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Expression of CYP2A6, CYP2D6 and CYP4A11 Polymorphisms in COS7 Mammalian Cell Line

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The cytochrome P450 (P450, CYP) are the superfamily of heme-containing monooxygenase enzymes, found throughout all nature including mammals, plants, and microorganisms. Mammalian P450 enzymes are involved in oxidative metabolism of a wide range of endo- and exogenous chemicals. Especially P450s involved in drug metabolisms are important for drug efficacy and polymorphisms of P450s in individuals reflect differences of drug responses between people. To study the functional differences of CYP2A6, CYP2D6, and CYP4A11 variants, we cloned the four CYP2A6, three CYP2D6, and three CYP4A11 variants, which were found in Korean populations, in mammalian expression vector pcDNA by PCR and examined their expressions in COS-7 mammalian cells using immunoblots using P450 specific polyclonal antibodies. Three of four CYP2A6, two of three CYP4A11, and two of three CYP2D6 variants showed expressions in COS-7 cells but the relative levels of expressions are remarkably different in those of each variants. Our findings may help to study and explain the differences between functions of CYP variants and drug responses in Korean populations.

***Key words*:** CYP2A6, CYP2D6, CYP4A11, Drug metabolism, COS-7 mammalian cells

INTRODUCTION

The cytochrome P450 (CYP or P450) superfamily represents a group of enzymes that are involved in the oxidative metabolism of both endogenous and foreign compounds (McFadyen *et al.*, 2004). In mammalian, P450 enzymes are key players in the phase I-dependent metabolism of drugs and other xenobiotic substances such as drug and environmental toxins. As a result of the P450-dependent metabolism, intermediates that often exert toxicity or carcinogenicity, but which also are targets for phase II enzyme dependent conjugation reactions are formed, rendering them inactive polar products suitable for excretion via the kidneys (Rodriguez-Antona and Ingelman-Sundberg, 2006; Wrighton and Stevens, 1992).

P450 enzymes are classified into subfamilies based on their sequence similarities. Fifty-seven genes and five pseudogenes have been identified in the human genome, and together these enzymes are responsible for the metabolism of thousands of endogenous and xenobiotic substrates, including environmental pollutants, pharmaceuticals, steroids, prostaglandins, and fatty acids (Nelson *et al.*, 2004). P450 families 1-3 are particularly active in the detoxification of exogenous chemicals, whereas P450s in families 4-51 are mainly active in the metabolism of endogenous compounds like sterols, steroids, bile acids and fatty acids (Danielson, 2002; Guengerich, 2008).

Genetic polymorphisms affecting the function and expression of enzymes involved in drug metabolism are important factors for interindividual variability in drug response and susceptibility to adverse drug effects (Daly, 1995). P450s particularly involved in drug metabolisms are important for drug efficacy and polymorphisms of P450s in individuals reflect differences of drug responses between people. The purpose of this study was to resolve the functional differences of P450 and its variants within populations of Korean origin by examined the expression of these P450 variants in COS-7 mammalian cells with immunoblots using P450 spe-

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cific polyclonal antibodies.

Especially we are interested in CYP2A6, CY2D6 alleles, and CYP4A11 alleles which are observed in Korean populations and of which the functional studies are not finished. CYP2A6 enzymes are known to metabolize nicotine, coumarine, and cotinine. CYP2D6 enzymes metabolize debrisoquine and bufralolol. CYP4A11 enzymes utilize arachidonic acid and lauric acid as substrates.

In this paper, the clonings and expressions of CYP2A6 alleles, CYP2D6 allelles, and CYP4A11 alleles are studied in COS-7 mammalian cell lines. Three of four CYP2A6, two of three CYP4A11, and two of three CYP2D6 variants showed expressions in COS-7 cells but the relative levels of expressions are different in those of each variants. Our findings might help to study and explain the differences between functions of CYP450 variants and drug responses in Korean and Asian populations.

MATERIAL AND METHODS

Reagents. Fetal bovine serum (FBS) and DMEM were obtained from WelGENE Inc. (Daegu, South Korea). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibody against CYP2A6, CYP2D6, and CYP4A11 were made by Professor Chul-Ho Yun's laboratory (Chonnam National University, Chonnam, Korea) and the antibody to β-actin was from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. African green monkey kidney cells (COS-7 cells) were purchased from Korean Cell Line Bank (KCLB). Cells were grown and maintained at 37°C in an atmosphere of 95% air, 5% CO_2 in DMEM supplemented to a final concentration of 10% heat-inactivated fetal bovine serum, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin.

CYP variants cloned from CYP2A6, or CYP2D6, or CYP4A11. Plasmids containing CYP2A6 alleles, CYP2D6 alleles, and CYP4A11 alleles are constructed by Professor Donghak Kim's laboratory (Konkuk University, Seoul, Korea). Plasmids are subcloned into pcDNA3.1 by PCR cloning. Primers used are listed in followings. CYP2A6-F: ATGGCTGCTTCAGGAATGTTATTAGTG, CYP2A6-R: GTGATGGTGATGGTGGCGG, CYP2D6-F: ATGGCTCTTGAAGCACTTGTACCA, CYP2D6-R: TTAATGGGTGATGGTGATGGCG, CYP4A11-F: ATGGCTCTGTTAGCAGTTTTTCTGCT, CYP4A11-R: GTGATGGTGATGGTGATGTCCAAG.

Transfection. COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 at 37°C. The cells were plated at 1.8×10^6 cells in 100-mm dishes 24 h before transfection. Subsequently, jetPEI™ (Polyplus-transfection, NY, USA) was added to the culture medium, and the cells were transfected with 5 μg of CYP 2A6, CYP2D6, or CYP4A11 plasmids by using 150 mM NaCl solution, according to the manufacturer's instructions. Cells were incubated for 24 h at 37°C.

Western blot analysis. Cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% triton X-100, 2 mM EDTA, 1% DOC (Deoxycholic acid), 0.1% SDS, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT] and centrifuged to yield whole-cell lysates. Protein concentration was measured using the Bradford method. Aliquots of the lysates (20~30 μg of protein) were separated on a 4~12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 10% MeOH (v/v)]. After blocking the nonspecific site with 2% non-fat dry milk, the membrane was then incubated with specific primary antibody in non-fat dry milk at room temperature for 2 h. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1 : 5000, Santa Cruz, CA, USA) at room temperature. Immunoactive proteins were detected using the WEST-ZOL (plus) Western Blot Detection System (iNtRON, Gyeonggi, Korea).

Protein levels were quantified by densitometry using the Image J software. The levels of the three markers (CYP2A6, or CYP2D6, or CYP4A11) were normalized against β-actin. The analysis was based on the comparison of cells expressing a wild type protein at each point.

RESULTS AND DISCUSSION

Specific expression of CYP2A6, CYP2D6, and CYP4A11 in COS-7 cells. To examine that the P450 antibody reacts strongly and specifically with the cloned P450 genes, COS-7 cells were transfected with P450 vectors (CYP2A6, or CYP2D6, or CYP4A11). After 24 h, expressions of P450 were determined by specific antibodies, respectively. As shown in Fig. 1, CYP2A6, or CYP2D6, or CYP4A11 clone react specifically with their antibodies.

Expression of CYP2A6 and its allelic variants in COS-7 cells. In the human CYP2A family, three genes (CYP2A6, CYP2A7 and CYP2A13) and two pseudogenes (CYP2A7PC and CYP2A7PT) have been reported (Fernandez-Salguero *et al.*, 1995). Among them, only the CYP2A6 gene encodes an active protein, while two other genes produce catalytically defective enzymes. The CYP2A6 protein has 494 amino acids and is an important hepatic Phase I enzyme that metabolizes approximately 3% of therapeutic drugs such as nicotine (van Schaik, 2005). Because of CYP2A6 is responsible for 70~80% of the initial metabolism of nicotine, CYP2A6 has been proposed to be a novel target for

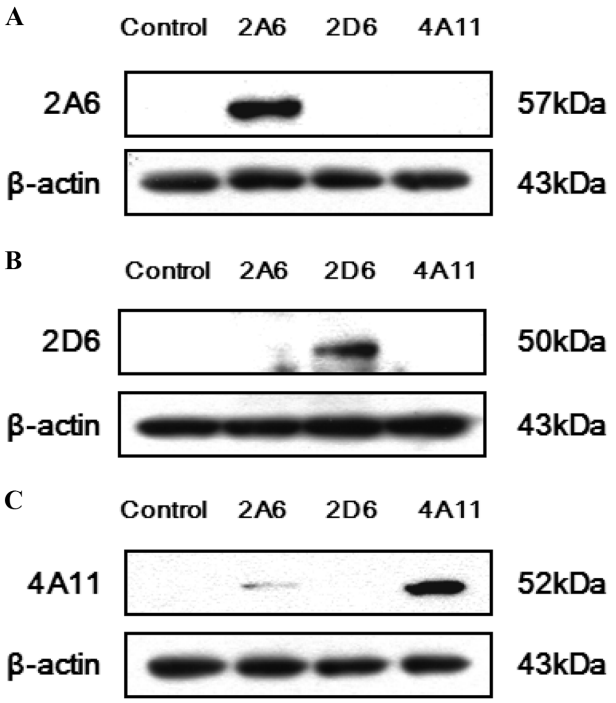

Fig. 1. Expression of CYP2A6, CYP2D6, and CYP4A11 in COS-7 cells. (A, B, and C) COS-7 cells were transfected with 5 μg of control vector and CYP vectors (CYP2A6, or CYP2D6, or CYP4A11). After 24 h, whole-cell lysates (30 μg) were prepared and the protein level was subjected to 4~12% SDS-PAGE, and expression of CYPs determined by CYP2A6 (A), or CYP2D6 (B), or CYP4A11 (C) specific polyclonal antibodies. β-actin was used here as an internal control.

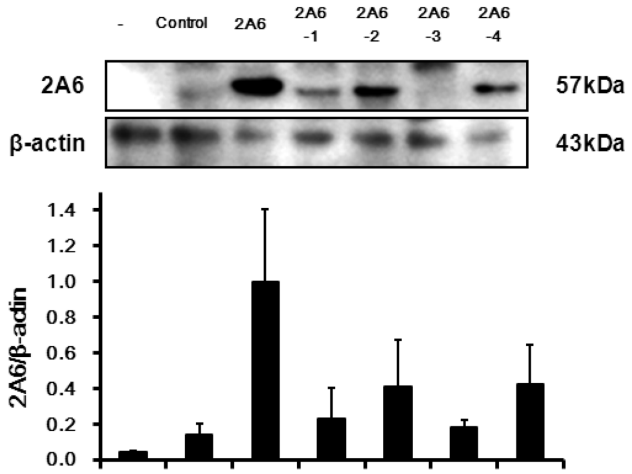

Fig. 2. Expression of CYP2A6 and its allelic variants in COS-7 cells. COS-7 cells were transfected with 5 μg of control vector, or CYP2A6, or its mutation vectors (CYP2A6-1, CYP2A6-2, CYP2A6-3, CYP2A6-4). After 24 h, whole-cell lysates (30 μg) were prepared and the protein level was subjected to 4~12% SDS-PAGE, and expression of CYP2A6 was determined by anti-CYP2A6 antibody. β-actin was used here as an internal control.

smoking cessation (Di *et al.*, 2009).

The CYP2A6 gene is highly polymorphic; over 30 alleles have been reported although many are at low frequency or have not been characterized for functional impact. Comprehensive evaluation of CYP2A6 polymorphic alleles in four ethnic populations, CYP2A6*5 and CYP2A6*19 are discovered and characterized in Korean population (Kwon *et al.*, 2001; Nakajima *et al.*, 2006). CYP2A6*5 allele has a point mutation in G479V and CYP2A6*19 allele has double mutation (Y392F and I471T).

To examine the functional differences of CYP2A6*5 allele and CYP2A6*19 allele mutants, we cloned the four mutant genes (G479V, Y392F, I471T, Y392F and I471T) (Table 1) and investigated expressions of these cloned variants in COS-7 cells with western blotting using CYP2A6 specific polyclonal antibodies. The result showed that wild-type protein (CYP2A6) was highly expressed. Compared with the wild-type protein, CYP2A6-2 (Y392F mutant) and CYP2A6-4 (Y392F; I471T mutant) were expressed up to 30% density (Fig. 2).

Expression of CYP2D6 and its allelic variants in COS-7 cells. CYP2D6 is involved in the metabolism of 20~25% of all drugs in clinical use though the enzyme represents a small proportion of the total P450 content of human liver. The genetic polymorphisms of CYP2D6 are defined in 67

Table 1. CYP450 variants cloned from CYP2A6, or CYP2D6, or CYP4A11

Mutants numbers	Allelic variants	Nucleotides	Amino acid	Korean frequency	Substrates
2A6-1	CYP2A6*5	6582G>T	G479V	0.5%	Coumarin, Nicotine, Cotinine
2A6-2	CYP2A6*19	5668A>T	Y392F		
2A6-3	CYP2A6*19	6558T>C	I471T		
2A6-4	CYP2A6*19	5668A>T; 6558T>C	Y392F; I471T	1%	
2D6-1	CYP2D6*52	100C>T	P34S		Debrisoquine, Bufuralolol
2D6-2	CYP2D6*52	3877G>A	E418K		
2D6-3	CYP2D6*52	100C>T; 3877G>A	P34S; E418K	0.33%	
4A11-1	haplotype ND	7227A>G	S353G	0.429%	Trachidonic acid, Lauric acid
4A11-2		4126T>C	W126R		
4A11-3		6911A>C	K276T		

allelic variants, many of which are associated with increased, decreased, or abolished enzyme functions (Ingelman-Sundberg *et al.*, 2007; Owen *et al.*, 2009). A system of assigned patients into four categories based on their ability to metabolize CYP2D6 substrates began to emerge. They are, listed in the order of highest functioning to lowest: ultrarapid metabolizers (UM), extensive metabolizers (EM), intermediate metabolizers (IM), and poor metabolizers (PM) (Gardiner and Begg, 2006; Zanger *et al.*, 2004).

The *CYP2D6* gene locus is highly polymorphic, and various point mutations, nucleotide deletions or insertions, gene rearrangements, and multiplication/deletion of the entire *CYP2D6* gene, resulting in more than 106 different alleles (http://www.cypalleles.ki.se/cyp2d6.htm), have been reported. Recent studies have shown that CYP2D6*52 and CYP2D6*60 were newly identified in a Korean population (Lee *et al.*, 2009) and the newly identified E418K and S183Stop were assigned as CYP2D6*52 and CYP2D6*60, respectively, by the Human P450 (CYP) Allele Nomenclature Committee.

In order to investigate the functional differences of CYP2D6 and CYP2D6*52 mutants, we cloned the three mutant (P34S, E418K, P34S; E418K) (Table 1) and investigated expressions of these cloned variants in COS-7 cells with western blotting using CYP2D6 specific polyclonal antibodies. The result showed that wild-type protein (CYP2D6) was highly expressed. Compared with the wild-type protein, CYP2D6-2 (E418K mutant) was expressed up to 60% density (Fig. 3).

Expression of CYP4A11 and its allelic variants in COS-7 cells. CYP4A subfamily enzymes are highly expressed in the cardiovascular and renal tissues. They are primarily involved in the ù-hydroxylation of medium- and long-chain fatty acids such as lauric and arachidonic acids (Elbekai and El-Kadi, 2006). In humans, two members of CYP4A subfamily have been detected in the kidney, CYP4A11 and CYP4A22. CYP4A11 has been reported to encode an active enzyme that converts arachidonic acid to 20-HETE primarily in the kidney (Lasker *et al.*, 2000; Zordoky and El-Kadi, 2010).

Nine variants of CYP4A11 [4126T>C (W126R), 4648G>A (G130S), 4714T>A (Y152N), 5829G>T (V185F), 6911A>C (K276T), 7227A>G (S353G), 8447C>T (P428L), 8610T >C (F434S), 11284C>T (L509F)] were previously identified (Cho *et al.*, 2005; Gainer *et al.*, 2005), however, their functional study has not been fully examined. To determine the functional differences of CYP4A11 and its variants, we cloned the three variant mutant (S353G, W126R, K276T) (Table 1) and investigated expressions of these cloned variants in COS-7 cells using CYP4A11 specific polyclonal antibodies. The result showed that wild-type protein (CYP4A11) was highly expressed. Compared with the wild-type protein, CYP4A11-2 (W126R mutant) and CYP4A11-2 (K276T mutant) were expressed up to 40% and 20% respectively (Fig. 4).

In the last couple of years, information on genetic polymorphisms in P450 enzymes is rapidly increasing. In the present study, we evaluated the expressional differences of

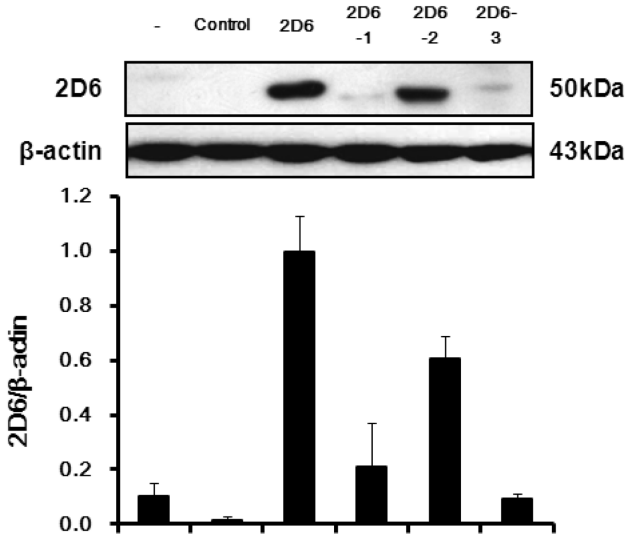

Fig. 3. Expression of CYP2D6 and its allelic variants in COS-7 cells. COS-7 cells were transfected with 5 μg of control vector, or CYP2D6, or its mutation vectors (CYP2D6-1, CYP2D6-2, CYP2D6-3). After 24 h, whole-cell lysates (30 μg) were prepared and the protein level was subjected to 4~12% SDS-PAGE, and expression of CYP2D6 was determined by anti-CYP2D6 antibody. β-actin was used here as an internal control.

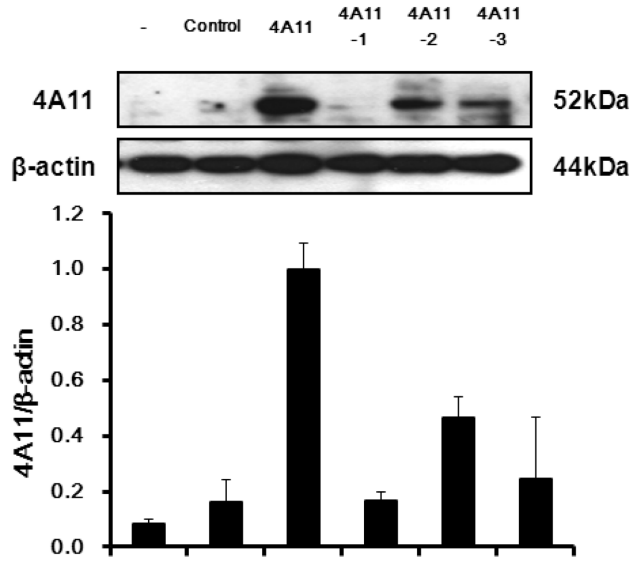

Fig. 4. Expression of CYP4A11 and its allelic variants in COS-7 cells. COS-7 cells were transfected with 5 μg of control vector, or CYP4A11, or its mutation vectors (CYP4A11-1, CYP4A11-2, CYP4A11-3). After 24 h, whole-cell lysates (30 μg) were prepared and the protein level was subjected to 4~12% SDS-PAGE, and expression of CYP4A11 were determined by anti-CYP4A11 antibody β-actin was used here as an internal control.

CYP2A6, CYP2D6, and CYP4A11 variants in COS-7 mammalian cells. Three of four CYP2A6, two of three CYP4A11, and two of three CYP2D6 variants showed expressions in COS-7 cells but the relative levels of expressions are different in those of each variants.

The causes of different expressions between alleles of P450 genes are not known in COS-7 cells. It might be resulted from the different stability of mRNA although the change of sequences of mRNA is only one base. Similar mechanisms are already reported in case of CYP1B1 variants (G61E and R469W) (Jansson *et al.*, 2001). Other possibility might be contributed to the different stabilities of proteins. One amino acid change might lead to different stability of proteins of P450 gene alleles. Similar case is also reported in case of CYP1B1 wild type and CYP1B1 R453S mutant (Bandiera *et al.*, 2005). But exact mechanisms involved in different expression of P450 alleles are required to extensive studies.

Our findings may help to explain the differences between the functional roles of P450 variants and the drug responses in Korean populations.

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