Characterization of a Novel CYP2C9 Mutation (1009C>A) Detected in a Warfarin-Sensitive Patient

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Abstract. Warfarin is the most frequently prescribed anticoagulant for the long-term treatment in the clinic. Recent studies have shown that polymorphic alleles within the CYP2C9, VKORC1, and CYP4F2 genes are related to the warfarin dosage requirement. In this study, a novel non-synonymous mutation (1009C>A) in CYP2C9 was detected in a warfarin-hypersensitive patient, while the other two candidate genes were both found to be homozygous for the wild-type alleles. The newly identified point mutation results in an amino acid substitution at position 337 of the CYP2C9 protein (P337T) and has been designated as the novel allele CYP2C9*58. When expressed in insect cell microsomes, the relative intrinsic clearance values of the CYP2C9.58 variant for tolbutamide and losartan were quite similar to those of the typical defective variant CYP2C9.3, whereas the clearance value of CYP2C9.58 for diclofenac was slightly higher than that of another typical defective variant CYP2C9.2. These data suggested that when compared with wild-type CYP2C9.1, the enzymatic activity of the novel allelic variant has been greatly reduced by the 1009C>A mutation. If patients carrying this allele take drugs metabolized by CYP2C9, their metabolic rate might be slower than that of wild-type allele carriers and thus much more attention should be paid to their clinical care.

[Supplementary methods and Figure: available only at http://dx.doi.org/10.1254/jphs.13189FP]

Keywords: CYP2C9, allelic variant, insect cell microsome, functional analysis in vitro

Introduction

Warfarin is a widely prescribed oral anticoagulant for the prevention of thromboembolic events. However, warfarin is also a problematic drug due to the narrow therapeutic index and frequent bleeding complications (1, 2). It is well known that estimation of the optimal warfarin dose for each patient is notably difficult because significant variability in warfarin sensitivity exists among individuals. A number of factors have been shown to contribute to the variability in warfarin requirement, such as age, weight, height, drug interaction, and diet. Additionally, a significant portion (approximately 40%) of this variability appears to be genetically based (3 – 6).

Warfarin exists as a racemic mixture of S-warfarin and R-warfarin, with S-warfarin being the more active isomer and having a greater therapeutic effect. It has
previously been shown that S-warfarin is predominantly metabolized by cytochrome P450 2C9 (CYP2C9), while R-warfarin is mainly metabolized by CYP3A4 in vivo. Thus, structural changes in the CYP2C9 gene is one of the most important genetic factors influencing the oral dosage of warfarin (7). Multiple studies have clearly demonstrated that patients with alleles of CYP2C9 that result in decreased metabolic activity, such as CYP2C9*2 and CYP2C9*3, are usually at a higher risk for serious bleeding that is associated with higher plasma level of S-warfarin. Such patients therefore require lower doses of warfarin to attain a therapeutic international normalized ratio (INR) that is comparable to that of the general population (8, 9). In addition to CYP2C9, several other gene variants have also been implicated to have an impact on therapeutic warfarin dosage, including VKORC1, CYP4F2, APOE2, and APOE4, in which VKORC1 is regarded as the other highly important genetic determinant of warfarin dosing and has been studied extensively over the last decade (10 – 12). To date, a total of 57 CYP2C9 allelic variants have been reported and deposited in the website of Human CYP Allele Nomenclature Committee (http://www.cypalleles.ki.se/cyp2c9.htm). In vitro studies have revealed that most of these allelic isoforms exhibit significantly reduced enzyme activities to CYP2C9-specific substrates as compared with that of wild-type protein (13 – 17). In this study, we found a novel point mutation in exon 7 of CYP2C9 in a patient with high warfarin hypersensitivity and performed the in vitro functional analysis of this novel allelic variant by highly expressing it in the insect cell microsomes. Our results indicated that this new non-synonymous mutation could significantly reduce the enzymatic activity of CYP2C9 both in vitro and in vivo.

Materials and Methods

Chemicals and materials

TIANamp Blood DNA Midi Kit was obtained from TIANGEN (Beijing, China). LA Taq with GC buffer was purchased from Takara Bio, Inc. (Otsu). CEQ DTCS Quick Start Sequencing Kit was obtained from Beckman & Coulter, Inc. (Brea, CA, USA). Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). Spodoptera frugiperda (Sf)21 insect cells, SF-900™ III SFM insect culture medium, fetal bovine serum, and the Bac-to-Bac Baculovirus Expression System were purchased from Invitrogen (Carlsbad, CA, USA). Baculosomes co-expressing human CYP2C9 and NADPH-cytochrome P450 oxidoreductase (OR) or cytochrome b5 and OR were purchased from BD Gentest (Woburn, MA, USA). The rabbit polyclonal anti-CYP2C9 antibody was from AbD Serotec (Kidlington, Oxford, UK). The mouse monoclonal anti-OR antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA). The Super Signal West Pico Trial Kit was obtained from Thermo Scientific (Rockford, IL, USA). Tolbutamide, carbamazepine, losartan and E-3174 were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxytolbutamide, 4-hydroxydiclofenac, and telmisartan were obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). Diclofenac and chlorpropamide were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo). The NADPH-regenerating system was purchased from Promega (Madison, WI, USA). High-pressure liquid chromatography-grade solvents were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). All of the other chemicals and solvents that were used were of analytical grade or the highest grade that was commercially available.

Study subject

The study subject was a 69-year-old, 155 cm, 45 kg female Han Chinese patient, who was treated with warfarin due to atherosclerotic cardiomyopathy with atrial fibrillation. Initially, the patient had been treated with 3 mg of warfarin daily, which is the normal oral amount for most Chinese subjects, for 3 days, and the INR value was found to be as high as 3.72. The warfarin dose was subsequently reduced to 2 mg/day in the following 3 days, and the INR value dropped to 3.25, which was still excessive. In the following weeks the dose of warfarin was progressively reduced to 7.5 – 12.0 mg/week, which resulted in INR values of 1.59 – 3.05. After several weeks of adjustment, the dose of warfarin was stabilized at 9.0 mg/week, and the INR reached the recommended therapeutic target value of 2 – 3.

DNA extraction and genotyping

Informed written consent was obtained from the patient during blood sample collection, and this study was approved by the Institutional Ethical Committee of Beijing Hospital. DNA was extracted from white blood cells using the TIANamp Blood DNA Midi Kit, and final concentration of the DNA was diluted to approximately 25 – 50 ng/μL for PCR amplification. All nine exons of CYP2C9, the promoter and coding regions of VKORC1, and the coding region of CYP4F2 were amplified and directly sequenced according to previously described methods (13, 18, 19). The acquired sequences were aligned and blasted against the reference sequences in the NCBI database or on the Human CYP Allele Nomenclature Committee website to verify whether any reported or novel mutations were present in the patient DNA.
Expression vector construction and recombinant CYP2C9 protein expression

Site-directed mutagenesis of the typical defective CYP2C9 allelic variants (CYP2C9*2 and CYP2C9*3) was accomplished using an overlap-extension PCR amplification method as previously described (13). Primer sequences for introducing the 1009C>A mutation into the cDNA fragment of wild-type CYP2C9 were as follows: 5′-ACCGGAGC4CTGCATGCAAGACAG-3′ (sense) and 5′-CATGCCAGOTGTCGCCGTTCCTGCCA-3′ (antisense). The full-length PCR product of each variant was gel-purified, double-digested with the EcoR I and Sal I enzymes, and ligated into the pFastBac-OR vector to produce the dual-expression baculovirus vector pFastBac-OR-CYP2C9. To ensure that no errors were introduced during PCR amplification, all of the cDNA regions in the vectors were sequenced using the CEQ DTCS Quick Start Kit on the CEQ 8000 Genetic Analysis System (Beckman & Coulter, Inc.). The vectors were then used for the generation of baculoviruses and the overexpression of CYP2C9 and OR in insect cell microsomes using the Bac-to-Bac Baculovirus Expression System as previously described (14).

Assay for CYP2C9-mediated enzymatic activity

CYP2C9 activities for the wild-type and 3 variants were assessed with 3 different CYP2C9-specific substrates, in which tolbutamide and losartan hydroxylation analyses were performed as described previously (14, 15). For the diclofenac hydroxylation analysis, the incubation mixture contained diclofenac (1 – 100 μM), 4 – 10 pmol of P450 from insect microsomes, and 8 to 20 pmol of purified cytochrome b5 (P450:b5 = 1:2) in 100 mM Tris-HCl buffer (pH 7.5). The reactions were allowed to pre-incubate for 5 min at 37°C and an NADPH regenerating system (1.3 mM NADP+, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 0.4 unit/mL glucose-6-phosphate dehydrogenase) was added to start the reaction at 37°C in a final volume of 200 μL. The reaction proceeded for 20 min and was terminated by the addition of 40 μL of 0.1 M HCl and 10 μL of 20 ng/μL carbamazepine as an internal standard. Reaction samples were extracted with 800 μL acetic ether, and the organic layer was then evaporated completely under a nitrogen stream at 45°C. The dried samples were reconstituted in 100 μL of mobile phase. The incubations were performed in triplicate, and the data are presented as the mean ± S.D. from 3 experiments.

High Performance Liquid Chromatography (HPLC) analysis was carried out on a Waters e2695 separation module (Miford, MA, USA). Separation was conducted on a ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm; Agilent, Santa Clara, CA, USA) using a mobile phrase of 0.1% trifluoroacetic acid–water–acetonitrile (20:35:45, V/V) at a flow rate of 1.0 mL/min. The retention times of 4′-hydroxydiclofenac and diclofenac were 5.1 min and 14.0 min, respectively.

Statistical analysis

The kinetic parameters \( K_m \) and \( V_{max} \) were estimated using a software program designed for non-linear regression analysis with the hyperbolic Michaelis-Menten equation (Prism version 6.01; GraphPad Software, San Diego, CA, USA). The intrinsic clearance value of each variant was calculated using the following formula: \( \text{Clint} = \frac{V_{max}}{K_m} \). Kinetic data for each variant are presented as the mean ± S.D. for 3 microsomal preparations that were derived from separate transfections, and the significance difference of variants from wild-type were evaluated by one-way ANOVA followed by post-hoc test using SPSS software (version 16.0).

Homology modeling

The CYP2C9 crystal structure (20) was obtained from the Protein Data Bank (Accession: 1OG2) and used as the template to predict the significance of Pro337Thr on SWISS-MODEL Server using Automatic Modelling Mode (21).

Results

To determine the genetic basis for the low dose required for the tested patient to achieve recommended target therapeutic range, genetic polymorphism analyses were performed in 3 candidate genes that are known to be associated with warfarin dosing. As a result, no reported or new mutations were detected in VKORC1 and CYP4F2, while one novel point mutation at position 1009 of the coding region of CYP2C9 gene was found in the patient DNA. The sequencing result showed the presence of a novel mutation C>A in heterozygote status, which could give rise to one amino acid substitution Pro > Thr at codon 337 (Fig. 1). This newly found non-synonymous variant had been designated as novel allele CYP2C9*58 by the Human CYP Allele Nomenclature Committee.

When expressed in insect cell microsomes, the novel CYP2C9.58 allelic isoform showed a similar protein expression level but exhibited significantly decreased intrinsic clearance values when compared with the wild-type protein CYP2C9.1 (Fig. 2). As shown in Table 1, novel variant CYP2C9.58 showed much lower \( V_{max} \) values for all three CYP2C9 specific substrates than the wild-type protein. Simultaneously, CYP2C9.58 also exhibited higher \( K_m \) values for both tolbutamide and losartan but a similar value for diclofenac compared...
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Overall, the enzymatic activity of novel variant CYP2C9.58 was significantly decreased compared with that of the wild-type, and the intrinsic clearance values of this novel variant for both tolbutamide and losartan were quite similar to those of the typical defective variant CYP2C9.3, while the value for diclofenac was slightly higher than that of another typical defective variant CYP2C9.2. Michaelis-Menten plots for the wild-type protein and each of the allelic isoforms are shown in Fig. 3.

Discussion

CYP2C9 is a clinically important enzyme that metabolizes approximately 15% of all clinically used drugs. The CYP2C9 gene is known to be highly polymorphic and at least 57 CYP2C9 allelic isoforms have been defined by the Human CYP Allele Nomenclature Committee to date. The current study describes the identification and characterization of a novel mutation in exon 7 of the CYP2C9 gene in a Chinese patient with warfarin hypersensitivity. A C>A transversion at nucleotide position 1009 leads to a missense mutation at codon 337, which results in the substitution of a proline residue with threonine.

For the Chinese population, previous reports have revealed that the mean warfarin maintenance dose is approximately 3.3 – 3.58 mg daily, while the usual warfarin daily dose for Caucasians is 4 – 6 mg (22, 23). For the studied subject, the maintenance dose of warfarin was 1.286 mg/day (or 9 mg/week), which is only 36% – 42% of the normal dosing for the Han Chinese population. By pharmacogenetics analysis, we found that only one novel point mutation was presented in the CYP2C9 gene of this patient, while the other 2 candidate genes that are linked to warfarin dosage sensitivity were both

Table 1. Enzyme kinetic values of recombinant wild-type and mutant CYP2C9 isoforms

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Allelic protein</th>
<th>$V_{\text{max}}$ (pmol/min per nmol P450)</th>
<th>$K_m$ ($\mu$M)</th>
<th>Clearance ($V_{\text{max}}/K_m$)</th>
<th>Relative clearance (CYP2C9.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolbutamide</td>
<td>CYP2C9.1</td>
<td>4.601 ± 0.16</td>
<td>49.82 ± 6.85</td>
<td>(9.24 ± 1.68)E-02</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.2</td>
<td>6.253 ± 0.137*</td>
<td>141.4 ± 8.96*</td>
<td>(4.42 ± 0.72)E-02*</td>
<td>47.88%*</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.3</td>
<td>2.327 ± 0.193*</td>
<td>252.8 ± 50.62*</td>
<td>(0.92 ± 0.031)E-02*</td>
<td>9.97%*</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.58</td>
<td>1.427 ± 0.082*</td>
<td>96.83 ± 18*</td>
<td>(1.47 ± 0.13)E-02*</td>
<td>15.96%*</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>CYP2C9.1</td>
<td>19.15 ± 0.41</td>
<td>4.729 ± 0.44</td>
<td>4.05 ± 0.18</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.2</td>
<td>17.56 ± 0.94</td>
<td>8.427 ± 1.64*</td>
<td>2.09 ± 0.1*</td>
<td>51.46%*</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.3</td>
<td>16.66 ± 0.60*</td>
<td>10.97 ± 1.39*</td>
<td>1.52 ± 0.17*</td>
<td>37.50%*</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.58</td>
<td>10.3 ± 0.47*</td>
<td>4.73 ± 0.95</td>
<td>2.18 ± 0.19*</td>
<td>53.77%*</td>
</tr>
<tr>
<td>Losartan</td>
<td>CYP2C9.1</td>
<td>299.1 ± 8.85</td>
<td>1.011 ± 0.128</td>
<td>295.85 ± 27.42</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.2</td>
<td>198.4 ± 11.34*</td>
<td>1.268 ± 0.29</td>
<td>156.47 ± 10.99*</td>
<td>52.89%*</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.3</td>
<td>92.82 ± 7.34*</td>
<td>1.717 ± 0.249*</td>
<td>52.50 ± 7.59*</td>
<td>18.56%*</td>
</tr>
<tr>
<td></td>
<td>CYP2C9.58</td>
<td>96.73 ± 3.72*</td>
<td>1.394 ± 0.209</td>
<td>69.39 ± 5.02*</td>
<td>23.45%*</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.D. of 3 different expression experiments. *$P < 0.05$ vs. wild-type.
homozygous wild-type. Further in vitro expression studies revealed that this amino acid residue substitution could greatly reduce the enzymatic activity of the translated product CYP2C9.58. These data suggest that the newly found CYP2C9 isoform P337T should be classified into the poor metabolizer allele group and novel allele CYP2C9*58 is another distinct defective allele in the Chinese population.

Pro337 is a highly conserved residue in the CYP2C family and is located on the exterior of the protein with a distance of 25 Å from the heme group. In the crystal structure of wild-type CYP2C9, Pro337 is located in the loop region between the J and J' helices and forms a hydrogen bond with the adjacent residue Ser336 (20). We used the automated protein structure modeling algorithm at SWISS-MODEL Workspace to further investigate the impact of the Pro337Thr substitution on protein structure and function. As shown in Fig. 4A, the substitution results in the loss of hydrogen-bonding with Ser336, but three additional hydrogen bonds within the helix J-loop-helix J' motif are established between the nitrogen atom of Cys338 and the oxygen atom of Asp341; the oxygen atom of Cys338 and the nitrogen atom of Asp341; and the oxygen atom of Gln340 and the nitrogen atom of Arg342. In addition, the substitution of Pro337Thr also affects the spatial conformation of residues Ser343 and His344, resulting in the truncation of the J'-K loop and the elongation of the J' helix (Fig. 4B). Therefore, the protein secondary structure might be affected by the Pro337Thr substitution. It has been reported that the substitution of the adjacent residue 335 from Arg to Trp results in another poor metabolizer allele CYP2C9*11, exhibiting a threefold increase in the $K_m$ and a greater than twofold decrease in the intrinsic clearance value for tolbutamide (24). Compared with wild-type subjects, people carrying the *1/*11 genotype exhibit a 33% reduction in warfarin maintenance dose. However, when expressed in insect cells, much lower protein expression was detected for the CYP2C9.11 variant, but a similar level of (S)-warfarin hydroxylation activity was found for this allelic isoform compared with the wild-type enzyme (25). Molecular modeling analysis revealed that the substitution of R335W in CYP2C9 can disrupt the hydrogen bond bridge among residues 335, 341, and 336, thus decreasing the stability of protein secondary structure and altering substrate affinity and intrinsic clearance (24). In the current study, we found that the novel variant P337T could not only disrupt the hydrogen bond between Pro337 and Ser336 but also facilitate the formation of three novel hydrogen bonds among Cys338, Gln340, Asp341, and Arg342. We speculate that, similar to variant R335W changes in the intermolecular interactions and the spatial conformation of the J'-K loop in novel variant P337T might have significantly influenced substrate affinity, thus contributing to the decrease of enzymatic activity both in vitro and in vivo.

When we expressed the CYP2C9 variants in insect cells, all the variants metabolized (S)-warfarin into an unexpected product. The retention time for this metabolite was 5.3 min in the HPLC analysis, which was quite different from that of the reported metabolite 7-hydroxy warfarin (4.8 min). Our data showed that all the expressed variants had similar enzymatic activities for (S)-warfarin hydroxylation even for the commercially available insect microsomes and the intact insect microsomes (Supplementary Fig. 1, available in the online version only), which inferred that the Sf21 insect cell is not a suitable expression system for in vitro warfarin metabolic analysis. To evaluate the impact of the novel point mutation, three other typical CYP2C9-specific substrates including
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tolbutamide, losartan, and diclofenac were used in our enzymatic activity analysis.

Recently, we have performed a large scale polymorphic analysis of CYP2C9 gene structure in 2127 Chinese subjects and found as many as 22 novel non-synonymous mutations in 9 exons of the CYP2C9 gene (13). However, CYP2C9*58 is not included in these novel alleles. Thus we estimate that the frequency of this novel mutation in the common healthy Han Chinese population may be no more than 0.024%. In spite of this fact, the detection of this novel mutation still has great importance to clinical practice in China because the novel variant CYP2C9.58 exhibits significantly decreased activity both in vitro and in vivo, and special care must be paid to patients carrying this mutation when they take medicines metabolized by CYP2C9.

In summary, we found a novel CYP2C9 point mutation (1009C>A transversion in exon 7) that results in the P337T substitution, and in vitro functional analysis revealed that this novel sequence variant could significantly decrease the enzymatic activity of translated protein toward three different CYP2C9-specific substrates. Given that the studied patient required a low maintenance dose of warfarin, our data suggested that subjects carrying this allele should be regarded as poor metabolizers, and precautions must be taken when prescribing drugs that are metabolized by CYP2C9, especially drugs with a narrow therapeutic index, such as warfarin, tolbutamide, and phenytoin.

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Fig. 4. Magnified view of the three-dimensional structures of the CYP2C9 variants. A) Hydrogen-bonding among the residues that lie between Arg335 and Arg342 of the wild-type protein and the P337T variant. The yellow arrows indicate the hydrogen bonds that are either lost or gained among Ser336, Pro337, Cys338, Gln340, Asp341, and Arg342. B) A partial view of the three-dimensional structures of the J, J’, and K helices in the wild-type protein and the P337T variant.
Conflicts of Interest

The authors declare no conflicts of interest.

References