Identification and functional assessment of a new CYP2C9 allelic variant CYP2C9*59

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Running title: A Novel CYP2C9 allelic Variant

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Non-standard Abbreviations: P450, cytochrome P450; PM, poor metabolizer; OR, NADPH P450 reductase; PCR, polymerase chain reaction; ORF, open reading frame; HPLC, high-performance liquid chromatography; $K_m$, Michaelis constant; $V_{max}$, maximal velocity; $CL_{int}$, intrinsic clearance
Abstract

Cytochrome P450 2C9 (CYP2C9), one of the most important drug-metabolizing enzymes, is responsible for metabolizing approximately 15% of clinically important drugs, including warfarin, diclofenac and losartan. Similar to other CYP members, human CYP2C9 exhibits marked genetic polymorphisms among individuals of different ethnicities. In this study, a novel missense mutation (1300A>T) was identified in a warfarin sensitive patient after a genetic screen of three candidate genes related to high variability in response to warfarin doses. This base transversion leads to an Ile-to-Phe amino acid substitution at codon 434 within the CYP2C9 protein, and this new variant has been named a novel allele, CYP2C9*59, by the Human CYP Allele Nomenclature Committee. The exogenous expression of CYP2C9.59 in insect cell microsomes revealed that, despite a similar protein expression level as wild-type CYP2C9, variant CYP2C9.59 exhibited significantly reduced \( V_{\text{max}} \) and/or increased \( K_m \) values toward three CYP2C9-specific substrates. Our data suggest that the 1300A>T mutation can greatly decrease the enzymatic activity of the CYP2C9 protein both \textit{in vitro} and \textit{in vivo}.
Introduction

Cytochrome P450 2C9 (CYP2C9) is the major CYP2C isoform in humans and is responsible for the metabolism of approximately 15% of all clinically important drugs (Samer et al., 2013; Chen et al., 2014). Similar to other CYP members, CYP2C9 is highly polymorphic across various racial and ethnic populations. These genetic polymorphisms have been shown to have clinical importance, leading to great inter-individual variability and serious adverse effects in the efficacies of many therapeutic drugs (Chaudhry et al., 2014). Alleles CYP2C9*2 (containing a R144C substitution) and CYP2C9*3 (containing an I359L substitution) have been well studied among different ethnic populations. They have been identified as the most common defective alleles and exhibited reduced metabolic activities, both in vitro and in vivo, for the CYP2C9 substrates, such as warfarin. In addition to these two common alleles, other allelic variants, such as CYP2C9*4 (Lee et al., 2007), CYP2C9*5 (Dickmann et al., 2001), CYP2C9*8 (Liu et al., 2012), CYP2C9*11 (Tai et al., 2005), CYP2C9*12 (O’Brien et al., 2013), CYP2C9*14 (Lee et al., 2014), CYP2C9*35 (Ciccacci et al., 2011), CYP2C9*57 (Nahar et al., 2013) and CYP2C9*58 (Luo et al., 2014), were reported to affect warfarin sensitivity. In this study, we present the case of a Chinese warfarin-sensitive patient who requires only one-half of the normal oral warfarin dose. Genetic analysis in that patient revealed an absence of the common mutations in CYP2C9 (*2, *3), VKORC1 (rs9923231 and rs7294) and CYP4F2 (rs2108622) that are usually related with warfarin sensitivity. However, a novel missense mutation (1300A>T) was identified in exon 9 of the CYP2C9 gene. The in vitro functional assessment of this newly found CYP2C9 variant revealed that the substitution of an Ile to a Phe at codon 434 significantly reduced the enzymatic activity of this variant toward all of the tested substrates.
**Materials and Methods**

**Study subject**

The study subject was a 47-year-old male Han Chinese patient who has taken warfarin since his mitral valve replacement surgery in 2012. Initially, the patient was prescribed with an average oral dose of warfarin that is suitable for most Chinese individuals (3.0 mg/day). Three days later, the INR value reached 4.35, which is higher than the recommended value. Subsequently, the patient stopped taking the drug for several days to let the INR value decrease to a value of approximately 2. Next, his dosage was reduced to 2 mg/day for the following three days. Nonetheless, this lower dose resulted in an INR value that was as high as 3.23. Over the next three weeks, the warfarin dose was gradually decreased and adjusted down to 1.5 mg/day for maintenance, stabilizing the INR value between 2.00 and 2.80.

**Genotyping**

This study was approved by the Institutional Ethical Committee of Beijing Hospital and informed written consent was obtained from the patient during blood sample collection. The genomic DNA extracted from the peripheral blood cells of the subject, using the TIANamp Blood DNA Midi Kit (TIANGEN, Beijing China), was used for the PCR amplification of the promoters or exons of the **CYP2C9**, **VKORC1** and **CYP4F2** genes according to the methods reported previously (Yuan et al., 2005; Burmester et al., 2011; Dai et al., 2014). To avoid sequencing errors, bi-directional sequencing was performed for the putatively mutated sites.

**Construction of the expression vectors**

Site-directed mutagenesis of each CYP2C9 variant was accomplished using the
overlap-extension PCR amplification method according to the protocols reported previously (Dai et al., 2014; Luo et al., 2014). Detailed information for all of the other primers are illustrated in the Supplemental Table 1. The full-length sequence of each CYP2C9 allele was purified, digested and cloned into the receptor vector pFastBac-OR to produce the ultimate dual-expression vector pFastBac-OR-CYP2C9, as described previously (Dai et al., 2013).

Recombinant CYP expression in insect microsomes

The pFastBac-OR-CYP2C9 vectors were packaged into baculoviruses. Then, the CYP2C9 variants and OR were simultaneously expressed at high levels in insect microsomes, according to the manufacturer's procedure. The determination and quantification of expressed proteins were performed via immunoblotting, as reported previously (Dai et al., 2013). Briefly, two micrograms of microsomes were used for the immunoblotting analysis using a rabbit polyclonal anti-CYP2C9 antibody (AbD Serotec, Oxford, UK) as the primary antibody at a dilution of 1:1000. The recombinant CYP2C9 Baculosome reagent from BD Gentest (Woburn, MA, USA) was co-analyzed as the microsomal protein standard. The immunoblots were visualized using a Super Signal West Pico Trial kit, and the band densities were determined using the ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Enzymatic activity assay using typical CYP2C9 probe substrates

To gain a better understanding of the enzymatic characteristics of the newly identified CYP2C9 variants, three different CYP2C9 specific probe substrates (tolbutamide, losartan and diclofenac) were included in the in vitro experiments, as described previously (Dai et al., 2013; Luo et al., 2014; Wang et al., 2014). The reaction mixtures contained 4-20 pmol of CYP2C9 insect microsome, 8-40 pmol of cytochrome b5 (CYP2C9/b5=1:2), 50 mM (for losartan) or 100 mM (for
tolbutamide and diclofenac) of Tris-HCl (pH 7.5) and a series of different concentration of drugs (10-1000 μM for tolbutamide, 0.5–25 mM for losartan, 1-100 μM for diclofenac). Briefly, the reaction mixtures were pre-incubated for 5 min at 37°C. Then, an NADPH regenerating system was added to start the reaction in a final volume of 200 μL, and the hydroxylation reactions were incubated at 37°C for 60, 30 or 20 min for tolbutamide, losartan or diclofenac, respectively. Then, the reaction was terminated by the addition of 1 mL of acidified ethyl acetate for extraction. The organic layer was evaporated to dryness and reconstituted in 100 μL of mobile phase for HPLC analysis. The kinetic parameters (K_m and V_max) were estimated using the GraphPad Prism program (version 6.01, San Diego, USA) with non-linear regression analysis using a hyperbolic Michaelis-Menten equation. The kinetic data are presented as the means ± S.D. for three independent microsomal preparations derived from separate transfections for wild-type and variant CYPs, and significant differences in CYP2C9s were analyzed by one-way ANOVA followed by a post-hoc test using the IBM SPSS software (version 16.0, Armonk, New York, USA).
Results and Discussion

Identification of new CYP2C9 variant *59

The subject only required half of the oral amount of warfarin that is commonly used for most Chinese individuals. It has been reported that the CYP2C9*2, *3 and VKORC1 (rs9923231) can explain approximately 49% of the dose requirement variations for long-term treatment with warfarin (Tatarunas et al., 2014). In the present study, we screened the coding regions and promoter sequences in CYP2C9, VKORC1 and CYP4F2 genes, and none of reported mutations (CYP2C9*2, CYP2C9*3, rs9923231, rs7294 and rs2108622) could not be detected in this warfarin-sensitive patient. However, a new non-synonymous mutation, 1300A>T, was identified in CYP2C9 gene. As illustrated in Figure 1A, the subject carried a heterozygous A-to-T mutation in exon 9 of CYP2C9 at position 1300, resulting in an Ile (I) to a Phe (F) substitution at codon 434. The newly found mutation was confirmed by sequence alignment and searching against the public databases in NCBI, then was submitted to the Human CYP Allele Nomenclature Committee and was named as the novel allele CYP2C9*59 (http://www.cypalleles.ki.se/cyp2c9.htm).

Expression of wild-type and variant CYP2C9s in insect cell microsomes.

To gain insights into the metabolic characteristics of the novel CYP2C9 variant, the insect cell expression system was used to simultaneously express the recombinant CYP2C9 variants and the NADPH-cytochrome P450 oxidoreductase (OR) according to the methods described previously (Dai et al., 2013). As shown in Figure 1B, both OR and CYP2C9s could be highly expressed in insect microsomes, with a similar expression level between the wild-type and the other two variants. These data suggest that the Ile-to-Phe amino acid residue substitution at
position 434 has no effect on the expression level of CYP2C9 enzyme in insect cells.

**Functional characterizations of CYP2C9 allelic variants**

As reported previously, the \( S/21 \) insect cell is not a perfect expression system for *in vitro* warfarin metabolic analysis because an unexpected product could be produced after the incubation of \( S \)-warfarin with the recombinant CYPs, even with intact \( S/21 \) insect cells (Luo et al., 2014). Therefore, three other typical CYP2C9-specific substrates were included in the metabolic activity assessments. As shown in Figure 1 and Table 1, the typical variant CYP2C9.3 exhibited approximately 8.6-38.9% of the enzymatic activity of wild-type CYP2C9.1 towards tolbutamide, diclofenac and losartan. When expressed in insect cell microsomes, previous reports revealed that typically defective variant CYP2C9.3 exhibited higher \( K_m \) and/or decreased \( V_{max} \) values than wild-type enzyme for the hydroxylation of probe drugs diclofenac and losartan (Dickmann et al., 2001; Maekawa et al., 2009). Our data were quite consistent with those observed in previous studies. For the novel variant CYP2C9.59, our results showed that only 6.1-18.7% of activity of wild-type enzyme were retained for the hydroxylation of probe drugs tolbutamide, diclofenac or losartan. These data suggested that the Ile-to-Phe substitution at position 434 significantly decreased the metabolic activity of the CYP2C9 protein. The present subject carrying the \( CYP2C9^{*1/*59} \) genotype only requires 50% of the warfarin dose that is required in Chinese individuals harboring the wild-type genotype, whereas previous reports revealed that carriers with \( CYP2C9^{*1/*3} \) genotype required a 33.7% lower warfarin dose than individuals harboring the wild-type \(^*1/*1\) genotype (Lam and Cheung, 2012). This result suggests that subjects carrying the \( CYP2C9^{*59} \) allele need lower doses of warfarin than those with the defective allele \( CYP2C9^{*3} \).

Consequently, the variant CYP2C9.59, containing an I434F substitution, might exhibit a lower...
enzymatic activity than variant CYP2C9.3 \textit{in vivo}, which is in well accordance with the results that we have obtained \textit{in vitro}.

An inspection of the crystal structure of the human CYP2C9 protein (PDB ID: 1OG5) revealed that Cys435 has a central role in the interaction with the heme group, and that the residue Arg433 also contributes to the stabilization of heme group via hydrogen bonds (Williams et al., 2003). In addition to these two residues, Val436 and G437 can also interact with heme group when the crystal structure was analyzed using the protein structure homology modeling system SWISS-MODEL Workspace (Biasini et al., 2014). Figure 2 illustrates that Ile434 is located near the heme group and forms a hydrogen bonding ion pair with Val436. Therefore, the I434F substitution might abolish this interaction and destabilize the regional spatial conformation, which may be the direct cause for the reduction in the catalytic activity of variant CYP2C9.59.

\textbf{Genetic distribution of CYP2C9*59}

To better investigate the allele distribution pattern of this newly identified mutation, another group of 50 patients with lower warfarin dosage were enrolled for sequencing of exon 9 in \textit{CYP2C9}. No carriers were found to harbor the novel allele \textit{CYP2C9*59}. NHLBI GO ESP Exome variant server (http://evs.gs.washington.edu/EVS/) is one public website for listing novel rare variants found in recent sequencing studies on very large numbers of individuals. However, newly found CYP2C9 allelic variant *59 is not included in this database, which infers that this novel variant is absent or hard to be detected in European and African Americans. Additionally, we recently analyzed the \textit{CYP2C9} genetic polymorphisms in 2127 healthy Chinese individuals and failed to identify the \textit{CYP2C9*59} allele in this population (Dai et al., 2014). These data suggest that the estimated allele frequency of this newly found \textit{CYP2C9} variant is no more than 0.1%.
among the general Chinese subjects and should be considered as a rare allele in the Chinese population. Despite this fact, the identification of this novel mutation is still significant for clinical practice in China because more than 1.3 billion people live in the Chinese mainland, and the novel variant CYP2C9.59 exhibits a dramatically reduced enzymatic activity that is even lower than that of the typically defective variant CYP2C9.3, as shown both \textit{in vitro} and \textit{in vivo.}

In summary, \textit{CYP2C9*59} (1300A>T, I434F), a new variant allele, was identified in a warfarin-sensitive Chinese patient. By expression in insect cell microsome, recombinant variant CYP2C9.59 exhibited significantly reduced catalytic activities toward tolbutamide, diclofenac and losartan, and these activities were even lower than those measured for the common defective variant CYP2C9.3. Our data suggest that the substitution of an Ile to a Phe at position 434 in CYP2C9 could significantly decrease its enzymatic activity both \textit{in vitro} and \textit{in vivo}. Therefore, care must be provided to patients carrying this allele when prescribing CYP2C9-metabolized drugs. To better understand the overall functional characteristics of novel variant CYP2C9.59, the catalytic activities toward typical CYP2C9-mediated drugs, especially for warfarin, are scheduled to be further evaluated or validated in the future by expressing the variant in other P450 expression system, such as in yeast cells.

\textbf{Acknowledgments}

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Authorship Contributions.

Participated in research design: J.P. Cai, Hu, Dai

Conducted experiments: Dai; S.H. Wang, Li; Geng, J. Cai, H. Wang

Contributed new reagents or analytic tools: Dai, S.H. Wang

Performed data analysis: Dai, S.H. Wang

Wrote or contributed to the writing of the manuscript: Dai, S.H. Wang, Li
References


Footnotes

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* D.P.D., S.H.W. and C.B.L. contributed equally to this work.
Figure legends.

**Figure 1. Discovery and functional assessment of novel variant CYP2C9.59.** (A) Sanger sequencing result of CYP2C9*59 allele in a warfarin-sensitive Chinese Han patient. The red arrow shows the heterozygous site, the red rectangle shows codon 434, and the black rectangle shows the Ile-to-Phe substitution. (B) Immunoblotting analysis of exogenously expressed CYP2C9 and OR proteins in insect cell microsomes. Commercially available insect microsomes from BD Biosciences and the microsomes from cells transfected with the pFastBac-OR vector were used as the positive and negative controls, respectively. (C) Michaelis-Menten kinetics for the hydroxylation of tolbutamide by the recombinant wild-type and variant CYP2C9s. Plots for diclofenac and losartan were omitted in the figure and detailed kinetic parameters for all three probe substrates were listed in Table 1. Each point represents the means ± S.D. of three independent experiments.

**Figure 2. A magnified view of the crystal structure of human CYP2C9 (PDB ID: 1OG5) showing the interaction of the heme group with residues 433-438.** Each amino acid residue is shown with different colors and the hydrogen bond interactions are illustrated with green dashed lines.
Table 1 Kinetic parameters for the hydroxylation of tolbutamide, diclofenac and losartan by recombinant CYP2C9 variants.

<table>
<thead>
<tr>
<th></th>
<th>Tolbutamide</th>
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<tr>
<td></td>
<td></td>
<td>$V_{\text{max}}$ (pmol/min/pmol CYP2C9)</td>
<td>$K_{m}$ (μM)</td>
<td>Clearance ($V_{\text{max}} / K_{m}$)</td>
<td>$V_{\text{max}}$ (pmol/min/pmol CYP2C9)</td>
<td>$K_{m}$ (μM)</td>
<td>Clearance ($V_{\text{max}} / K_{m}$)</td>
<td>$V_{\text{max}}$ (pmol/min/nmol CYP2C9)</td>
<td>$K_{m}$ (μM)</td>
</tr>
<tr>
<td>CYP2C9.1</td>
<td>9.22±0.62</td>
<td>46.24±6.21</td>
<td>(200.50±13.64)E-03</td>
<td>17.16±0.59</td>
<td>4.73±0.04</td>
<td>3.63±0.16</td>
<td>210.30±19.52</td>
<td>1.58±0.35</td>
<td>134.90±17.27</td>
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<tr>
<td>CYP2C9.3</td>
<td>4.45±0.05*</td>
<td>256.10±7.60*</td>
<td>(17.40±0.70)E-03*</td>
<td>15.43±0.74</td>
<td>11.00±0.64*</td>
<td>1.40±0.01*</td>
<td>92.86±19.83*</td>
<td>1.76±0.13</td>
<td>52.53±7.59*</td>
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<tr>
<td>CYP2C9.59</td>
<td>0.81±0.01*</td>
<td>66.09±5.53*</td>
<td>(12.30±1.10)E-03*</td>
<td>3.04±0.14*</td>
<td>4.59±0.81</td>
<td>0.68±0.15*</td>
<td>20.49±1.43*</td>
<td>0.93±0.01*</td>
<td>21.94±1.71*</td>
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</table>

Data are presented as the means ± S.D. of three different expression experiments.

*Represents $P<0.05$ vs. wild-type
Figure 1
Identification and functional assessment of a new CYP2C9 allelic variant CYP2C9*59

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Drug Metabolism and Disposition
**Supplemental Table 1.** PCR primers used for the construction of expression plasmids

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Note</th>
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<td>M3-1R</td>
<td>AAGGTCAGGTATCTCTGGACCTCGT</td>
<td>for CYP2C9*3</td>
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<tr>
<td>M3-2F</td>
<td>AGAGATAACCTTGACCTTCTCCCCACCA</td>
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<tr>
<td>M59-1R</td>
<td>CACACAAAAACCGTTTTTCTGCTGAG</td>
<td>for CYP2C9*59</td>
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<tr>
<td>M59-2F</td>
<td>GAAAACGGTTTTGTGTGGGAGAAGC</td>
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<tr>
<td>CYP2C9-F</td>
<td>GCCTGAATTCATGGATTCTTCTTGTGTT</td>
<td>for amplification of ORF region</td>
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<tr>
<td>CYP2C9-R</td>
<td>GAACGTCGACTCAGACAGGAATGAAGCA</td>
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</table>

The positions where the nucleotides are exchanged are indicated with red italic bold characters