Functional characterization of 34 CYP2A6 allelic variants by assessment of nicotine C-oxidation and coumarin 7-hydroxylation activities

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Abstract

Cytochrome P450 2A6 (CYP2A6) is one of the enzymes responsible for the metabolism of therapeutic drugs and tobacco components, such as nicotine, 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone, and N-nitrosodiethylamine. Genetic polymorphisms in CYP2A6 are associated with individual variation in smoking behavior, drug toxicities and the risk of developing several cancers. In this study, we conducted an in vitro analysis of 34 allelic variants of CYP2A6 using nicotine and coumarin as representative CYP2A6 substrates. These variant CYP2A6 proteins were heterologously expressed in 293FT cells, and their enzymatic activities were assessed based on nicotine C-oxidation and coumarin 7-hydroxylation activities. Among the 34 CYP2A6 variants, CYP2A6.2, CYP2A6.5, CYP2A6.6, CYP2A6.10, CYP2A6.26, CYP2A6.36, and CYP2A6.37 exhibited no enzymatic activity, while 14 other variants exhibited markedly reduced activity toward both nicotine and coumarin. These comprehensive in vitro findings may provide useful insight into individual differences in smoking behavior, drug efficacy, and cancer susceptibility.
Introduction

Cytochrome P450 (CYP) 2A6 is the primary enzyme involved in the oxidative metabolism of nicotine and clinically useful drugs such as metronidazole, methoxyflurane, tegafur, valproic acid, and fadrozole (Kharasch et al., 1995; Nakajima et al., 1996; Sadeque et al., 1997; Komatsu et al., 2000; Pelkonen et al., 2000; Pearce et al., 2013). This enzyme also plays an important role in the metabolic activation of 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosodiethylamine (NNN), major constituents in tobacco smoke (Crespi et al., 1991). Nicotine, the main driver in the development and maintenance of tobacco dependence, is primarily metabolized to cotinine (70–80%) by CYP2A6 (Fig. 1) (Benowitz et al., 1995; Fagerstrom and Balfour, 2006). A phenotype study using nicotine as the CYP2A6 probe substrate discovered significant individual variability in CYP2A6 activity (Nakajima et al., 2006). A meta-analysis suggested that reduced metabolizers (patients with low CYP2A6 activity) smoke fewer cigarettes per day than normal metabolizers (Pan et al., 2015). The presence of several CYP2A6 variants explained as to why large deviation fractions where observed in estimated nicotine biomarker metabolism rates, identified in a genome-wide association study (Loukola et al., 2015). Furthermore, CYP2A6 activity was significantly correlated with not only increased nicotine uptake...
but also with the effect that causes smokers to smoke more extensively (Patel et al., 2016). Tegafur, a prodrug of 5-fluorouracil (5-FU), is another clinically important CYP2A6 substrate in human liver microsomes (Yamamiya et al., 2014). CYP2A6 enzymatic activity variation would affect the therapeutic effect through 5-FU biokinetics.

Investigating the enzymatic functions of individual CYP2A6 allelic variants in vivo is difficult being that the number of patients carrying low-frequency alleles is limited (Soriano et al., 2011). Therefore, in vitro characterization of CYP2A6 variants activity, using a recombinant enzyme system is essential for a deeper understanding of the individual variability in drug response and toxicity. Various heterologous expression systems, including bacteria, yeast, insect cells, and mammalian cells, have been used in previous studies (Hiratsuka, 2012). Through the use of these systems, several CYP2A6 variants have been found to exhibit reduced catalytic activities toward various CYP2A6 substrates compared with that of the wild type, CYP2A6.1 (Kitagawa et al., 2001; Daigo et al., 2002; Han et al., 2012; Yamamiya et al., 2014). However, these studies assessed different kinetic parameters ($K_m$, $V_{max}$, and $CL_{int}$). In addition, posttranslational processing of proteins expressed in bacteria, yeast, and baculoviruses differs from that in mammalian cells (Oldham, 1998; Hirathuka, 2012). Such differences in expression
systems may result in discrepancies in findings and conclusions.

A large number of genetic polymorphisms in the CYP2A6 gene have been identified in several ethnic groups and shown to contribute to altered enzyme activation and expression (Fernandez-Salguero et al., 1995; Oscarson et al., 1999; Kiyotani et al., 2002; Haberl et al., 2005; Al Koudsi et al., 2009; Piliguian et al., 2014). These polymorphisms are thought to produce individual variations in pharmacokinetics, drug efficacy, adverse drug reactions, and drug toxicities (Fujieda et al., 2004; Wang et al., 2011; Yamamiya et al., 2014; Hosono et al., 2015; Kumondai et al., 2016). To date, 45 CYP2A6 allelic variants have been identified (http://www.cypalleles.ki.se/cyp2a6.htm) [Accessed September 5th 2016]. Although most of these alleles carry amino acid substitutions, CYP2A6*3, CYP2A6*4, CYP2A6*12, CYP2A6*20, CYP2A6*27, and CYP2A6*34 are known to be inactive as a result of frame-shift mutations, gene conversion with the pseudogene CYP2A7 (another member of the human CYP2A subfamily), or whole-gene deletion. CYP2A6*9 is reported to exhibit reduced transcriptional activity due to a point mutation in the TATA box, located in the 5′ flanking region. Information regarding CYP2A6*29, CYP2A6*30, CYP2A6*32, and CYP2A6*33 is currently unavailable. After removal of the above mentioned alleles, 34 CYP2A6 variants remained for characterization. To characterize these 34 CYP2A6 variants, we assessed their functional activities over a nicotine substrate following protein expression in 293FT cells. Additionally, coumarin was used as another substrate for CYP2A6 to determine whether the observed functional changes were substrate-dependent (Miles et al., 1990).
Materials and Methods

Chemicals

(-)-Nicotine, (-)-cotinine, and (±)-cotinine-(methyl-d3) derivatives, as well as coumarin, 7-hydroxycoumarin, and 4-methyl-7-hydroxycoumarin were purchased from Sigma-Aldrich (Tokyo, Japan). CYP2A6 baculosomes and Ultrapool 150 Human Liver Cytosol were purchased from Corning Incorporated (Corning, NY, USA). Oxidized β-nicotinamide-adenine dinucleotide phosphate (NADP⁺), glucose-6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Oriental Yeast (Tokyo, Japan). The polyclonal anti-human CYP2A6 antibody was purchased from Nosan Corporation (Kanagawa, Japan). The polyclonal anti-calnexin antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

CYP2A6 cDNA cloning and construction of expression vectors

CYP2A6 cDNA fragments, obtained from a human liver complementary DNA (cDNA) library, (Takara, Shiga, Japan) were amplified by PCR with a forward primer (5’-CACCATGCTGGCCTCAGGGATGCTTC-3’) and reverse primer (5’-
-TCAGCGGGGCAGGAAGCTCATGGTGTAG-3′) using PfuUltra High-Fidelity DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA). The underlined sequences in the forward primer were introduced for directional TOPO cloning. The amplified fragments were subcloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids carrying CYP2A6*1 cDNA (wild-type) were used as a template to generate various CYP2A6 constructs (CYP2A6*2, CYP2A6*5–CYP2A6*8, CYP2A6*11, CYP2A6*13–CYP2A6*18, CYP2A6*21–CYP2A6*23, CYP2A6*25, CYP2A6*28, CYP2A6*31, CYP2A6*35, CYP2A6*38–CYP2A6*43, and CYP2A6*45) using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions. Other CYP2A6 constructs were generated from plasmids carrying other cDNA templates: CYP2A6*10, CYP2A6*19, and CYP2A6*36 from CYP2A6*7 cDNA; CYP2A6*24 from CYP2A6*35 cDNA; CYP2A6*26 from CYP2A6*25 cDNA; CYP2A6*37 from CYP2A6*10 cDNA; and CYP2A6*44 from CYP2A6*28 cDNA. All prepared constructs were confirmed by direct sequencing. The Wild-type and variant CYP2A6 cDNA sequences were subsequently subcloned into the mammalian expression vector pcDNA3.4 (Thermo Fisher Scientific).
Expression of CYP2A6 variant proteins in 293FT cells

We cultured 293FT cells in Dulbecco’s modified Eagle’s medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum at 37°C in the presence of 5% CO₂. Cells were transfected with plasmid (5 μg) carrying CYP2A6 cDNA using TransFectin lipid reagent (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s instructions. After incubation for 24 h at 37°C, cells were scraped off, centrifuged at 1500 g for 5 min, and re-suspended in homogenization buffer [10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% glycerol]. Microsomal fractions were prepared by differential centrifugation at 9000g for 20 min, followed by centrifugation of the resulting supernatant at 105,000g for 60 min. The microsomal pellet was re-suspended in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 20% glycerol, 150 mM KCl, and Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extract (Nacalai Tesque) and stored at -80°C. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific).

Determination of protein expression levels by immunoblotting

We separated 293FT microsomal fractions (4 μg of microsomal protein) by
electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels. Western blotting was performed in triplicate for each CYP2A6 variant protein in accordance with standard procedures. Recombinant CYP2A6 supersome was used as the standard (0.06–0.50 pmol) in each gel to quantify the CYP2A6 protein level. CYP2A6 protein was detected using a polyclonal anti-human CYP2A6 antibody (diluted at 1:10,000), and calnexin, used as a loading control, was detected using a polyclonal anti-calnexin antibody (diluted at 1:5000). Secondary detection was carried out with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted at 1:10,000). Immunoblots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Chemiluminescence was quantified using a ChemiDoc XRS+ with the help of Image Lab Software (Bio-Rad Laboratories).

**Nicotine C-oxidation assay**

Nicotine C-oxidation by CYP2A6 was measured as reported previously (Nakajima et al., 1996) with several modifications. The incubation mixture consisted of the microsomal fraction (20 μg), Ultrapool 150 Human Liver Cytosol as a source of aldehyde oxidase (10 μg), nicotine (10, 25, 50, 100, 250, 500, or 1000 μM), and 50 mM potassium phosphate buffer (pH 7.4) in a total volume of 100 μL. Following
pre-incubation at 37°C for 3 min, reactions were initiated by addition of
NADPH-generating medium, consisting of 0.5 mM NADP⁺, 5 mM G-6-P, 5 mM MgCl₂,
and 1 U/mL G-6-PDH. The mixture was incubated at 37°C for 20 min. Reactions were
terminated by adding 100 μL of acetonitrile containing 1 μM of cotinine-(methyl-d3)
(internal standard). Measurement of nicotine C-oxidation using 100 μM of nicotine and
20 μg of microsomal fraction containing the wild-type and variant CYP2A6 showed that
cotinine formation was linear for incubations of up to 20 min. Moreover, when the
reaction containing 100 μM of nicotine was incubated for 20 min, cotinine formation
was linear in the presence of up to 20 μg of microsomal protein (data not shown).

After protein removal by centrifugation at 15,400 g for 10 min), the
supernatant was diluted twice with water, and 10 μL of diluted solution was injected
into a liquid chromatography tandem mass spectrometry (LC-MS/MS) system. Cotinine
was measured using the LC-MS/MS system in the positive ion detection mode at the
electrospray ionization interface (TSQ Vantage Triple Stage Quadrupole Mass
Spectrometer; Thermo Fisher Scientific). Separation by ultra-high-performance liquid
chromatography (HPLC) was conducted using the Nexera Ultra High Performance
Liquid Chromatography system (SHIMADZU, Kyoto, Japan). Chromatographic
separation was performed using a CAPCELLPAK ADME S2 (2.1 × 50 mm, 2.0-μm
particle size; Shiseido, Tokyo, Japan) maintained at 35°C. Cotinine was eluted isocratically with a mobile phase consisting of 0.05% (v/v) formic acid in water and 0.05% (v/v) formic acid in acetonitrile (90:10, v/v) at a flow rate of 200 μL/min.

Quantitative MS/MS analyses were performed in the selective reaction monitoring mode. The each area under the peak of \( m/z \ 177 \rightarrow 80 \) (collision energy, 24 V; S-Lens RF amplitude voltage, 85 V) for cotinine and of \( m/z \ 180 \rightarrow 80 \) (collision energy, 29 V; S-Lens RF amplitude voltage, 55 V) for cotinine-(methyl-d3) was measured. The optimized parameters for MS were as follows: spray voltage, 4.5 kV; sheath gas pressure, 60 psi; vaporizer temperature, 200°C; capillary temperature, 205°C; and collision pressure, 1.7 mTorr. The sheath gas was nitrogen, and the collision gas was argon. The LC-MS/MS system was controlled by Xcalibur software (Thermo Fisher Scientific), which was also used to analyze the data. The lower limit of cotinine quantification was 10 nM. Standard curves for cotinine were constructed in the 0.01–30 μM range using authentic metabolite standards. The enzymatic activity was normalized to the corresponding CYP2A6 expression level.

**Coumarin 7-hydroxylation assay**

The coumarin 7-hydroxylation activity of CYP2A6 was determined using
previously reported methods (Ho et al., 2008) with minor modifications. The reaction mixture contained the microsomal fraction (30 μg), coumarin (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, or 25 μM), and 50 mM potassium phosphate buffer (pH 7.4) in a total volume of 100 μL. Following pre-incubation at 37°C for 3 min, reactions were initiated by the addition of NADPH-generating medium (see above). The mixture was incubated for an additional 10 min at 37°C. Reactions were terminated by adding 100 μL of acetonitrile containing 250 nM of 4-methyl-7-hydroxycoumarin (internal standard). Determination of coumarin 7-hydroxylation activity in the microsomal fraction containing CYP2A6.1 (30 μg of microsomal protein) and 2.5 μM of coumarin revealed that 7-hydroxycoumarin formation was linear for incubations of up to 10 min. When the reaction containing 2.5 μM of coumarin was incubated for 10 min, 7-hydroxycoumarin formation was linear in the presence of up to 30 μg of microsomal protein (data not shown).

After protein removal by centrifugation at 15,400 g for 10 min, the supernatant was diluted twice with 50 mM potassium phosphate buffer (pH 7.4), and 50 μL of the diluted solution was subjected to HPLC. The HPLC system consisted of a Waters 2695 Separations Module, a Waters 2475 multi λ fluorescence detector (Waters, Milford, MA, USA), and a SunFire C18 column (4.6 × 150 mm, 5-μm particle size; Waters)
maintained at 40°C. We eluted 7-hydroxycoumarin isocratically with 20 mM potassium phosphate buffer (pH 7.4) and acetonitrile (70:30, v/v) at a flow rate of 0.5 mL/min. The quantity of 7-hydroxycoumarin was measured at an excitation wavelength of 376 nm and emission wavelength of 454 nm. The lower limit of 7-hydroxycoumarin quantification was 25 nM. Standard curves for 7-hydroxycoumarin were constructed in the 25–2000 nM range using authentic metabolite. The enzymatic activity was normalized to the CYP2A6 expression level.

**Data analysis**

The kinetic data were analyzed using the Enzyme Kinetics Module of SigmaPlot 12.5 (Systat Software, Inc., Chicago, IL, USA), a curve-fitting program based on nonlinear regression analysis. $K_m$, maximum velocity ($V_{max}$), and intrinsic clearance ($CL_{int} = V_{max}/K_m$) values were determined by using this software. $V_{max}$ was calculated using the average CYP2A6 expression level values in triplicate. All values are expressed as the mean ± standard deviation (SD) of experiments performed in triplicate. Statistical analyses of enzymatic activity and kinetic parameters were performed by analysis of variance using Dunnett’s T3 test or the Kruskal-Wallis method (IBM SPSS Statistics Ver. 22, International Business Machines, Armonk, NY, USA).
The correlation between the $CL_{int}$ ratios of nicotine $C$-oxidation and coumarin 7-hydroxylation among CYP2A6 variants was analyzed using weighted linear regression analysis (IBM SPSS Statistics). Differences or correlations with $P$-values of less than 0.05 were defined as significant.

Three-dimensional structural modeling of CYP2A6

Three-dimensional (3D) structural modeling of CYP2A6 and nicotine binding was based on the DeVore and Scott (2012) CYP2A6 X-ray structure (Protein Data Bank code 4EJJ), while 3D structural modeling of CYP2A6 and coumarin docking was based on the Yano et al. (2005) CYP2A6 X-ray structure (Protein Data Bank code 1Z10). Maestro 9.7.012 (Schrodinger, LLC, and New York, USA) was used for 3D imaging of CYP2A6.
Results

Protein levels of CYP2A6 variants expressed in 293FT cells were measured by immunoblotting. Polyclonal CYP2A6 antibodies recognized all CYP2A6 variant proteins (Fig. 2). Only CYP2A6.13 exhibited a double band, the bands at both molecular weights were quantified. Expression levels for calnexin, an endoplasmic reticulum-resident protein, were virtually constant in the microsomes of transfected cells. CYP2A6 protein was not detected in cells transfected with empty vector (mock transfection).

The kinetic parameters of nicotine C-oxidation were determined for the wild type (CYP2A6.1) and 26 CYP2A6 variants (Table 1). $K_m$, $V_{max}$, and $CL_{int}$ values for nicotine C-oxidation by CYP2A6.1 were 33.3 μM, 5.91 pmol·min$^{-1}$·pmol$^{-1}$ CYP2A6, and 176 nL·min$^{-1}$·pmol$^{-1}$ CYP2A6, respectively. The kinetic parameters of four variants (CYP2A6.2, CYP2A6.5, CYP2A6.6, and CYP2A6.26) could not be determined because no nicotine C-oxidation activity was detected. The kinetic parameters of three variants (CYP2A6.10, CYP2A6.36, and CYP2A6.37) could not be determined because the amount of product was at or below the lower quantification limit at low substrate concentrations. The $K_m$ value of CYP2A6.38 (108 μM) was significantly higher than that of the wild type. $V_{max}$ values of CYP2A6 allelic variants were not significantly lower than those of the wild-type CYP2A6. The $CL_{int}$ value for CYP2A6.15 was 1.2
times higher than that of CYP2A6.1.

The kinetic parameters of coumarin 7-hydroxylation were determined for 25 CYP2A6 variants, as shown in Table 1. The $K_m$, $V_{\text{max}}$, and $CL_{\text{int}}$ values for coumarin 7-hydroxylation by the wild-type enzyme were 1.94 $\mu$M, 5.89 pmol·min$^{-1}$·pmol$^{-1}$ CYP2A6, and 3.04 $\mu$L·min$^{-1}$·pmol$^{-1}$ CYP2A6, respectively. The kinetic parameters of CYP2A6.2, CYP2A6.5, CYP2A6.6, CYP2A6.26, and CYP2A6.37 could not be determined because no coumarin 7-hydroxylation activity was detected. The kinetic parameters of four variants (CYP2A6.10, CYP2A6.25, CYP2A6.36, and CYP2A6.44) could not be determined because the amount of 7-hydroxycoumarin product produced by these variants was below the quantification limit at low substrate concentrations. Compared to the $K_m$ value for CYP2A6.1, that for CYP2A6.19 was significantly elevated by 2.4-fold. In contrast, the $K_m$ values of CYP2A6.13, CYP2A6.15, and CYP2A6.40 were significantly reduced. CYP2A6.15 exhibited a $V_{\text{max}}$ value that was 1.5-fold higher than that of the wild-type CYP2A6. The $V_{\text{max}}$ values of eleven variants (CYP2A6.8, CYP2A6.11, CYP2A6.17, CYP2A6.19, CYP2A6.38, CYP2A6.39, CYP2A6.40, CYP2A6.41, CYP2A6.42, CYP2A6.43, and CYP2A6.45) were significantly lower than that of CYP2A6.1. The $CL_{\text{int}}$ values for CYP2A6.14 and CYP2A6.15 were significantly higher than that of the wild type, and those of

After calculating the $CL_{int}$ values for nicotine C-oxidation and coumarin 7-hydroxylation, a significant correlation was found between these values among the different variants ($R^2 = 0.970$, $P < 0.001$), as shown in Fig. 3. CYP2A6.2, CYP2A6.5, CYP2A6.6, CYP2A6.10, CYP2A6.26, CYP2A6.36, and CYP2A6.37 were catalytically inactive toward both nicotine and coumarin.
Discussion

CYP2A6 is an important enzyme that metabolizes several tobacco components, including nicotine, NNK, and NNN. CYP2A6 genetic polymorphisms are believed to affect individual variation in smoking behavior and tobacco-related lung cancer risk. In the present study, the effects of 34 CYP2A6 variant alleles on enzyme activity were characterized by overexpressing the variant proteins in 293FT cells. Among the CYP2A6 variants, the kinetic parameters for nicotine $C_\text{Ox}$-oxidation and coumarin 7-hydroxylation were determined for 27 and 25 variants, respectively. Thus, it is likely that the measured enzymatic properties were affected by the allelic polymorphisms present in each variant.

Our results revealed that there was a significant correlation between the $CL_{\text{int}}$ values for nicotine $C_\text{Ox}$-oxidation and coumarin 7-hydroxylation among CYP2A6 variants. In particular, metabolic activity on both nicotine and coumarin substrates was virtually abolished in CYP2A6.2, CYP2A6.5, CYP2A6.6, CYP2A6.10, CYP2A6.26, CYP2A6.36, and CYP2A6.37. Similarly, the $CL_{\text{int}}$ of CYP2A6.10 expressed in Sf9 cells was shown to reduce the metabolism of tegafur to 0.6% of the wild-type CYP2A6 (Yamamiya et al., 2014). Thus, these inactive variant enzymes are predicted to exhibit markedly reduced catalytic metabolism in the presence of other clinical drugs and
activation of procarcinogens similar to nicotine and coumarin. Such variants may influence individual differences in drug efficacies, adverse effects, and cancer risks.

The nicotine $C_\text{oxydation}$ and coumarin 7-hydroxylation activities of CYP2A6.17, CYP2A6.19, CYP2A6.25, CYP2A6.26, CYP2A6.35, CYP2A6.36, CYP2A6.37, and CYP2A6.43 were reduced compared to that of CYP2A6.1. The amino acid substitutions in these variant alleles exists in conserved regions among CYP isoforms and are critical to enzymatic function. CYP2A6*17, carrying the Val365Met amino acid substitution, was found in African-American subjects with a frequency of 9.4% (Fukami et al., 2004).

The substituted residue is located in the substrate recognition site (SRS)-5, some of these amino acid chains form the heme pocket. CYP2A6*25 and CYP2A6*26 were identified in an African population and include the Phe118Leu substitution (Mwenifumbo et al., 2008). The nicotine metabolic activities for these variants were reduced to 90% when expressed in *Escherichia coli* cells (CYP2A6*25) and to 0% (CYP2A6*26) for the wild-type. Phe118 is situated in SRS-1 and is predicted to be involved in substrate stability. Moreover, CYP2A6*26 contains the Arg128Leu substitution, which is thought to be related to holoprotein formation. CYP2A6*35, CYP2A6*36, and CYP2A6*37 were also discovered in an African population.
These variant alleles include Asn438Tyr, caused by the 6458A>T mutation. Asn438 is located in a heme binding site, suggesting the possibility that the amino acid substitution alters the binding stability of heme. CYP2A6*43 (4406C>T, Thr303Ile) was identified in African Americans, and its catalytic efficiency was reported to be reduced compared to that of CYP2A6.1 (Piliguian et al., 2014). Thr303 is positioned in SRS-4, and its substitution may negatively impact function and stability. Thus, these mutations in highly conserved CYP regions may affect enzyme activity by altering the stability of the enzyme and/or its affinity to substrates and heme. However, the quantitative protein expression levels differ between CYP2A6 variants due to alterations in protein stability caused by amino acid replacement variations (Fig 4). However, it is the limitation of this study that the transfection efficiency for expressing CYP2A6 protein was not evaluated.

The CYP2A6*7 allele contains a 6558T>C nucleotide substitution causing the Ile471Thr substitution in the β4(1) strand near SRS-6; this substitution is known to decrease the catalytic activity of the enzyme (Ariyoshi et al., 2001; Uno et al., 2013). This amino acid may be involved in determining the conformation of SRS-6. In our study, CYP2A6.8, including the Arg485Leu substitution, exhibited reduced enzymatic activity. This substitution also occurs near SRS-6 and may alter the folding of SRS-6.
Both of the above amino acid changes are also included in several other variant alleles (CYP2A6*10, CYP2A6*19, CYP2A6*36, and CYP2A6*37) and are likely to affect the enzymatic activities for these alleles as well. For CYP2A6*13, two protein bands were detected by immunoblotting. The Gly5Arg amino acid substitution in CYP2A6.13, occurring in the membrane anchor region, may alter the protein’s digestion by proteases explaining the additional band. Further studies are needed to confirm these findings.

The 1703G>A single point mutation in CYP2A6*6 leads to the Arg128Gln substitution, which is present in the Japanese population with a frequency of 0.4% (Kitagawa et al., 2001). The Arg128 residue is located in a highly conserved region of the CYP superfamily and results in a reduced CO difference spectrum for hemin-fortified CYP2A6.6 compared with that of the wild-type enzyme. In the 3D structure model of the human CYP2A6, this residue forms hydrogen bonds with Asn438, which is located next to the heme-binding Cys439 (Fig. 5). By eliminating this hydrogen bond, the Arg128Gln substitution may affect the binding of heme and disorder the holoprotein structure. The CYP2A6*11 allele (3391T>C, Ser224Pro) was identified in a Japanese patient exhibiting a high plasma tegafur concentration-time curve (Daigo et al., 2002). According to our analysis, the coumarin 7-hydroxylation activity of
CYP2A6.11 was significantly reduced by 26% compared to that of CYP2A6.1. While Ser224 is not located in a highly conserved region, this amino acid forms a hydrogen bond with Tyr220 and contributes to the folding of an α-helix. The Ser224Pro substitution may abrogate the hydrogen bond and alter the 3D structure of the CYP2A6 enzyme. The single-nucleotide polymorphism in CYP2A6*15 results in the Lys194Glu substitution and significantly higher metabolic activity with both substrates. While Lys194 is not located within a highly conserved region, the substitution is considered to be indirectly adjacent to the F-helix and SRS-2 (Uno et al., 2013). CYP2A6.44 was also found to exhibit markedly reduced enzymatic activity in the presence of both substrates. CYP2A6*44 was identified in an African American and contains the Glu390Lys amino acid substitution (Piliguian et al., 2014). Glu390 is positioned in a β-sheet and forms hydrogen bonds with Glu103 in SRS-1 and Arg76. CYP2A6*44 alleles may affect the protein conformation of SRS-1, resulting in reduced affinity to CYP2A6 substrates.

In conclusion, we expressed 34 CYP2A6 protein variants in 293FT cell and determined their metabolic activities in vitro. Our results demonstrated the presence of functional variability in enzyme activities due to amino acid substitutions caused by genetic polymorphisms. CYP2A6 is an important CYP isoform involved in the metabolism of nicotine, many clinical drugs and the activation of procarcinogens. These
findings provide further insight into individual differences in smoking behaviors, drug efficacies, and cancer susceptibilities.
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Authorship Contributions

Participated in research design: Hosono, Kumondai, and Hiratsuka

Conducted experiments: Hosono and Kumondai

Contributed new reagents or analytic tools: Hirasawa, Maekawa, Yamaguchi, Mano, and Hiratsuka

Performed data analysis: Hosono, Kumondai, Maekawa, Oda, and Hiratsuka

Wrote or contributed to the writing of the manuscript: Hosono, Kumondai, and Hiratsuka
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Foot notes

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Figure Legends

Fig. 1. Metabolic pathways for nicotine showing the first reaction, catalyzed by cytochrome P450 2A6 (CYP2A6) and the second reaction, catalyzed by aldehyde oxidase (left). Primary metabolic pathway of coumarin (right).

Fig. 2. Western blots showing immunoreactive CYP2A6 proteins (upper panel) and calnexin (lower panel). 4 μg of microsomal protein fraction was loaded in each lane. Microsomal proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and then subjected to western blotting according to standard procedures. The CYP2A6 variant proteins were detected using a polyclonal antibody against CYP2A6 and calnexin was detected using a polyclonal antibody against calnexin. Numbers correspond to CYP2A6 variants.

Fig. 3. Correlation between the $CL_{int}$ ratios (relative to the wild type CYP2A6.1) for nicotine C-oxidation and coumarin 7-hydroxylation among CYP2A6 variants. Nicotine C-oxidation $CL_{int}$ ratios are plotted on the horizontal axis and coumarin 7-hydroxylation $CL_{int}$ ratios are plotted on the vertical axis. Numbers correspond to CYP2A6 variants. Correlation was analyzed using weighted linear regression analysis with IBM SPSS Statistics.

Fig. 4. Average levels of CYP2A6 proteins expressed in 293FT cells. Each bar is presented as the mean ± SD of three independently performed immunoblotting. Numbers correspond to CYP2A6 variants.

Fig. 5. Diagram of a portion of the cytochrome P450 2A6 (CYP2A6) crystal structure.
showing the Arg128Gln substitution in CYP2A6.6. Ionic bonding differs between the wild-type enzyme and the CYP2A6.6 variant.
Table 1. Kinetic parameters of nicotine C-oxidation and coumarin 7-hydroxylation by microsomes from 293FT cells expressing CYP2A6 wild-type and variant proteins.

<table>
<thead>
<tr>
<th>Variants</th>
<th>Nucleotide changes</th>
<th>Amino acid changes</th>
<th>Nicotine C-oxidation</th>
<th>Coumarin 7-hydroxylation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max}$</td>
<td>$CL_{int} (V_{max}/K_m)$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_m$ (μM)</td>
<td>(nL/min/pmol CYP2A6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$V_{max}$ (pmol/min/pmol CYP2A6)</td>
<td>(% of wild-type)</td>
</tr>
</tbody>
</table>

- **CYP2A6*1** 33.3 ± 5.44 5.91 ± 1.26 176 ± 11.2 1.94 ± 0.06 5.89 ± 0.34 3.04 ± 0.08
- **CYP2A6*2** 1799T>A Leu160His N.D. N.D. N.D. N.D. N.D. N.D.
- **CYP2A6*5** 6582G>T Gly479Val N.D. N.D. N.D. N.D. N.D. N.D.
- **CYP2A6*6** 1703G>A Arg128Gln N.D. N.D. N.D. N.D. N.D. N.D.
- **CYP2A6*7** 6558T>C Ile471Thr 267 ± 59.9 2.36 ± 0.41 9.14 ± 2.44* (5%) 4.55 ± 1.94 3.33 ± 0.62 0.80 ± 0.17* (26%)
- **CYP2A6*8** 6600G>T Arg485Leu 103 ± 16.3 2.10 ± 0.24 20.6 ± 2.44* (12%) 3.13 ± 0.30 2.22 ± 0.16* (23%)
<table>
<thead>
<tr>
<th>CYP2A6*10</th>
<th>6558T&gt;C</th>
<th>Ile471Thr</th>
<th>N.D.</th>
<th>N.D.</th>
<th>N.D.</th>
<th>N.D.</th>
<th>N.D.</th>
<th>N.D.</th>
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<tr>
<td></td>
<td>6600G&gt;T</td>
<td>Arg485Leu</td>
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<tr>
<td>CYP2A6*11</td>
<td>3391T&gt;C</td>
<td>Ser224Pro</td>
<td>142 ± 31.3</td>
<td>4.31 ± 0.34</td>
<td>31.6 ± 5.88* (18%)</td>
<td>3.30 ± 0.37</td>
<td>2.57 ± 0.03*</td>
<td>0.79 ± 0.09***</td>
</tr>
<tr>
<td>CYP2A6*13</td>
<td>13G&gt;A</td>
<td>Gly5Arg</td>
<td>30.8 ± 0.80</td>
<td>3.94 ± 0.20</td>
<td>128 ± 5.36 (73%)</td>
<td>1.37 ± 0.03*</td>
<td>2.61 ± 0.19</td>
<td>1.90 ± 0.17 (63%)</td>
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<tr>
<td>CYP2A6*14</td>
<td>86G&gt;A</td>
<td>Ser29Asn</td>
<td>28.8 ± 0.65</td>
<td>7.01 ± 0.12</td>
<td>243 ± 5.67 (138%)</td>
<td>1.77 ± 0.15</td>
<td>8.02 ± 0.43</td>
<td>4.53 ± 0.16* (149%)</td>
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<tr>
<td>CYP2A6*15</td>
<td>2134A&gt;G</td>
<td>Lys194Glu</td>
<td>23.4 ± 0.74</td>
<td>9.03 ± 0.09</td>
<td>385 ± 8.46*** (219%)</td>
<td>1.27 ± 0.03*</td>
<td>9.10 ± 0.31*</td>
<td>7.17 ± 0.28* (236%)</td>
</tr>
<tr>
<td>CYP2A6*16</td>
<td>21611C&gt;A</td>
<td>Arg203Ser</td>
<td>39.0 ± 2.52</td>
<td>4.58 ± 0.26</td>
<td>118 ± 3.60 (67%)</td>
<td>1.93 ± 0.15</td>
<td>4.72 ± 0.14</td>
<td>2.46 ± 0.21 (81%)</td>
</tr>
<tr>
<td>CYP2A6*17</td>
<td>5065G&gt;A</td>
<td>Val365Met</td>
<td>25.9 ± 6.16</td>
<td>1.70 ± 0.21</td>
<td>68.3 ± 12.2* (39%)</td>
<td>1.72 ± 0.03</td>
<td>2.76 ± 0.08*</td>
<td>1.60 ± 0.02** (53%)</td>
</tr>
<tr>
<td>CYP2A6*18</td>
<td>5668A&gt;T</td>
<td>Tyr392Phe</td>
<td>82.1 ± 7.42</td>
<td>7.94 ± 0.63</td>
<td>97.1 ± 6.86 (55%)</td>
<td>2.77 ± 0.18</td>
<td>8.03 ± 0.43</td>
<td>2.90 ± 0.05 (95%)</td>
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<tr>
<td>CYP2A6*19</td>
<td>5668A&gt;T</td>
<td>Tyr392Phe</td>
<td>405 ± 118</td>
<td>2.29 ± 0.46</td>
<td>5.80 ± 0.53* (3%)</td>
<td>4.75 ±</td>
<td>2.18 ± 0.08*</td>
<td>0.46 ± 0.01***</td>
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<td></td>
<td>6558T&gt;C</td>
<td>Ile471Thr</td>
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<tr>
<td>CYP2A6*21</td>
<td>6573A&gt;G</td>
<td>Lys476Arg</td>
<td>40.3 ± 3.69</td>
<td>6.15 ± 0.33</td>
<td>153 ± 9.35 (87%)</td>
<td>2.34 ± 0.29</td>
<td>6.20 ± 0.35</td>
<td>2.67 ± 0.20 (88%)</td>
</tr>
<tr>
<td>CYP2A6*22</td>
<td>1794C&gt;G</td>
<td>Asp158Glu</td>
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<tr>
<td></td>
<td>1798C&gt;A</td>
<td>Leu160Ile</td>
<td>53.8 ± 1.70</td>
<td>6.26 ± 0.36</td>
<td>116 ± 6.49 (66%)</td>
<td>2.40 ± 0.10</td>
<td>5.28 ± 0.20</td>
<td>2.20 ± 0.06* (72%)</td>
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<td>Variation</td>
<td>Effect</td>
<td>Control</td>
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<tr>
<td>CYP2A6*23</td>
<td>594G&gt;A</td>
<td>Val110Leu</td>
<td>52.4 ± 8.56</td>
<td>93.4 ± 12.1</td>
<td>2.22 ± 0.18</td>
<td>3.23 ± 0.11</td>
<td>(48%)</td>
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<td>CYP2A6*24</td>
<td>1672T&gt;C</td>
<td>Phe118Leu</td>
<td>59.9 ± 5.94</td>
<td>28.0 ± 2.48</td>
<td>N.D.</td>
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<tr>
<td>CYP2A6*25</td>
<td>1703G&gt;T</td>
<td>Arg128Leu</td>
<td>N.D.</td>
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<td>N.D.</td>
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<tr>
<td>CYP2A6*26</td>
<td>1711T&gt;G</td>
<td>Ser131Ala</td>
<td>N.D.</td>
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<tr>
<td>CYP2A6*28</td>
<td>5745A&gt;G</td>
<td>Asn418Asp</td>
<td>35.0 ± 4.19</td>
<td>181 ± 14.7</td>
<td>1.43 ± 0.12</td>
<td>5.05 ± 0.25</td>
<td>3.55 ± 0.23</td>
<td>(117%)</td>
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<tr>
<td>CYP2A6*31</td>
<td>16A&gt;C</td>
<td>Met6Leu</td>
<td>35.4 ± 6.15</td>
<td>184 ± 36.3</td>
<td>1.61 ± 0.07</td>
<td>5.64 ± 0.13</td>
<td>3.50 ± 0.12</td>
<td>(115%)</td>
</tr>
<tr>
<td>CYP2A6*35</td>
<td>6458A&gt;T</td>
<td>Asn438Tyr</td>
<td>75.7 ± 14.9</td>
<td>53.5 ± 5.31</td>
<td>2.50 ± 0.28</td>
<td>4.63 ± 0.35</td>
<td>1.86 ± 0.08</td>
<td>(61%)</td>
</tr>
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<td>CYP2A6*36</td>
<td>6458A&gt;T</td>
<td>Asn438Tyr</td>
<td>N.D.</td>
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<td>Ile471Thr</td>
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<td>6458A&gt;T</td>
<td>Asn438Tyr</td>
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<tr>
<td>6558T&gt;C</td>
<td>108</td>
<td>65T&gt;C</td>
<td>Ile471Thr</td>
<td>2.37 ± 0.12</td>
<td>22.2 ± 2.84* (13%)</td>
<td>1.51 ± 0.08</td>
<td>2.07 ± 0.02*</td>
<td>1.37 ± 0.07*** (45%)</td>
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<tr>
<td>6600G&gt;T</td>
<td>263</td>
<td>66T&gt;C</td>
<td>Tyr351His</td>
<td>2.15 ± 0.43</td>
<td>8.43 ± 0.95* (5%)</td>
<td>2.32 ± 0.23</td>
<td>1.26 ± 0.04* (18%)</td>
<td></td>
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<tr>
<td>CYP2A6*38</td>
<td>5023T&gt;C</td>
<td>50T&gt;C</td>
<td>Tyr351His</td>
<td>108 ± 9.63*</td>
<td>2.37 ± 0.12</td>
<td>22.2 ± 2.84* (13%)</td>
<td>1.51 ± 0.08</td>
<td>2.07 ± 0.02*</td>
</tr>
<tr>
<td>CYP2A6*39</td>
<td>468G&gt;A</td>
<td>46V&gt;G</td>
<td>Val68Met</td>
<td>93.7 ± 20.4</td>
<td>21.4 ± 4.72* (12%)</td>
<td>1.50 ± 0.20</td>
<td>1.85 ± 0.02*</td>
<td>1.26 ± 0.16* (41%)</td>
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<tr>
<td>CYP2A6*40</td>
<td>1767C&gt;G</td>
<td>17I&gt;Q</td>
<td>Ile149Met</td>
<td>168 ± 72.0</td>
<td>13.0 ± 2.93* (7%)</td>
<td>1.34 ± 0.08*</td>
<td>1.33 ± 0.08*</td>
<td>0.99 ± 0.01** (33%)</td>
</tr>
<tr>
<td>CYP2A6*41</td>
<td>3515G&gt;A</td>
<td>35Q&gt;E</td>
<td>Arg265Gln</td>
<td>263 ± 82.3</td>
<td>8.43 ± 0.95* (5%)</td>
<td>2.32 ± 0.23</td>
<td>1.26 ± 0.04* (18%)</td>
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<tr>
<td>CYP2A6*42</td>
<td>3524T&gt;C</td>
<td>35I&gt;Q</td>
<td>Ile149Met</td>
<td>450 ± 72.9</td>
<td>6.07 ± 0.24* (3%)</td>
<td>3.25 ± 0.44</td>
<td>0.63 ± 0.02*</td>
<td>0.20 ± 0.02*** (6%)</td>
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<tr>
<td>CYP2A6*43</td>
<td>4406C&gt;T</td>
<td>44H&gt;Q</td>
<td>Thr303Ile</td>
<td>1049 ± 683</td>
<td>5.33 ± 1.28* (3%)</td>
<td>8.55 ± 1.47</td>
<td>1.34 ± 0.09*</td>
<td>0.16 ± 0.02*** (5%)</td>
</tr>
<tr>
<td>CYP2A6*44</td>
<td>5661G&gt;A</td>
<td>56R&gt;Q</td>
<td>Glu390Lys</td>
<td>1097 ± 284</td>
<td>2.39 ± 0.43</td>
<td>2.22 ± 0.16* (1%)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5745A&gt;G</td>
<td>574N&gt;Q</td>
<td>Asn418Asp</td>
<td>1097 ± 284</td>
<td>2.39 ± 0.43</td>
<td>2.22 ± 0.16* (1%)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5750G&gt;C</td>
<td>575E&gt;Q</td>
<td>Glu419Asp</td>
<td>1097 ± 284</td>
<td>2.39 ± 0.43</td>
<td>2.22 ± 0.16* (1%)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2A6*45</td>
<td>6531C&gt;T</td>
<td>65P&gt;Q</td>
<td>Leu462Pro</td>
<td>55.1 ± 11.3</td>
<td>22.0 ± 2.24* (13%)</td>
<td>1.65 ± 0.16</td>
<td>1.07 ± 0.02*</td>
<td>0.65 ± 0.05*** (21%)</td>
</tr>
</tbody>
</table>

This article has not been copyedited and formatted. The final version may differ from this version.
These data represent the mean ± SD of three independently performed catalytic assays.

\*P < 0.05, \**P < 0.01, and \***P < 0.005 compared to CYP2A6.1. N.D. represents not determined.

The kinetic parameters for nicotine C-oxidation of CYP2A6.2, CYP2A6.5, CYP2A6.6, and CYP2A6.26 could not be determined because the enzymatic activity of CYP was not detected at the highest substrate concentration (1000 μM nicotine).

The kinetic parameters for nicotine C-oxidation of CYP2A6.10, CYP2A6.36, and CYP2A6.37 could not be determined because the amount of product, cotinine, produced by these variants was below the quantification limit at low substrate concentrations.

The kinetic parameters of CYP2A6.2, CYP2A6.5, CYP2A6.6, CYP2A6.26, and CYP2A6.37 could not be determined because the enzymatic activity of CYP was not detected at the highest substrate concentration (25 μM coumarin).

The kinetic parameters for nicotine C-oxidation of CYP2A6.10, CYP2A6.25, CYP2A6.36, and CYP2A6.44 could not be determined because the amount of product, 7-hydroxycoumarin, produced by these variants was below the quantification limit at low substrate concentrations.
Figure 1

Nicotine is metabolized by CYP2A6 to form nicotine-$\Delta^{1,5'}$-iminium ion. This intermediate is further metabolized by aldehyde oxidase to form cotinine.

Coumarin is also metabolized by CYP2A6 to form 7-hydroxyxycoumarin.
Figure 2

Mock .1 .2 .5 .6 .7 .8 .10 .11 .13 .14 .15

CYP2A6

Calnexin

CYP2A6

Calnexin

CYP2A6

Calnexin
Figure 4

CYP2A6 expression level (pmol CYP2A6/mg protein)

CYP2A6 variants