



Functional Characterization of Pharmacogenetic Variants of Human Cytochrome P450 2C9 in Korean Populations

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Abstract

Human cytochrome P450 2C9 is a highly polymorphic enzyme that is required for drug and xenobiotic metabolism. Here, we studied eleven P450 2C9 genetic variants—including three novel variants F69S, L310V, and Q324X—that were clinically identified in Korean patients. P450 2C9 variant enzymes were expressed in *Escherichia coli* and their bicistronic membrane fractions were prepared. The CO-binding spectra were obtained for nine enzyme variants, indicating P450 holoenzymes, but not for the M02 (L90P) variant. The M11 (Q324X) variant could not be expressed due to an early nonsense mutation. LC-MS/MS analysis was performed to measure the catalytic activities of the P450 2C9 variants, using diclofenac as a substrate. Steady-state kinetic analysis revealed that the catalytic efficiency of all nine P450 2C9 variants was lower than that of the wild type P450 2C9 enzyme. The M05 (R150L) and M06 (P279T) variants showed high k_{cat} values; however, their K_m values were also high. As the M01 (F69S), M03 (R124Q), M04 (R125H), M08 (I359L), M09 (I359T), and M10 (A477T) variants exhibited higher K_m and lower k_{cat} values than that of the wild type enzyme, their catalytic efficiency decreased by approximately 50-fold compared to the wild type enzyme. Furthermore, the novel variant M07 (L310V) showed lower k_{cat} and K_m values than the wild type enzyme, which resulted in its decreased (80%) catalytic efficiency. The X-ray crystal structure of P450 2C9 revealed the presence of mutations in the residues surrounding the substrate-binding cavity. Functional characterization of these genetic variants can help understand the pharmacogenetic outcomes.

Key Words: Cytochrome P450, P450 2C9, Diclofenac, Polymorphism, Pharmacogenetics

INTRODUCTION

Cytochrome P450 (P450, CYP) is a superfamily of heme-containing enzymes and is evolutionary conserved across species (Danielson, 2002; Cho *et al.*, 2019). Over 6,500 sequences of P450 enzymes have been reported and the number is still increasing (<http://drnelson.utmem.edu/cytochromeP450.html>). P450 enzymes can utilize both endogenous and exogenous compounds, including fatty acids, sterols, carcinogens, and medicines, as their substrates (Guengerich *et al.*, 2016). The human genome contains 57 P450 genes and 16 P450 enzymes that are primarily expressed in the liver to metabolize xenobiotics, including drugs (Guengerich, 2015).

Genetic variants of metabolic enzymes can affect drug response in patients by affecting the efficacy or toxicity of the drug. In particular, polymorphisms in human P450 enzymes can affect catalytic activity. Therefore, the metabolic out-

comes of P450 genetic variants can have pharmacological and toxicological impacts on the therapeutic drugs (Lee and Kim, 2011; Jeong *et al.*, 2018; Kim *et al.*, 2018). Oscarson has supported the systematic nomenclature of the human P450 alleles (<https://www.pharmvar.org/>).

The human CYP2C gene cluster is located on chromosome 10q24 with the P450s 2C8 and 2C19 (Gray *et al.*, 1995). P450 2C9 exhibits the highest expression level in the liver along with P450 3A4 and is expressed in the small intestine, aorta, and coronary artery (Shimada *et al.*, 1994; Delozier *et al.*, 2007; Guengerich, 2015). P450 2C9 is also responsible for the metabolic clearance of a few drugs (15% to 20%), such as tolbutamide, diclofenac, ibuprofen, and S-warfarin (Guengerich, 2015).

P450 2C9 can be regulated on multiple levels. Inducers, such as rifampicin, dexamethasone, and phenobarbital, increase the gene expression of P450 2C9, whereas aminoda-

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rone, sulphaphenazole, and fluconazole, have been reported as a selective, competitive inhibitor of P450 2C9 (Miners and Birkett, 1998; Danielson, 2002; Nebert and Russell, 2002; Guengerich, 2015). These drugs can accelerate or delay the clearance of drugs metabolized by P450 2C9. The crystal structure of P450 2C9 published by Williams *et al.* (2003), can help understand the structure-function relationship of the protein. The relatively large active site of the enzyme can interact with many drug substrates. The Arg108 residue interacts with the carboxylates of some substrates (Williams *et al.*, 2003). Structural analyses have revealed a new binding pocket for P450 2C9 resulting from an allosteric change upon interaction with warfarin (Williams *et al.*, 2003).

The gene encoding P450 2C9 is highly polymorphic, and at least 65 alleles have been reported (<https://www.pharmvar.org/>). These polymorphisms include functional variants, and pharmacogenetic studies regarding the clinical effect of the alleles are important to decrease adverse drug reactions (Van Booven *et al.*, 2010). Two of the most studied alleles are P450 2C9*2 (R144C, rs1799853) and P450 2C9*3 (I359L, rs1057910). The P450 2C9*2 and P450 2C9*3 variant alleles are present in 35% Caucasian populations, while African and Asian populations exhibit less frequencies (Van Booven *et al.*, 2010). The P450 2C9*2 variant allele has been shown to affect the interaction between the P450 enzyme and a reductase. Moreover, the P450 2C9*3 variant allele is located in the active site of the enzyme. Thus, both alleles reduce the metabolic activity of the P450 2C9 enzyme (Crespi and Miller, 1997; Steward *et al.*, 1997).

In this study, the genetic screening of 433 Korean patients with P450 2C9 polymorphisms was conducted and 11 allelic variants of P450 2C9, including three novel variants (F69S, L310V, and Q324X), were detected (Table 1) (Han *et al.*, 2017). We performed functional characterization of these allelic variants of P450 2C9 containing nonsynonymous single-nucleotide polymorphisms.

MATERIALS AND METHODS

Chemicals

Diclofenac, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP⁺ were purchased from Sigma-Aldrich

(St. Louis, MO, USA). All chemicals were of the highest commercially available grade. *Escherichia coli* DH5 α cells were purchased from Invitrogen (Carlsbad, CA, USA).

Construction of expression vector for P450 2C9 allelic variants

The pBL vector containing the wild type P450 2C9 gene with 6 \times His-tag was used to construct allelic variant clones. Site-directed mutagenesis (Quick Change Lightning Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA, USA) with PCR was performed and 11 allelic variant clones were obtained using specific primers. The constructed clones with desired mutations were subcloned into the pCW bicistronic expression vector containing the NADPH-P450 reductase (NPR) gene, using *Nde*I and *Xba*I restriction enzymes (Parikh *et al.*, 1997). The insert sequences of all constructed clones were verified by nucleotide sequencing analysis.

Expression of P450 2C9 allelic variants and preparation of bicistronic membranes

P450 2C9 wild type and its allelic variants were expressed as previously described, with minor modifications (Jeong *et al.*, 2018; Lee *et al.*, 2018). The pCW vectors for the P450 2C9 variants were transformed into the *E. coli* DH5 α strain. Transformed clones were cultivated in 5 ml Luria Bertani media with 50 μ g/ml ampicillin for 16 h at 37°C with shaking of 200 rpm. The clonal cultures were transferred to 500 ml terrific broth (TB) expression media with 50 μ g/ml ampicillin and incubated at 37°C until the OD₆₀₀ value reached 0.5. The culture was induced with 0.5 mM 5-aminolevulinic acid, 1.0 mM IPTG, 1.0 mM thiamine, and trace elements. After induction, the expression media were cultured at 25°C with shaking at 200 rpm, followed by harvesting by centrifugation after 36 h. The harvested cells were resuspended in TES buffer (pH 7.4) containing lysozyme and incubated at 4°C for 20 min. Cell pellets were centrifuged for 20 min and sonicated in 30 ml sonication buffer that contained 0.1 M potassium phosphate (pH 7.4), 20% glycerol (v/v), 6.0 mM magnesium acetate, 100 μ M dithiothreitol, and 200 mM phenylmethanesulfonyl fluoride. Sonicated samples were centrifuged for 10 min at 4,000 rpm and the supernatants were transferred and ultracentrifuged for 2 h at 100,000 \times g. The pellet was resuspended in 10 ml of 200 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol

Table 1. Genetic variations of P450 2C9 in Korean populations^a

P450 2C9 Variants	rsID	Nucleotides Changes	Amino Acids Changes	Reported Alleles	Frequency
M01	10:96701652	T206C	F69S	-	0.231%
M02	rs72558187	T269C	L90P	*13	0.693%
M03	rs12414460	G371A	R124Q	*42	0.231%
M04	rs72558189	G374A	R125H	*14	0.231%
M05	rs7900194	G449T	R150L	*27	1.155%
M06	rs182132442	C835A	P279T	*29	0.693%
M07	10:96731969	C928G	L310V	-	0.231%
M08	rs1057910	A1075C	I359L	*3	6.928%
M09	rs56165452	T1076C	I359T	*4	0.231%
M10	10:96748741	G1429A	A477T	*30	0.426%
M11	10:96740948	C970T	Q324X	-	0.462%

^aHan *et al.* (2017).

(v/v) and was homogenized in a Dounce homogenizer. The homogenized membrane fractions were stored on ice until analysis. The CO-binding spectra of the purified membrane fractions were analyzed as previously described, with minor modifications (Guengerich *et al.*, 2009).

Catalytic activity analysis

The catalytic activity of P450 2C9 variants was determined by measuring diclofenac hydroxylation reaction. The reaction mixture contained 0.1 μM P450 2C9 BC membrane fraction, 0.1 M potassium phosphate buffer (pH 7.4) and various concentrations of diclofenac substrate in total volume of 0.1 ml. After preincubation at 37°C for 3 min, the reaction was initiated by adding the NADPH-generating system [100 mM glucose 6-phosphate, 1 mg/ml glucose-6-phosphate dehydrogenase, and 10 mM NADP⁺]. Reaction mixtures were incubated for 10 min at 37°C and were then stopped by adding two volumes of pre-chilled CH₃CN containing 200 ng/ml chlorpropamide as an internal standard. After vortexing, the mixture was centrifuged at 3,000 rpm for 20 min and then 100 μl of the supernatant was transferred to a glass vial and analyzed by LC-MS/MS.

The concentration of 4-hydroxydiclofenac was measured by LC-MS/MS using Waters ACQUITY UPLC™ and Waters Quattro Premier™. ACQUITY UPLC™ BEH C18 (2.1×50 mm, 1.7 μm) was equipped with Waters ACQUITY UPLC™. The isocratic mobile phase comprising CH₃CN and water in the ratio of 75:25 with 0.1% formic acid was pumped at a flow rate of 0.2 ml/min. An electrospray ionization source was used in the positive ion mode. The operation conditions were as follows: capillary voltage 3.9 kV, extractor voltage 3 V, RF lens 0.1 V, source temperature 150°C, dislocation gas flow 600 L/H, cone gas flow 0.0 L/hr, and collision gas flow 0.2 ml/min. The system was operated in the multiple reaction mode (MRM) to measure the peak area of compounds. The transitions of the 4-hydroxydiclofenac-H⁺ adduct (m/z 314.15>231.15), diclofenac-H⁺ adduct (m/z 296>214), and chlorpropamide-H⁺ adduct (m/z 277>111) were monitored at collision energies of 34, 32, and 20 eV, respectively. The QuanLynx software was used to calculate the peak area.

RESULTS

Expression of recombinant P450 2C9 variants in bicistronic membrane fractions

Eleven variants of P450 2C9 were successfully constructed in a pCW bicistronic vector and the recombinant P450 enzymes were expressed in *E. coli*. Nine variants exhibited CO-binding spectra, indicating functional P450 holoenzyme in *E. coli* whole cells. However, the holoenzymes for M02 variant (L90P) was not detected (data not shown). The P450 expression level of the nine variants was 80-360 nmol per liter culture, whereas that of wild type P450 2C9 was approximately 380 nmol per liter culture. In particular, the M07 (L310V) and M10 (A477T) variants exhibited decreased P450 expression, with their P450 expression of 110 and 80 nmol per liter culture, respectively. Bacterial membrane fractions containing the wild type P450 2C9, the nine variants as well as P450-NADPH reductase, were isolated (Fig. 1). The final concentrations of P450 in bicistronic membrane fractions were 0.3-2.1 μM (Fig. 1).

Diclofenac hydroxylation by P450 2C9 variants

The enzyme activity of P450 2C9 can be measured from the 4-hydroxylation reaction of diclofenac (Guengerich, 2015). LC-MS/MS analysis showed the diclofenac substrate at 1.77 min with an MRM of 296>214 and 4-hydroxydiclofenac at 1.27 min with an MRM of 314.15>231.15 (Fig. 2). The catalytic activity of the P450 2C9 variant enzymes was determined by measuring the rates of diclofenac 4-hydroxylation using bicistronic membrane fractions. Steady-state kinetic analysis of diclofenac 4-hydroxylation indicated that the catalytic efficiencies (k_{cat}/K_m) of all the variants were lower than that of the wild-type P450 2C9 (Fig. 3, Table 2). Six variants of P450 2C9, namely, M01 (F69S), M03 (R124Q), M04 (R125H), M08 (I359L), M09 (I359T), and M10 (A477T), exhibited increased K_m values and decreased k_{cat} values, thereby resulting in decreased catalytic efficiency (Fig. 3, Table 2). Of note, the M03 variant showed a dramatic decrease in its k_{cat} value (approximately 9% of the wild type k_{cat} value). Hence, its catalytic ef-

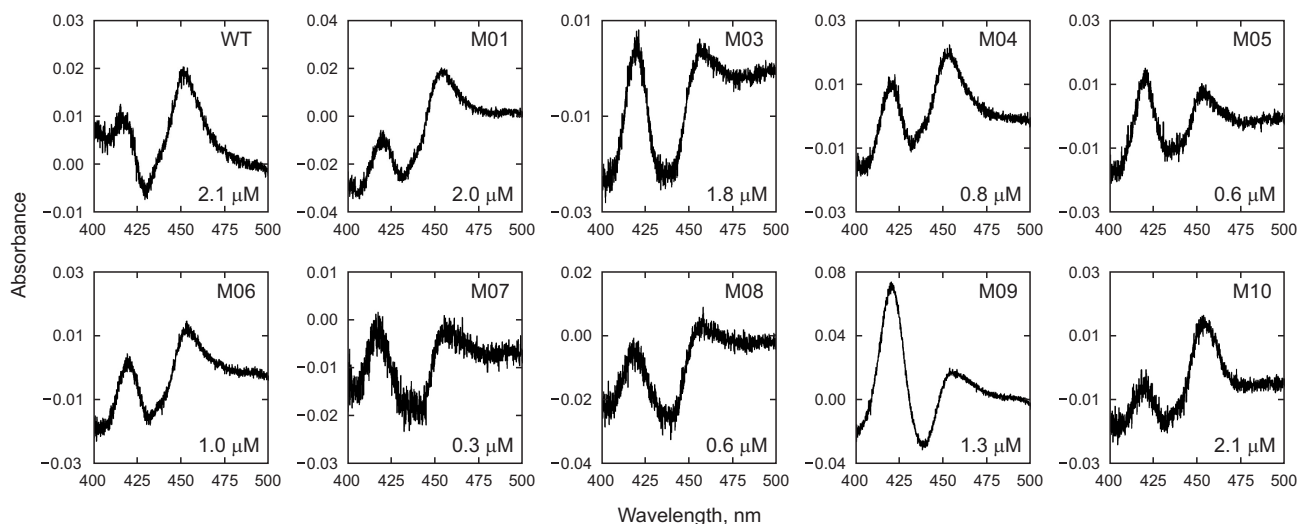


Fig. 1. CO-binding spectra of P450 2C9 allelic variant enzymes in bicistronic membrane fractions. CO-binding spectra of P450 2C9 wild type and variants in bicistronic membrane fractions were measured, and the final concentration of the P450 holoenzyme were calculated.

efficiency was 50-fold lower than that of wild type P450 2C9. A novel variant—M07 (L310V)—showed decreased k_{cat} , but the K_m value was also decreased. Therefore, its catalytic efficiency was minimally affected (approximately 80% of wild type catalytic efficiency) (Fig. 3, Table 2). Interestingly, the M05 (R150L) and M06 (P279T) variants showed increased k_{cat} values. However, their K_m values were also higher than that of the wild type enzyme (Fig. 3, Table 2).

Positions of mutated amino acids in P450 2C9 structures

Previously, the X-ray crystal structure of human P450 2C9

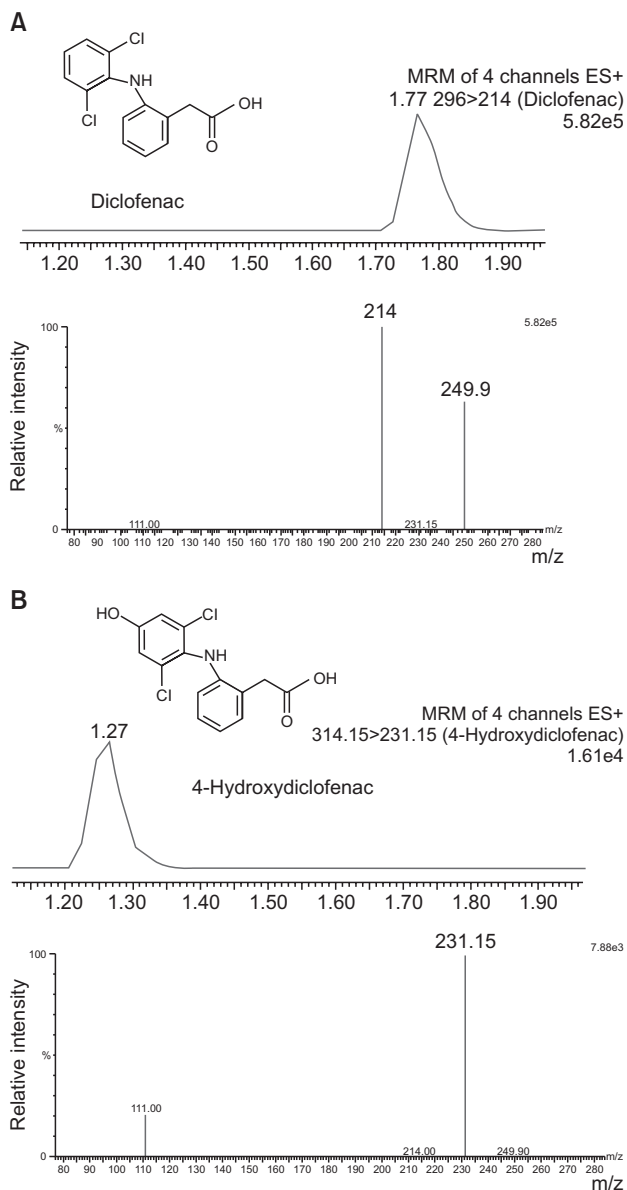


Fig. 2. LC-MS/MS analysis of diclofenac hydroxylation by P450 2C9. (A) Chromatogram and fragmentation of diclofenac in the multiple reaction mode (MRM), (B) Chromatogram and fragmentation of 4-hydroxydiclofenac in the MRM. The transitions of the 4-hydroxydiclofenac- H^+ adduct (m/z 314.15>231.15) and the diclofenac- H^+ adduct (m/z 296>214) were monitored.

with bound warfarin was determined by Williams *et al.* (2003) (PDB ID: 1OG5). The positions of mutated amino acid residues in the P450 2C9 variants were located using the crystal structure of human P450 2C9 (Fig. 4). F69S mutation in the M01 variant is located in the loop region at the first two β strands of N-terminus (Fig. 4). L90P mutation in the M02 variant is located at the end of the B-helix and a proline mutation may induce a profound effect on the structure near the heme group, resulting in failure to express the P450 holoenzyme (Fig. 4A). The M03 and M04 variants possess mutations in the C-helix region (Fig. 4A). The R150L mutation in the M05 variant is located in the center of the D-helix (Fig. 4A). The P279T mutation in the M06 variant is located in the H-I loop (Fig. 4A). The L310V mutation in the M07 variant is located in the I-helix, but not close to the active site (Fig. 4B). The I359L or I359T mutations in the M08 and M09 variants are located at the end of K-helix and the A477T mutation in the M10 variant is present on the loop of the C-terminal β strands (Fig. 4B). Ile359 and Ala477 residues appear to form the active site of P450.

DISCUSSION

Five human P450 enzymes—CYP1A2, 2C9, 2C19, 2D6, and 3A4—are responsible for the metabolism of more than 90% clinical drugs and P450 2C9 is the second major hepatic P450 enzyme that is responsible for the metabolic clearance of 15% to 20% of drugs (Si *et al.*, 2004; Dai *et al.*, 2014). In particular, P450 2C9 is a polymorphic enzyme with more than 65 reported allelic variations. The high frequency of P450 2C9 polymorphism and its wide spectrum of drug metabolism makes it important to predict the outcomes of allelic variations of P450 2C9.

In this study, the recombinant variant enzymes of P450 2C9 found in Korean populations (M01-M11) were heterologously expressed in *E. coli* and their functional outcomes of diclofenac hydroxylation were evaluated using bicistronic membrane fractions. Three allelic variants—M01 (F69S), M07 (L310V), and M11 (Q324X)—were first found in Korean populations. One of most studied polymorphisms of P450 2C9, *3 (I359L, rs1057910), was reported with much lower frequency in Asian and African populations (Van Booven *et al.*, 2010; Guengerich,

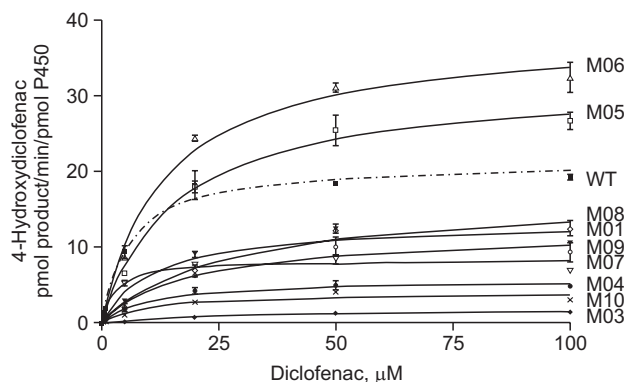


Fig. 3. Steady-state kinetic analysis of diclofenac hydroxylation by P450 2C9. Each data point is presented as the mean \pm SD of triplicate assays. Steady-state kinetic parameters of diclofenac hydroxylation were calculated (Table 2).

Table 2. Steady-state kinetic analysis of the bicistronic membranes of P450 2C9 variants

P450 2C9 Variants	4-Hydroxylation of Diclofenac			
	k_{cat} , min ⁻¹	K_m , μ M	Catalytic Efficiency (k_{cat}/K_m)	Folds
WT	21.2 \pm 1.0	6.0 \pm 1.3	3.5 \pm 0.8	1
M01	13.5 \pm 1.0	11.6 \pm 3.3	1.2 \pm 0.3	0.33
M02	ND ^a	ND ^a	ND ^a	ND ^a
M03	1.9 \pm 0.2	25.7 \pm 7.4	0.07 \pm 0.02	0.02
M04	5.7 \pm 0.4	9.6 \pm 2.5	0.6 \pm 0.2	0.17
M05	32.3 \pm 1.5	16.6 \pm 2.6	1.9 \pm 0.3	0.55
M06	38.3 \pm 1.6	13.5 \pm 2.0	2.8 \pm 0.4	0.80
M07	8.5 \pm 0.4	3.0 \pm 0.7	2.8 \pm 0.7	0.80
M08	16.7 \pm 1.4	26.2 \pm 6.3	0.6 \pm 0.2	0.18
M09	12.6 \pm 1.3	18.4 \pm 6.3	0.7 \pm 0.2	0.19
M10	4.1 \pm 0.4	10.4 \pm 4.1	0.4 \pm 0.2	0.11
M11	ND ^a	ND ^a	ND ^a	ND ^a

^aNot determined.

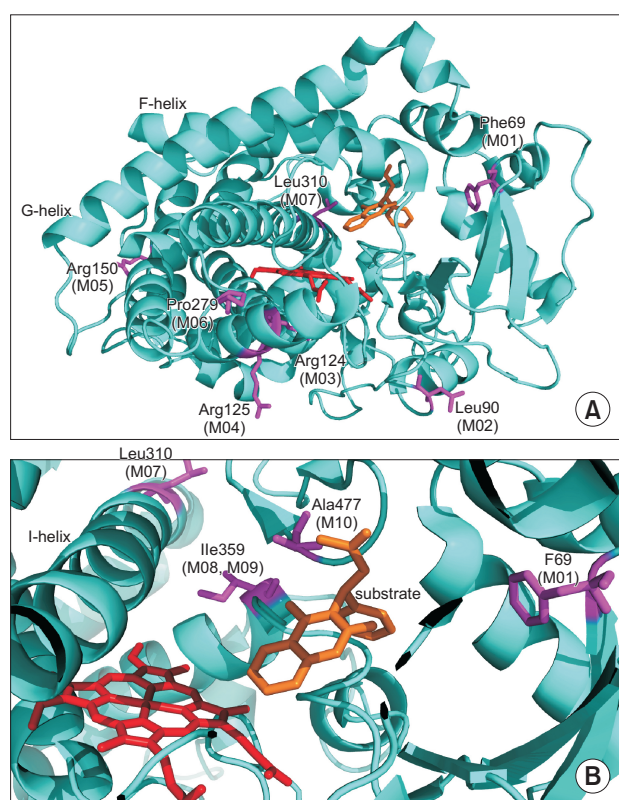


Fig. 4. Positions of mutated amino acid residues of variants in P450 2C9 structural model. (A) Overall structure of P450 2C9, (B) Structure of the substrate-binding site. The molecular structural model of P450 2C9 was constructed using the X-ray crystal structure of human P450 2C9 with bound warfarin (PDB ID: 1OG5). The P450 2C9 structural model and mutation sites were constructed using the PyMOL software (DeLano Scientific, South San Francisco, CA, USA).

2015). However, our study showed the highest frequency of 6.928% in this M08 variant (Table 1).

The novel variant M01 exhibited similar expression level to that of the wild type enzyme. However, a two-fold decrease in

its k_{cat} value and a two-fold increase in its K_m value resulted in 32.7% lower catalytic efficiency than the wild type enzyme. The M02 variant is P450 2C9*13 allele and was previously shown to exhibit reduced catalytic activity for all known P450 2C9 substrates (Guo *et al.*, 2005). In this study, its recombinant holoenzyme was not expressed in *E. coli*. As the L90P mutation in the M02 variant at the end of the B-helix is in proximity to the heme-interacting residues (Fig. 4A), the mutation of unique proline residues may affect coordination with heme and might result in the disruption of proper folding of the P450 enzyme.

The M03 and M04 variants showed similar expression levels compared to the wild type, but their catalytic efficiency was dramatically reduced (Fig. 3, Table 2). According to a previous study, the M04 variant in the P450 2C9*14 allele showed increased K_m value and significantly reduced intrinsic clearance of tolbutamide compared to the wild type enzyme (DeLozier *et al.*, 2005). In this study, the M04 variant showed a 75% decrease in the k_{cat} value and a two-fold increase in the K_m value, which resulted in a similar decrease in the catalytic efficiency of diclofenac oxidation. The C-helix is very close to the P450-conserved heme-binding motif. Moreover, the mutations in the M03 and M04 variants are located in the C-helix (Fig. 4). Therefore, these mutations could affect the productive alignment of heme and result in marked reduction in the catalytic efficiency.

Interestingly, the M05 and M06 variants exhibited higher turn-over numbers than the wild type enzyme (Table 2). The clinical outcomes of 50% to 80% increases in turn-over numbers (albeit increases in K_m values) may be critical for the metabolism of drugs with a narrow spectrum of pharmacological efficacy. The novel M07 variant contains mutations in the I-helix (Fig. 4). In P450 2C9, the I-helix is marginally kinked by interaction with the heme group, and the active site cavity is extended up from the I-helix (Fig. 4). Therefore, the substitution in the M07 variant might affect the active site cavity to alter interactions with diclofenac.

The M08 and M09 variants contain Ile359 mutations in the K-helix and are called P450 2C9*3 and *4 alleles, respectively (Sullivan-Klose *et al.*, 1996; Imai *et al.*, 2000). These variants exhibit poor metabolism phenotypes, such as hypersensitivity

of warfarin or adverse reaction to phenytoin (Sullivan-Klose *et al.*, 1996; Imai *et al.*, 2000). In our study, the bicistronic membrane systems of these variants also showed a significant reduction in their catalytic efficiency (Table 2). Structural analyses revealed that the mutations in these variants could alter the residues near the active site or the substrate access channel, and result in reduced catalytic activity (Fig. 4) (Maekawa *et al.*, 2017). In addition, the previous clinical study reported the importance of the I359L mutation in CYP2C9*3 allele, which is one of major polymorphisms affecting warfarin dose in UK Caucasians (King *et al.*, 2004).

The M10 variant (P450 2C9*30 allele) showed a 3-fold lower expression than the wild type enzyme, a 5-fold decrease in the k_{cat} value, and a 2-fold increase in the K_m value, all of which are similar to a previous report (Maekawa *et al.*, 2006). The catalytic efficiency of the M10 variant was much lower than that of the M04, M08, and M09 variants (Table 2). A recent study on the structural analysis of the M10 variant showed that the Ala477 residue is in the active site cavity and this substitution could alter hydrogen-bond interaction with the reoriented side chain, Gln214 (Maekawa *et al.*, 2017).

In the M11 variant, Glu324 is substituted with a stop codon. Thus, this mutation is incapable of producing proteins. Because Glu324 is downstream of the heme-binding signature sequences of P450 2C9, the holoenzyme containing the heme prosthetic group cannot be obtained for this variant.

In conclusion, 11 allelic variants of P450 2C9 were constructed and their functional attributes were examined. Nine variants and the wild type enzyme were successfully expressed and their functional alterations toward diclofenac were analyzed. Most of these allelic variants reduced catalytic efficiency; in particular, the M03 and M10 variants showed significantly reduced catalytic efficiency. Three novel variants—M01, M07, and M11—demonstrated significant alterations in their expression or catalytic activity. As P450 2C9 is important for the metabolism of various drugs, the study of 11 variants found in Korean populations can provide valuable information on their pharmacogenetic effects.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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