tested). The remaining 22 SNPs formed four haplotype blocks with high LD between block 1, block 2 and block 4 (Supplemental Figure 1). Two haplotypes containing completely linked SNPs (LD R^2=100%) were identified. Haplotype 1 (H1) contains nine SNPs: SNP2, 3, 4, 13, 14, 16, 18, 23, and 26; while haplotype 2 (H2) contains three SNPs: SNP7, SNP9 and SNP10. H1 and H2 are also in LD with each other, with R^2=0.79. We then correlated AEI status (displaying AEI or not displaying AEI) with heterozygosity of SNPs/haplotypes by calculating a Kappa coefficient. SNP7, SNP9 and SNP10 in haplotype 2 yielded the highest K coefficient (k=0.953, Table 2), with all 12 samples heterozygous for these three SNPs showing AEI (Supplemental Figure 2). However, one sample with AEI (L114) was homozygous for the major allele, suggesting that SNP7, SNP9 or SNP10 cannot fully account for the AEI and might not be responsible. H1 encompasses a previously identified haplotype (*3A and 3B, with SNP13, 14, 16 and 18) that has been associated with reduced *CYP2C9* promoter activity in reporter gene assays (Kramer *et al.*, 2008; Shintani *et al.*, 2001). However, the AEI data again failed to fully agree with heterozygosity of this haplotype (k coefficient = 0.915). Specifically, two samples heterozygous for H1 SNPs did not show AEI (L17 and L163, Supplemental Figure 2), arguing against H1 haplotype or any SNPs in H1 as causative factors. SNP6 was previously shown to reduce promoter activity in a reporter gene assay (Kramer *et al.*, 2008), but did not correlate with AEI status (k = -0.136) (Supplemental Figure 3A). SNP11 and SNP12 were shown to reduce rifampicin or phenytoin-induced promoter activity in reporter gene assays (Chaudhry *et al.*, 2010; Kramer *et al.*, 2008) and were associated with phenytoin maintenance dose (Chaudhry *et al.*, 2010), but they did not associate with AEI status (k coefficient 0.066 and 0.096 respectively) (Supplemental Figure 3B, 3C), indicating these two SNPs did not affect constitutive CYP2C9

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expression in livers, consistent with the earlier studies by Kramer et al and Chaudhry et al (Chaudhry *et al.*, 2010; Kramer *et al.*, 2008) that these variants do not affect constitutive CYP2C9 promoter activity, but only inducible activity

To search for the causative polymorphism, we sequenced > 6 kb of the promoter region in two samples with AEI and two samples without AEI. Besides known SNPs genotyped in this study and identified by Kramer et al (Kramer *et al.*, 2008), a region between nucleotide positions 388753 to 388890 in reference sequence NT0030059.13 (-3979bp upstream of translation start site) was found to contain a variable number tandem repeat polymorphism (pVNTR). To measure the length and distribution of pVNTR in liver samples, we PCR-amplified genomic DNA using a fluorescently labeled primer (Supplemental Table 1) followed by analysis on an ABI 3730 sequencer. This yielded a pattern of three fragment types varying in length: a short allele (pVNTR-S) ranging from 417bp to 438bp in length, medium allele with 446 – 488bp (pVNTR-M) and long allele with 512 – 522bp (pVNTR-L). Of 159 liver DNA samples tested, 62% samples were homozygous for pVNTR-M and 23% were heterozygous for pVNTR-M/pVNTR-L. Therefore, pVNTR-M was considered as reference allele.

Linkage disequilibrium analysis showed pVNTR-S is in high LD with H1 and H2, with LD R^2 of 0.87 and 0.93, respectively. All 13 samples displaying AEI were heterozygous for pVNTR-S, while all samples lacking AEI either did not carry the pVNTR-S or were homozygous for pVNTR-S (Supplemental Figure 2). K coefficient analysis showed a complete agreement between heterozygosity of the pVNTR-S and displaying AEI (K=1, Table 2). This result strongly suggests that pVNTR-S is the causative polymorphism and responsible for allelic

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CYP2C9 mRNA expression imbalance. In contrast, pVNTR-M and pVNTR-L alleles showed similar RNA level and did not display AEI (Supplemental Figure 3D).

Structure of the promoter variable number tandem repeat polymorphism in CYP2C9

After PCR amplification of the pVNTR region from 10 individuals and TOPO cloning the PCR products into pCR 2.1-TOPO vector, 16 clones representing variable lengths of pVNTR-S, pVNTR-M and pVNTR-L alleles were sequenced. As shown in Figure 1, pVNTR-S, pVNTR-M and pVNTR-L alleles not only have different lengths, but have distinct motif patterns (nTGnTAnTG(or CA)nTA(+/-CG)). pVNTR-L contains 4 motif copies, pVNTR-M has two copies (the first and third motifs are deleted), while pVNTR-S contains only the most 3' motif common to all three alleles (Figure 1). All four motifs have variable lengths in different individuals due to variable numbers of dinucleotide repeats (TG 1 to 4 and TA 5 to 9, CG being always 1). Moreover, there is a variable number of poly A and TA stretch preceding this repeat region (ranging from 11-31 'A's and 9-13 'TA's in the clones sequenced). Because of the variable length of the preceding poly A and TA stretch and variable lengths of the motifs, all three alleles show variable lengths in different individuals with pVNTR-L ranging from 168bp to 178bp, pVNTR-M from 107bp to 138bp and pVNTR-S from 79bp to 90bp.

2. *Promoter activity of pVNTR polymorphism with reporter gene assays:*

Since pVNTR-S allele completely accounts for the observed AEI results (reducing allelic mRNA levels 38% compared to the pVNTR-M or pVNTR-L alleles), we tested all three pVNTR alleles on *CYP2C9* promoter activity using reporter gene assays. A *CYP2C9* fragment of 4465bp upstream of the translation start site was PCR amplified and cloned into pGL3 reporter gene

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vector upstream of fly luciferase cDNA. To test for interactions between the pVNTR-S and previously reported SNPs or haplotypes, different reporter gene plasmids were constructed containing pVNTR-S, pVNTR-M or pVNTR-L alleles in combination with other SNPs or haplotypes as they occur in the 4465bp region of the test subjects (Table 3). Shown in Figure 2, in the presence of transcription factor expression cocktail (HNF1α 50 ng + pCDB1 25 ng + HNF4α 50ng), reporter plasmids containing pVNTR-S in any combination with other SNPs or haplotypes all displayed lower basal promoter activity compared to the reference construct (M1), while there were no difference between pVNTR-M and pVNTR-L alleles in any combination with other SNPs or haplotypes.

Testing the effect of enzyme induction, in additional to transcription factor expression cocktail, co-transfection of CAR, PXR and GATA4 but not CEPBA increased reporter gene activity across all constructs, consistent with earlier study by Chen et al (Chen *et al.*, 2005) with HNF4a, CAR and PXR. However, only pVNTR-S allele maintained lower promoter activity compared to pVNTR-M allele (Figure 3) to the same extent as observed with basal promoter activity (in the presence of transcription factor expression cocktail). This result indicates that the reduced promoter activity of pVNTR-S is not caused by the lack of these transcription factors known to regulate CYP2C expression in the cell transfection system. We next tested whether the pVNTR-S allele affects promoter activity after drug induction. CAR or PXR co-transfected cells were treated with phenytoin or rifampicin for 24 hrs before luciferase activity was measured. Phenytoin and rifampicin generally increased promoter activity 1.7 to 2 fold over basal activity, but the induction ratio did not differ between pVNTR-S and pVNTR-M alleles after treatment with either phenytoin or rifampicin (Figure 4). In contrast, the reporter plasmid containing minor

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alleles of SNP11 and SNP 12 displayed lower induction after CAR/phenytoin but not PXR/rifampicin treatment (Figure 4). *Allele frequency of pVNTR-S*:

Since pVNTR-S is highly linked to H1, which contains the known loss-of-function allele **3*, we tested the allele frequency of *pVNTR-S* allele, **3* and *2 in DNA samples from a total of 804 Caucasians (146 from the liver cohort, 334 from the SMX cohort, 324 from UF warfarin study), 120 African Americans (96 from SMX cohort and 24 from UF warfarin study) and 207 Egyptians (Egyptian warfarin study). Of 72 pVNTR-S carriers, 59 subjects were similarly heterozygous or homozygous for **3*, indicating that most of the *pVNTR-S* carriers also carry **3*. However, the *pVNTR-S* is not always present concordantly with *3, and in African Americans pVNTR-S (5.1%) occurs at slightly higher allele frequency than *3 (2.5%) (Table 4). Two *pVNTR-S* allele homozygous and 9 heterozygous carriers did not carry **3*, while eleven *pVNTR-S* allele homozygotes were heterozygous for **3*, and six **3* heterozygotes and two *3 homozygotes did not carry the *pVNTR-S* allele, indicating incomplete concordance between **3* and *pVNTR-S*. The LD (R^2) between **3* and *pVNTR-S* is 0.75 for Caucasians, 0.53 for African Americans and 0.59 for Egyptians. In contrast, there is no LD between *2 and pVNTR-S in all cohorts (LD R^2=0). Since **3* reduces CYP2C9 enzyme activity and *pVNTR-S* reduces mRNA expression, it is possible that pVNTR-S may contribute to the *in vivo* effects attributed to *3 when they are co-exist.

Clinical significance of promoter short allele:

The clinical significance of the promoter pVNTR-S was tested by association of pVNTR-S with weekly warfarin maintenance dose in patients undergoing stable warfarin anticoagulation therapy (Aquilante *et al.*, 2006; Shahin *et al.*, 2011). pVNTR genotype was

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determined in 453 patients. The mean weekly warfarin dose was 38.2 ± 16.9, 30.7± 12.7 and 21.0 ± 7.7 mg/week for 0, 1 and 2 copies of short alleles, respectively (p =0.0001). Univariate linear regression showed that the short allele was a significant predictor for warfarin dose (p = $3.1*10^{-6}$, R^2 = 0.047, as was the CYP2C9*3 allele (p = $5.7*10^{-7}$, R^2 = 0.054 and CYP2C9*2 allele (p = 0.0027, R^2 = 0.0199). However, in multivariable regression models that included the CYP2C9*3 allele, pVNTR-S was no longer a significant predictor of the warfarin dose (p = 0.60). When the genotypes of CYP2C9*3 and pVNTR-S were considered together, the effect of pVNTR-S alone was not significant in the absence of CYP2C9*3 (Figure 5). On the other hand, the CYP2C9*2 allele did not affect the association of warfarin dose with pVNTR-S. When all three variants were evaluated together in the multiple regression model, the only significant predictors were CYP2C9*3 which explained 5.4% of the variance and CYP2C9*2 which explained 1.3% of variance in the warfarin dose required, while pVNTR-S was not an independent predictor.

Discussion:

In this study, we have identified and characterized a regulatory promoter/enhancer pVNTR, located nearly 4kb upstream of the translation start site that fully accounts for differences in the allelic CYP2C9 mRNA constitutive expression in human livers. This pVNTR consists of tandem-repeat motifs grouped into three alleles according to length and structure: short (*pVNTR-S*), medium (*pVNTR-M*) and long (*pVNTR-L*) alleles. *pVNTR-S* is associated with 25-60% (allelic RNA ratio = 1.38 ± 0.12, mean ± SD) reduced CYP2C9 mRNA expression, compared to *pVNTR*-M or *pVNTR-L*. However, because pVNTR-S is in high LD with the known loss-of-function allele *CYP2C9*3*, the *in vivo* effects of pVNTR-S on warfarin metabolism cannot be fully assessed in this study.

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Several *CYP2C9* promoter SNPs/haplotypes have been identified previously (Kramer *et al.*, 2008; Shintani *et al.*, 2001; Takahashi *et al.*, 2004), but none of them fully accounts for the observed allelic RNA expression in this study. This finding argues against these variants directly modulating constitutive CYP2C9 mRNA expression in human livers. In support of the allelic RNA expression results, reporter gene assays confirmed that only the *pVNTR-S* allele significantly reduces basal promoter activity, consistent with a ~38% allelic expression imbalance observed in human livers. Sequencing of 6kb upstream *CYP2C9* promoter region in two samples showing AEI and two samples lacking AEI did not reveal any other polymorphisms that associate with AEI. Since the *pVNTR-S* allele is in high LD with promoter haplotype containing 981G>A, -1537C>T, -1885C>G and -1911T>A (previously identified as *3A/*3B), it is possible that previously used reporter gene constructs inadvertently contained the *pVNTR-S allele* (Kramer *et al.*, 2008). To determine whether the *pVNTR-S* allele interacts with other SNPs/haplotypes, we tested the promoter activity of *pVNTR-S* in the context of other naturally occurring SNPs/haplotypes. The *pVNTR-S* allele consistently reduced promoter activity regardless of the presence or absence of other SNPs, indicating that the pVNTR-S allele is active independently. Overall, the *pVNTR-S* allele appears to be the only functional polymorphism present within the 6kb upstream promoter region that reduces constitutive CYP2C9 mRNA expression, at least in a Caucasian population, since a majority of our liver samples were from Caucasians. Because of ethnic differences in the frequency of *CYP2C9* variants and haplotypes, we cannot rule out the possibility that other regulatory polymorphisms exist in Asian and African populations, while none was detected in Caucasians (Lee *et al.*, 2010; Perera *et al.*, 2011).

The molecular mechanisms underlying *pVNTR-S* regulation of CYP2C9 mRNA expression remains to be resolved. Promoter variable tandem repeat polymorphisms can regulate

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gene expression *via* different ways: 1. Altering transcription factors or other protein binding sites, inhibiting or promoting gene expression; 2. changing DNA structure, such as varying the spacing between functional motif or altering the structure and melting properties of DNA in their proximity (Contente *et al.*, 2002; Lesch *et al.*, 1994). VNTRs have been shown frequently to affect gene expression (Albanese *et al.*, 2001; Amador *et al.*, 2004; Borrmann *et al.*, 2003; Donninger *et al.*, 2004; Okada *et al.*, 2006) and have been associated with disease risk (Heshmati *et al.*, 2009; Zarif Yeganeh *et al.*, 2010). Using Mfold calculations, we found that the pVNTR-S allele has a secondary structure of reduced complexity than pVNTR-M and pVNTR-L alleles, indicating that pVNTR-S may alter DNA structure necessary for transcription factors or other regulatory protein binding. Alternatively, use of the Matinspector program (Genomatix software suite) to search for transcription factors binding sites revealed that the pVNTR-M and pVNTR-L alleles contain a putative hypoxia inducible factor binding site (tatatata<u>CGTG</u>tgtg) located at the junction of the second and third motif, which is deleted in the pVNTR-S allele. This requires further investigation.

Because of the high LD between **3* and *pVNTR-S*, the independent *in vivo* effect of pVNTR-S may be limited and cannot be fully evaluated in this study, at least in the ethnic groups studied. Since the occurrence of *pVNTR-S* and **3* is not completely concordant, *pVNTR-S* may reduce *in vivo* drug metabolism independent of **3*, especially in populations where *3 and *pVNTR-S* are in relatively low LD (African Americans). Detecting an independent effect of *pVNTR-S* from *3 will require testing in a larger cohort. In 2005, Veenstra *et al.* (Veenstra *et al.*, 2005) reported that promoter haplotype *CYP2C9* *1D was significantly associated with decreased warfarin dose requirement, time to stable dosing, and time to bleeding event. Comparing the SNPs in **1D* with the *pVNTR-S* allele haplotype lacking the**3 allele*, both

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contain the minor allele of SNP rs61886769 (SNP10), which is a part of H2 we have determined here to be in high LD with pVNTR-S. Therefore, *1D may have affected warfarin dosage requirements by inclusion of *pVNTR-S*. Moreover, *CYP2C9*3* reduces enzyme activity in a substrate-specific fashion, with over tenfold differences in either Km or Vmax for different substrates, tested in a CYP2C9 yeast expression system (Takanashi *et al.*, 2000), suggesting variable effects of **3* with different substrates. Any independent effect of pVNTR-S would become more apparent with substrates that have less reduction of intrinsic clearance caused by coding SNP **3*.

In summary, the promoter variable number tandem repeat polymorphism identified here regulates CYP2C9 mRNA expression. Since the reduction in mRNA level is moderate and *pVNTR-S* is in high LD with the loss-of-function *CYP2C9*3* allele, the independent *in vivo* effect of pVNTR-S on warfarin metabolism appears to be limited. Thus, pVNTR-S is not considered as an additional biomarker for warfarin dosing. The independent *in vivo* effects of *pVNTR-S* on metabolism of other substrates requires further investigation. We also confirm the previously proposed reduction of CYP2C9 inducibility caused by the minor alleles of SNP11 and SNP 12 (Chaudhry *et al.*, 2010; Kramer *et al.*, 2008). Taken together, this study provides a comprehensive analysis of regulatory variants in *CYP2C9* promoter region that can serve as a guide for assessing regulatory genetic factors in CYP2C9 with potential impact on drug therapy.

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Authorship contributions:

Participated in research design: Wang, Johnson, Khalifa and Shahin

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Performed data analysis: Wang, Gong and Gawronski

Wrote or contributed to the writing of the manuscript: Wang, Gong and Johnson

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Figure legends:

Figure 1. Representative DNA sequence (A) and the motifs structure (B) of the long, medium and short alleles of promoter variable number tandem repeat (pVNTR) polymorphism in CYP2C9.

Figure 2. Effects of pVNTR-S, pVNTR-M and pVNTR-L alleles on constitutive CYP2C9 promoter activity. HepG2 cells were transfected with reporter gene constructs containing pVNTR-M, pVNTR-M or pVNTR-S in combination with other SNPs/haplotypes in naturally occurring combinations, as shown in Table 3. Luciferase activities were measured 48 hr post transfection. Data are mean ± SD, n=3. Compared to reference construct M1, * P<0.05, ANOVA with Dunnett post-hoc test.

Figure 3. Co-transfection of transcription factors did not alter pVNTR-S allele effects on CYP2C9 promoter activity. HepG2 cells were co-transfected with reporter gene constructs containing pVNTR-M, pVNTR-L or pVNTR-S allele, in combination with other SNPs/haplotypes as shown in Table 3, together with different transcription factors (CAR, PXR, GATA4 or CEPBA). Luciferase activities were measured 48 hr post-transcription. Data are mean ± SD, n=3. Compared to reference construct M1, ** P<0.01, ANOVA with Dunnett post-hoc test.

Figure 4. Effects of CAR-phenytoin or PXR-rifampicin-mediated induction on pVNTR-S modulation of CYP2C9 promoter activity. HepG2 cells were co-transfected with reporter gene constructs containing pVNTR-M, pVNTR-L or pVNTR-S allele, in the presence or absence of other SNPs/haplotypes, together with transcription factors CAR or PXR. Cells were then treated

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with either 50 μM phenytoin or 10 μM rifampicin, respectively, at 24 hours post-transfection, and luciferase activities measured another 24 hrs after treatment. Data are mean ± SD, n=3. Compared to reference construct M1, * $P<0.05$, ANOVA with Dunnett post-hoc test.

Figure 5. Weekly warfarin dose (mg/week) for each combination of pVNTR-S and CYP2C9*3 allele.

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Table1. CYP2C9 polymorphisms genotyped in liver samples. The translation start site A (in ATG) is taken as position 1.

SNP#	rs#	position	Gene region	Nucleotide or amino acid changes	Note
1		-8897	promoter	T>C	
2		-7419	promoter	A>G	link to *3, H1
3		-5813	promoter	A>G	link to *3, H1
4		-5661	promoter	C>A	link to *3, H1
5	rs74150723	-4877	promoter	G>A	
6	rs12251841	-4302	promoter	C>T	
7		-3849	promoter	G>A	H2
8	rs61886768	-3597	promoter	A>G	
9		-3579	promoter	G>A	H2
10	rs61886769	-3360	promoter	T>C	H2
11	rs12782374	-3089	promoter	G>A	
12	rs71486745	-2663	promoter	del GT	
13	rs9332092	-1911	promoter	T>C	link to *3, H1
14	rs9332093	-1885	promoter	C>G	link to *3, H1
15	rs9332096	-1565	promoter	C>T	monomorphic
16	rs61604699	-1537	promoter	G>A	link to *3, H1
17	rs4918758	-1188	promoter	T>C	
18	rs9332098	-981	promoter	G>A	link to *3, H1
19	rs1799853	cDNA 430	exon 3	C>T, R144C, *2	
20	rs7900194	cDNA 449	exon 3	G>A, R150H, *8	monomorphic
21	rs9332131	cDNA 818	exon 5	del A, frame shift	monomorphic
22	rs28371685	cDNA1003	exon 7	C>T, R335W, *11	monomorphic
23	rs1057910	cDNA1075	exon 7	A>C, I359L, *3	H1
24	rs28371686	cDNA1080	exon 7	C>G, D360E, *5	monomorphic
25	rs2298037		intron 8	C>T	marker SNP
26	rs1057911	cDNA1425	exon 9	A>T, G457G	marker SNP, link to *3, H1
27	rs9332239	cDNA1465	exon 9	C>T, P489S, *12	monomorphic
28	rs9332242	cDNA1581	exon 9	C>G, 3'UTR	marker SNP

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Table 2. Correlation between SNP heterozygosity and AEI positive status (allelic RNA ratio ≥1.25). K coefficient of 1 indicates a perfect agreement.

SNP #	K coefficient	p value	Haplotype
SNP1	0.869	<0.0001	
SNP2, SNP3, SNP4, SNP13, SNP14, SNP16, SNP18, SNP23, SNP26	0.915	<0.0001	H1
SNP5	0.876	<0.0001	
SNP6	-0.136	0.9719	
SNP7, SNP9, SNP10	0.953	<0.0001	H2
SNP8	-0.291	0.999	
SNP11	0.066	0.549	
SNP12	0.096	0.366	
SNP17	-0.067	0.972	
SNP19	-0.296	0.999	
SNP25	-0.270	0.998	
SNP28	-0.314	0.999	
pVNTR-S	1	<0.0001	

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Table 3. Reporter gene constructs

Construct	Nucleotide changes
M1	pVNTR-M, reference sequence
M2	pVNTR-M, -1911T>C, -1885C>G, -1537C>T, -1188T>C, -981G>A
M3	pVNTR-M, -3089G>A, -2663delTG
M4	pVNTR-M, -4302C>T
L1	pVNTR-L
L2	pVNTR-L, -1911T>C, -1885C>G, -1537C>T, -1188T>C, -981G>A
L3	pVNTR-L, -3089G>A, -2663delTG
S1	pVNTR-S
S2	pVNTR-S, -1911T>C, -1885C>G, -1537C>T, -1188T>C, -981G>A
S3	pVNTR-S, -3089G>A, -2663delTG

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Table 4. Minor allele frequency of CYP2C9 *3, *2 and promoter pVNTR alleles. The LD R^2 refers to the pVNTR-S and *3, determined in different cohorts. The LD R^2 between *2 and pVNTR-S is zero in all cohorts. The SMX cohort was used in another study in which patients were taking sulfomethexazole (SMX) as described in method section.

		Minor allele frequency					n	**LD R^2**
		***3**	***2**	**pVNTR-S**	**pVNTR-M**	**pVNTR-L**		
Caucasian	**Liver cohort**	**0.048**	**0.154**	**0.062**	**0.778**	**0.160**	**146**	**0.76**
	SMX cohort	**0.052**	**0.145**	**0.054**	**0.799**	**0.147**	**334**	**0.69**
	Warfarin cohort	**0.048**	**0.120**	**0.062**	**0.777**	**0.161**	**324**	**0.79**
	Combined	**0.050**	**0.137**	**0.058**	**0.789**	**0.152**	**804**	**0.75**
African American	**SMX cohort**	**0.031**	**0.036**	**0.052**	**0.784**	**0.164**	**96**	**0.58**
	Warfarin cohort	**0**	**0.042**	**0.045**	**0.954**	**0**	**24**	**na**
	combined	**0.025**	**0.038**	**0.051**	**0.883**	**0.065**	**120**	**0.53**
Egyptian cohort		**0.092**	**0.112**	**0.115**	**0.780**	**0.100**	**207**	**0.59**

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A

Long: 168-178bp

AAAAAAAAAAAAATATATATATATATATATATATATGTGTATATATATATATGTATATATATATATATATATGTGTATAT
ATATATATGTATATATATATATATATACGTGTGTGTATATATATATGTATATATATATATATATACGTGTGTGTATATATAT
ATATACATATATATA

Medium: 107-138bp

AAAAAAAAAAAAAAAAATATATATATATATATATATATATATGTGTATATATATATATATGTGTATATATATATATATATAT
ATATATACGTGTGTGTATATATATATATATACATATATATA

Short: 78-90bp

AAAAAAAAAAAAAAAAAAAAAAAAAGTATATATATATATATATATATATATATGTGTGTGTATATATATATATATATATAC
ATATATATA

B

Long :	nA nTA	1/2TG nTA 1TG nTA	2TG nTA 1/2TG nTA 1CG	3TG nTA 1TG nTA 1CG	3/4TG nTA 1CA 4TA
Medium:	nA nTA		2TG nTA 1/2TG nTA 1CG		3/4TG nTA 1CA 4TA
Short :	nA nTA 1G				3/4TG nTA 1CA 4TA

Figure 1.

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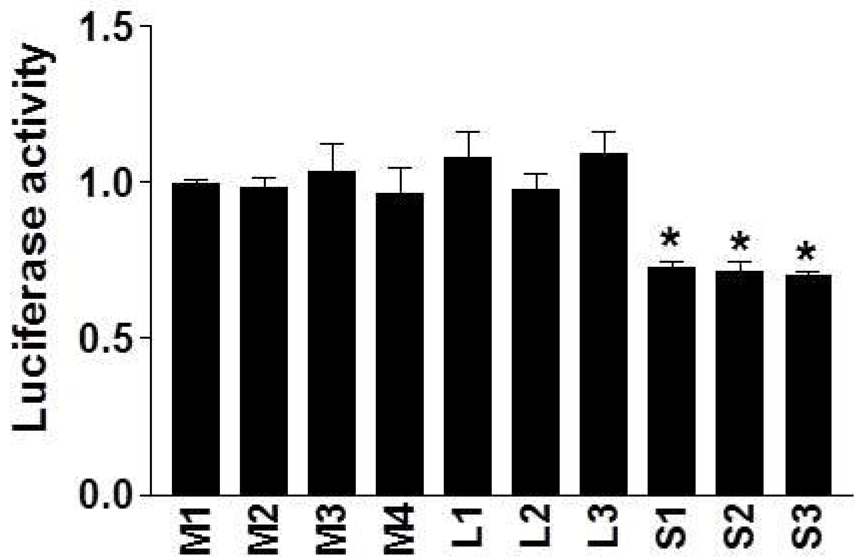

Figure 2

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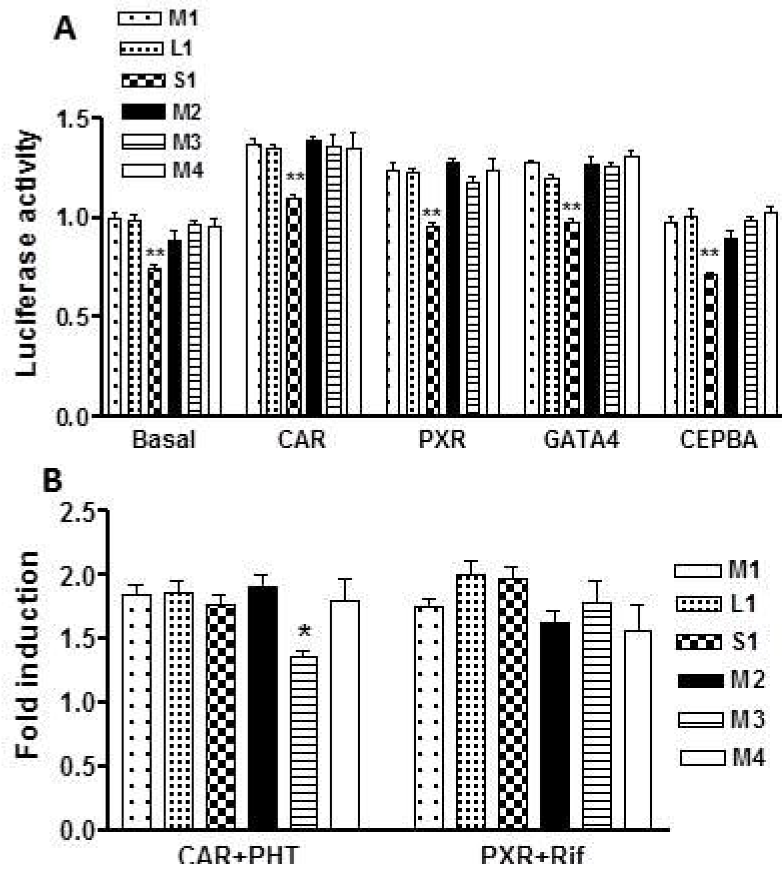

Figure 3

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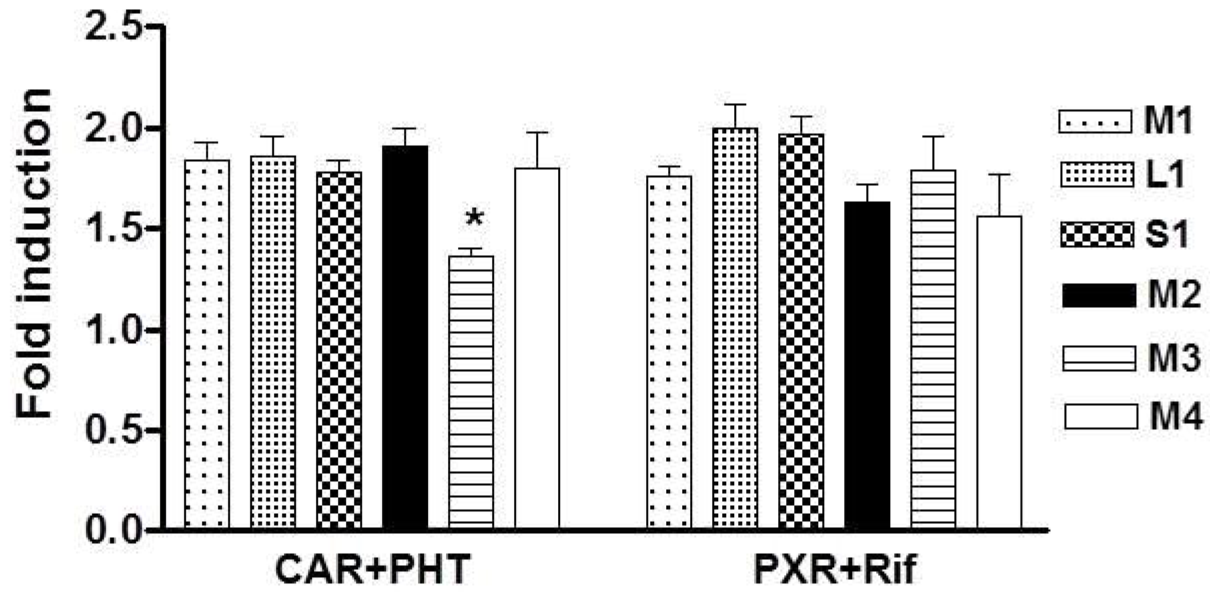

Figure 4

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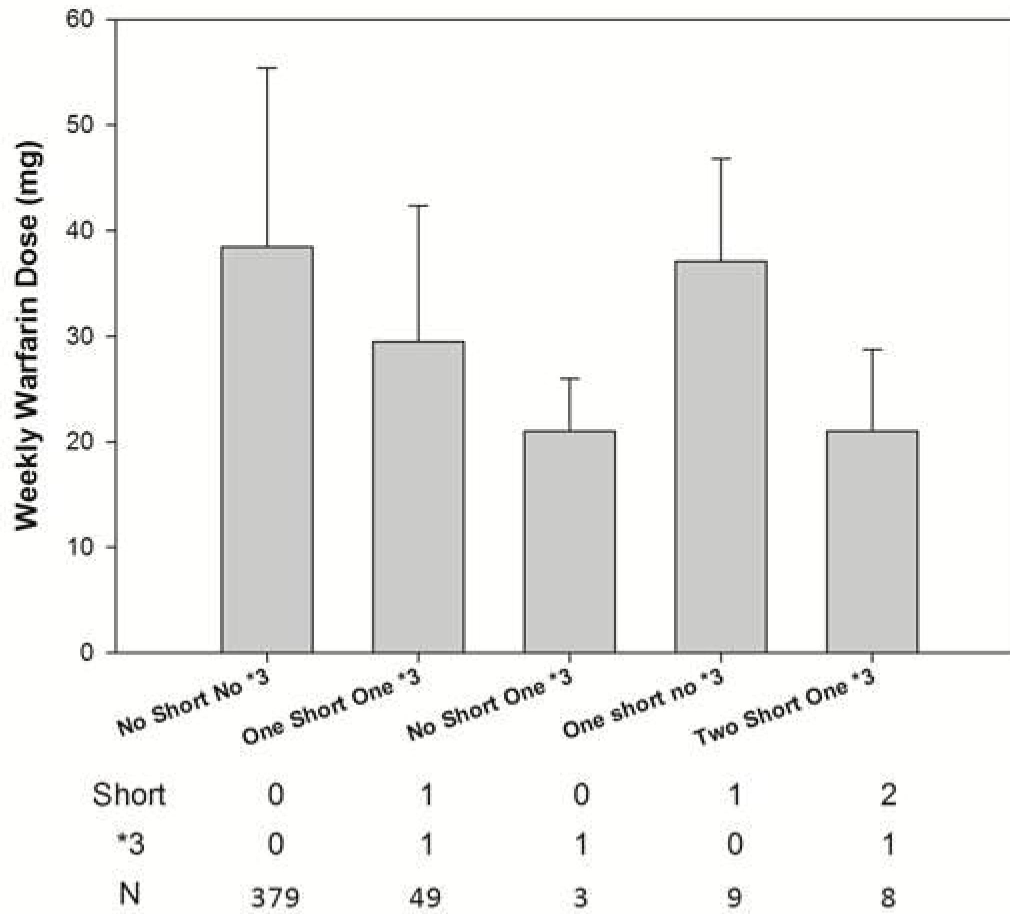

Figure 5

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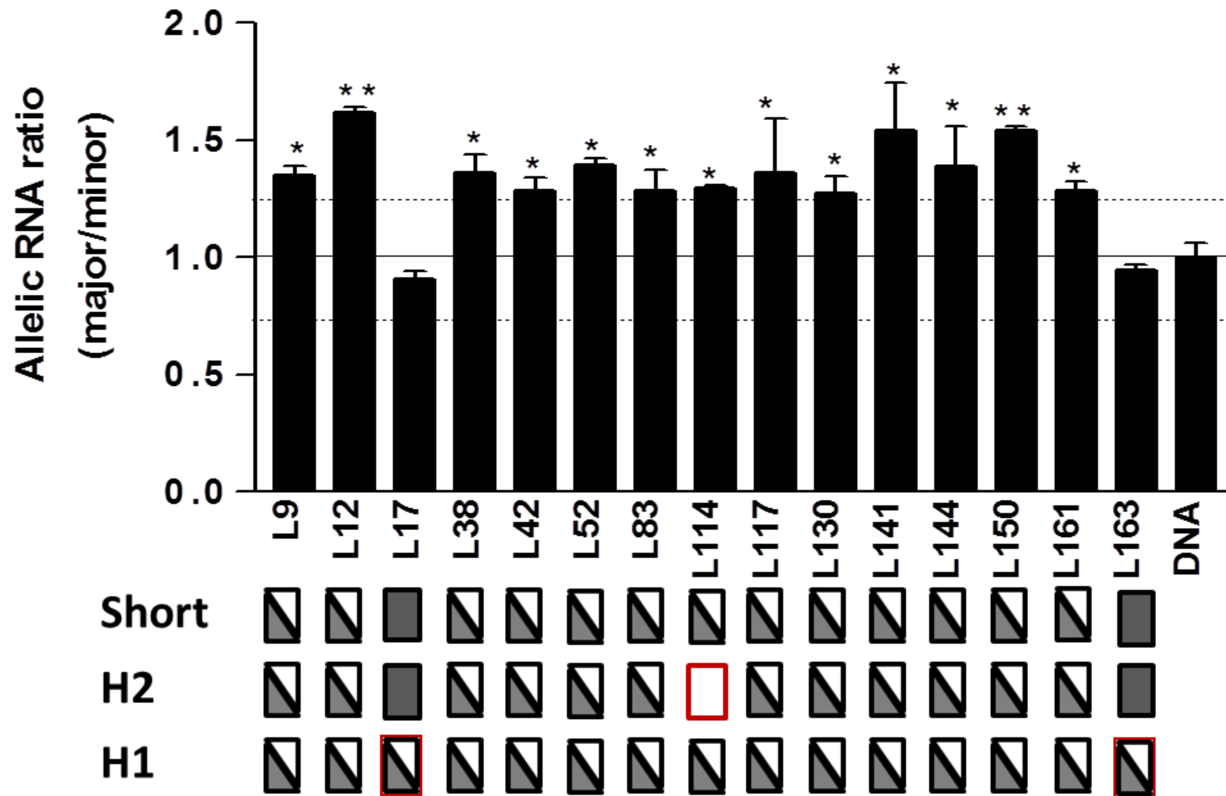
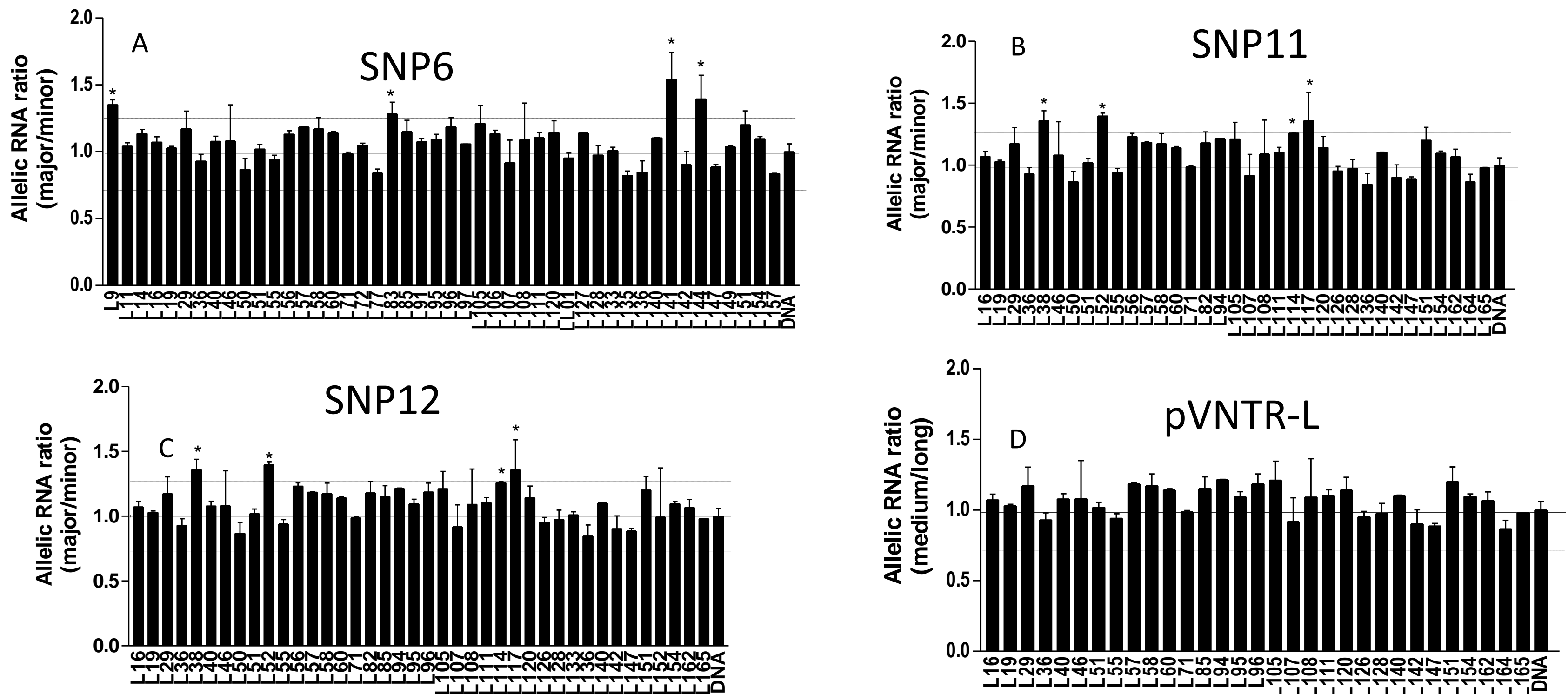
"CYP2C9 promoter variable number tandem repeat polymorphism (pVNTR) regulates mRNA expression in human livers" by D. Wang, X. Sun, Y. Gong, B.E. Gawronski, T.Y. Langaee, M.H.A. Shahin, S.I. Khalifa and J.A. Johnson, Drug Metabolism and Disposition.

Supplemental Figure 1. LD plot and haplotype structure of CYP2C9 promoter SNPs



"CYP2C9 promoter variable number tandem repeat polymorphism (pVNTR) regulates mRNA expression in human livers" by D. Wang, X. Sun, Y. Gong, B.E. Gawronski, T.Y. Langaee, M.H.A. Shahin, S.I. Khalifa and J.A. Johnson, Drug Metabolism and Disposition.

Supplemental Figure 2. Correlation between allelic CYP2C9 RNA expression imbalance and herozygosity of short allele, haplotype 1(H1) and haplotype 2 (H2). Wild-type homozygotes, heterozygotes and minor allele homozygotes show as
Note discrepancies for H1 (L17 and L163) and H2 (L114).



"CYP2C9 promoter variable number tandem repeat polymorphism (pVNTR) regulates mRNA expression in human livers" by D. Wang, X. Sun, Y. Gong, B.E. Gawronski, T.Y. Langaee, M.H.A. Shahin, S.I. Khalifa and J.A. Johnson, Drug Metabolism and Disposition.

Supplemental Figure 3. Allelic RNA ratios in samples heterozygous for SNP6 (A), SNP11 (B), SNP12 (C) and promoter long allele (D). Only eight samples (L9, L38, L52, L83, L114, L117, L141 and L144) showed allelic RNA ratio significantly different from 1. Each of these eight samples were heterozygous for the pVNTR-S allele (see Supplemental Figure 2).

"CYP2C9 promoter variable number tandem repeat polymorphism (pVNTR) regulates mRNA expression in human livers" by D. Wang, X. Sun, Y. Gong, B.E. Gawronski, T.Y. Langaee, M.H.A. Shahin, S.I. Khalifa and J.A. Johnson, Drug Metabolism and Disposition

Supplemental Table 1. Primers and PCR conditions

1. Genotyping using Snapshot

PCR primers

SNP1 & SNP2	forward	TGTCCCACTGTAAGGTTTTCAGG
	reverse	TGCCCTTGTTCGTTGTTTCTATT
	condition	Denville PCR mix, 60°C annealing, ext 2min
SNP3 & SNP4	forward	CAAAAGAGAAAACACCAGACCCATA
	reverse	CACCTGTGGATATGATGATGAGAC
	condition	Denville PCR mix, 60°C annealing, ext 1 min
SNP5 & SNP6	forward	AGTATCATTTATATTAGCACC
	reverse	TGCATTTTATATTTGGATCTA
	condition	Denvile PCR mix, 56°C annealing, ext 1.5 min
SNP7, SNP9-SNP11	forward	GGTCTTTTCAACGAAGACTAATGGAGT
	reverse	CAAGCCCTAGCAACAAATAATC
	condition	Takara Primestar HS, 68°C annealing, ext 1.5 min
SNP12	forward	TTTCAAAAGCCTACTCTAATCCACC
	reverse	CACATCTCAGTCCAAATGATCAGG
	condition	Sigma PCR mix, 60°C annealing, ext 1min
SNP13 - SNP18	forward	AAGGTCTAGGAAGGAGCCGC
	reverse	GCCACACAGCTCATAGCTGG
	condition	Sigma PCR mix, 60°C annealing, ext 1.5 min
SNP19 & SNP 20	forward	TCCCTCCTAGTTTCGTTTCTCTTC
	reverse	AAGGTCAGTGATATGGAGTAGGGT
	condition	Sigma PCR mix, 55°C annealing, ext 1min
SNP21	forward	TCTGGTTAGAATTGATCCTCTGGT
	reverse	ACAAATCACAAATTCACAAGCAG
	condition	Sigma PCR mix, 55°C annealing, ext 1min
SNP22 - SNP24	forward	TGTTTGGATACCTTCATGATTCA
	reverse	GGAGTTGCAGTGTAGGAGAAACA
	condition	Sigma PCR mix, 55°C annealing, ext 1min
SNP25	forward	CACTGTTCTGAATGCCTGTGTACA
	reverse	AAGAATTGGATTAACTCCCCAAAGT
	condition	Sigma PCR mix, 60°C annealing, ext 1min

SNP26 - SNP28	forward	AGGAGTAACTGCTCTCTGTGTTTGCTA
	reverse	TGAAACATAGGAAACTCTCCGTAAT
	condition	Sigma PCR mix, 55°C annealing, ext 1min

Primer extension primers

SNP1	TGTGTTCTTCTACGACTCATTTTCTTTTA
SNP2	GCACATAATACGGAAACTACAAAAAAGTA
SNP3	CCCCATAAAATGAGTTAAGAATAGAAGAATT
SNP4	TTTTTTTTTTTTTTAATAAAAGATTAGCAAATTGCATCCAAT
SNP5	GATATAAATAAATACACAAATATTTCATGTTCATG
SNP6	TCGAGACCATCCTGGCTAACA
SNP7	TTTTACTACAATGAAGGTATAATCCATGAAATAAAGAAT
SNP9	CATTGGGGTTTTAGTTTGCAGGTC
SNP10	TTTTTTTTTTTTTGTTTACCACATTATCTGGTAGTTGTGCTC
SNP11	AAGGCTACATACTGTATGATTCCAACC
SNP12	TTTTTTTTTTGGAGTATCAACATTAAGCCCTCCA
SNP13	TTTTTTTTTTTTTTCATGAGTCAGGGACCAAGTTA
SNP14	TTTTTTTTTTTTTTTTTTTTTTTTGCTTTTCTTTGCCCTGTATAAAGG
SNP15	TCAAGGCTCAGCTTCCTCATTC
SNP16	TTTTTTTTTTGTCCCTTTTGAATCTCTCAATTACCT
SNP17	TTTTTTTGTGATTTCCCTACCTCCCATCTT
SNP18	CGTTTCACTTCTGCAGTGATGGA
SNP19	TTTTTTTTTTTTTTTTTTTTGGGAAGAGGAGCATTGAGGAC
SNP20	TTTTTTTTTTTTTTTTTTTTTTTTTTCTCAACTCCTCCACAAGGCAG
SNP21	TTGCTTCCTGATGAAAATGGAGA
SNP22	TGCATGCAGGGGCTCC
SNP23	GTGCACGAGGTCCAGAGATAC
SNP24	CAGGCTGGTGGGGAGAAG
SNP25	GCCCAATAATTAGGATGTATCATGA
SNP26	TTTTTGGCGGCACAGAGGCAAA
SNP27	TTTTTTTTTTTTTTTCCTTCTACCAGCTGTGCTTCATT
SNP28	TTTTTTTTTTAATGCCTTTTCTCACCTGTCATCT

note: for multiplex purpose, different number of Ts were added to the 5' to adjust the length of primers

Multiplex groups:

group 1: SNP1+SNP3+SNP4

group2: SNP2+SNP5+SNP6

group3: SNP7+SNP9

group4: SNP10+SNP11+SNP12

group5: SNP13+SNP14+SNP15+SNP16+SNP17+SNP18

group6: SNP19+SNP20+SNP21+SNP22+SNP23

group7: SNP24+SNP26+SNP27+SNP28

2. Genotyping using allele specific PCR

SNP8	forward (common)	CTTGATTTAAAAAATGGGCAACAGAT
	reverse (wt)	CAGGTCCGTAGTGACGTTTAATCAT
	Reverse (SNP)	CAGGTCCGTAGTGACGTTTAATCAC
	condition	Cyber green mix, 60°C annealing, ext 1min

3. Genotyping using FAM labeled primer

promoter repeat	forward (FAM)	TGTAGTCCCAGGTTGTCAAGAGG
	reverse	CCAGTCTCTGTCTTTTCATCTCATTC
	condition	Sigma Jumpstart PCR mix, 60°C annealing, ext 1 min
	PCR produts	long=512~522, medium=453~461, short=417~434

4. Sequencing

PCR primers

Promoter fragment 1	forward	AGGGAACCAGAGAAAGAAGGACA
-6343 to-3518	reverse	TGGATTTTAGCCATTGTATAAGTGTGTAG
	condition	Takara prime star HS, 68°C annealing, ext 3min
Promoter fragment 2	forward	CTCACCCAAGAAGAAATACAGATGG
-3646 to -44	reverse	CCTTTTATAACACTCCATGCTAATTCG
	condition	Takara prime star HS, 68°C annealing, ext 4min

Sequencing primers

fragment 1	
seq 1	AGGGAACCAGAGAAAGAAGGACA
seq 2	GAAAAGTCTCATCATCATATCCACAGG
seq 3	AGTATCATTTATATTAGCACC
seq 4	TGGATTTTAGCCATTGTATAAGTGTGTAG
fragment 2	
seq 5	CTCACCCAAGAAGAAATACAGATGG
seq 6	TTTCAAAAGCCTACTCTAATCCACC
seq 7	AAGGTCTAGGAAGGAGCCGC
seq 8	GTTAGAATCCCTGTTAAAAATGACCAGTAA
seq 9	CCTTTTATAACACTCCATGCTAATTCG

5. Primers for cloning

reporter gene, 5P	forward (add XhoI site)	CCGCTCGAGCGGTCTTTTCAACGAAGACTAATGGAGT
-4512 to -3025	reverse (add NcoI site)	CATGCCATGGTGG CAAGCCCTAGCAACAAATAATC
	condition	Takara prime star HS, 68°C annealing, ext 1.5 min
reporter gene, 3P	forward (add XhoI site)	CCGCTCGAGCCTCACCCAAGAAGAAATACAGATGG
-3646 to -48	reverse (add NcoI site)	CATGCCATGGTGG CCTTTTATAACACTCCATGCTAATTCG
	condition	Takara prime star HS, 68°C annealing, ext 4 min
repeat region topo clon	forward	GGTCTTTTCAACGAAGACTAATGGAGT
	reverse	TGGATTTTAGCCATTGTATAAGTGTGTAG
	condition	Takara prime star HS, 68°C annealing, ext 1.5 min
CAR V1 cDNA	forward	AGATCAGAGGAAAACCAGCAACAG
	reverse	CCAGTGTATCCAGGGTGTTCCA
	condition	Sigma Jump start PCR mix, 60 C annealing, ext 1.5 min
HNF1α cDNA	forward	GGCAGCCGAGCCATGGTTTC
	reverse	GCCCAGGTGCCGTGGTTACTG

	condition	Takara prime star HS, 68°C annealing, ext 2 min
HNF4α cDNA	forward	GGCGTGGAGGCAGGG
	reverse	TTAGAACAGTGACTGGCACGTG
	condition	Sigma Jump start PCR mix, 60 C annealing, ext 1.5 min
CEBPA	forward	CTCGCCATGCCGGGAG
	reverse	AGATCCGGCGACCCCAA
	condition	Sigma Jump start PCR mix, 60 C annealing, ext 2min
PXR v1 cDNA	forward	GGAGCCGCTTAGTGCCTACA
	reverse	GAGGTAGCAATGAAAAGACTCAGGAAG
	condition	Takara prime star HS, 68°C annealing, ext 2 min
RXRα cDNA	forward	CGGGCATGAGTTAGTCGCA
	reverse	CAACACATCTCTTAGGCAGAGCA
	condition	Takara prime star HS, 68°C annealing, ext 2 min
GATA4 cDNA	forward	CATATTATCGTTGTTGCCGTCG
	reverse	GATTACGCAGTGATTATGTCCCC
	condition	Takara prime star HS, 68°C annealing, ext 2 min