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Regular Article

Functional characterization of 9 CYP2A13 allelic variants by assessment of nicotine *C*-oxidation and coumarin 7-hydroxylation☆

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ABSTRACT

Cytochrome P450 2A13 (CYP2A13) is responsible for the metabolism of chemical compounds such as nicotine, coumarin, and tobacco-specific nitrosamine. Several of these compounds have been recognized as procarcinogens activated by CYP2A13. We recently showed that *CYP2A13*2* contributes to inter-individual variations observed in bladder cancer susceptibility because *CYP2A13*2* might cause a decrease in enzymatic activity. Other CYP2A13 allelic variants may also affect cancer susceptibility. In this study, we performed an *in vitro* analysis of the wild-type enzyme (CYP2A13.1) and 8 CYP2A13 allelic variants, using nicotine and coumarin as representative CYP2A13 substrates. These CYP2A13 variant proteins were heterologously expressed in 293FT cells, and the kinetic parameters of nicotine *C*-oxidation and coumarin 7-hydroxylation were estimated. The quantities of CYP2A13 holoenzymes in microsomal fractions extracted from 293FT cells were determined by measuring reduced carbon monoxide-difference spectra. The kinetic parameters for CYP2A13.3, CYP2A13.4, and CYP2A13.10 could not be determined because of low metabolite concentrations. Five other CYP2A13 variants (CYP2A13.2, CYP2A13.5, CYP2A13.6, CYP2A13.8, and CYP2A13.9) showed markedly reduced enzymatic activity toward both substrates. These findings provide insights into the mechanism underlying inter-individual differences observed in genotoxicity and cancer susceptibility.

1. Introduction

Cytochrome P450 (CYP) 2A13 is an enzyme involved in the metabolism of several substrates, including coumarin, as well as nicotine and tobacco-specific nitrosoamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) (Fig. 1) [1–4]. CYP2A13 also contributes to the genotoxicity caused by metabolic activation of aflatoxins and NNK, which is a representative procarcinogen included in tobacco [5]. In A549 cell transfectants, CYP2A13 mediated NNK metabolism to induce γ-H2AX production, a sensitive marker of DNA adducts [6]. An aflatoxin-mediated increase in DNA-damage-response protein expression was reported in BEAS-2B cells, which express CYP2A13 [7]. The CYP2A13 mRNA is highly expressed in lung and bladder tissues (approximately 20 and 5×10^{-5} copies/0.1 μg RNA described as CYP2A13/GAPDH, respectively) [8]. In these tissues, bioactivation of procarcinogen caused by CYP2A13 had a much larger contribution to genotoxicity.

Several *CYP2A13* genetic polymorphisms have been identified in Japanese and French Caucasian populations [9–11]. The frequency of *CYP2A13*2*, specifically, has been reported with a frequency of 4.8–7.3% in Japanese individuals [9,11,12]. The Arg257Cys substitution caused by mutations in *CYP2A13*2* is located at the carboxyl end of the G helix, resulting in a 2–3-fold reduction in CYP2A13 enzymatic activity [13,14]. *CYP2A13*2* was associated with a decreased incidence of lung adenocarcinoma in smokers [15]. We previously found that the presence of *CYP2A13*2* was associated with a reduced risk for bladder cancer, given that it decreases DNA damage caused by CYP2A13 [12]. Therefore, the enzymatic activity of CYP2A13 plays an important role in cancer development.

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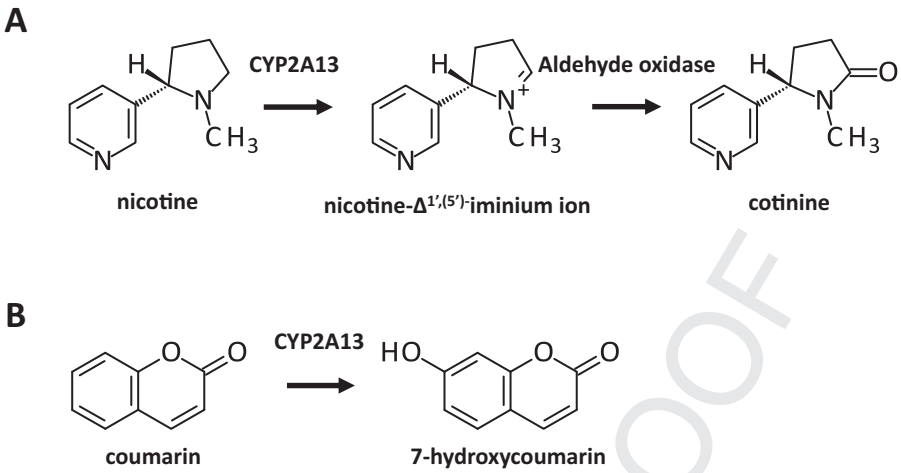

Fig. 1. Metabolic pathways from nicotine to cotinine catalyzed by CYP2A13 and aldehyde oxidase (A). Coumarin 7-hydroxylation metabolism catalyzed by CYP2A13 (B).

As shown in Table 1, several polymorphisms have been identified for the *CYP2A13* gene in addition to *CYP2A13*2* (http://www.cypalleles.ki.se/cyp2a13.htm) [Accessed Aug 9th, 2017]. The *in vitro* enzymatic activity of several of these variants has been characterized using a recombinant enzyme system such as *E. coli* cells [14,16,17]. Schlicht et al. reported that 3 substitutions (Asp158Glu, Arg257Cys, and Val323Leu) caused 2–3-fold decrease in enzymatic NNK α-hydroxylation activity [14]. *CYP2A13*4*, which did not show an absorbance increase near 450 nm, did not have the ability to metabolize 5-methoxypsoralen [16]. These polymorphisms may affect individual variations in cancer development.

To characterize the currently known 8 CYP2A13 variants, except for *CYP2A13*7*, which contains an immature stop codon (Arg101-Stop) that causes the disappearance of enzymatic activity, we evaluated their activity using nicotine as a substrate, following protein expression in 293FT cells. In addition, coumarin 7-hydroxylation catalyzed by CYP2A13 was measured to determine whether any functional changes observed were substrate-dependent.

2. Materials and methods

2.1. Chemicals

(−)-Nicotine, (−)-cotinine, (±)-cotinine-(methyl-d3) derivatives, coumarin, 7-hydroxycoumarin, and 4-methyl-7-hydroxycoumarin were purchased from Sigma-Aldrich (Tokyo, Japan). The UltraPool Human Liver Cytosol, 150-Donor Pool was purchased from Corning Incorporated (Corning, NY, USA). Oxidized β-nicotinamide-adenine dinucleotide phosphate oxidized form ($NADP^+$), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH), and β-nicotinamide-adenine dinucleotide phosphate reduced form (NADPH) were purchased from Oriental Yeast (Tokyo, Japan). The following reagents were purchased from the indicated sources: polyclonal anti-human CYP2A13 antibody (ab58740, Abcam, CB, UK); polyclonal anti-calnexin antibody (Enzo Life Sciences, Farmingdale, NY, USA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Triton N-101 and sodium cholate hydrate were obtained from Sigma-Aldrich. Sodium cyanide and cytochrome c from horse heart were purchased from Nacalai Tesque (Kyoto, Japan).

Table 1
Cytochrome P450 2A13 (CYP2A13) allelic variants characterized in this study.

Variants	Protein	Nucleotide changes	Amino acid changes
*CYP2A13*1*	CYP2A13.1		
*CYP2A13*2*	CYP2A13.2	74G > A	Arg25Gln
		3375C > T	Arg257Cys
*CYP2A13*3*	CYP2A13.3	1634_1635insACC	133_134insThr
		1706C > T	Asp158Glu
*CYP2A13*4*	CYP2A13.4	579G > A	Arg101Gln
*CYP2A13*5*	CYP2A13.5	7343T > A	Phe453Tyr
*CYP2A13*6*	CYP2A13.6	7465C > T	Arg494Cys
*CYP2A13*8*	CYP2A13.8	1706C > T	Asp158Glu
*CYP2A13*9*	CYP2A13.9	5294G > T	Val323Leu
*CYP2A13*10*	CYP2A13.10	74G > A	Arg25Gln
		3375C > T	Arg257Cys
		5792T > C	Ile331Thr

2.2. CYP2A13 cDNA cloning and construction of expression vectors

Plasmids carrying full-length *CYP2A13* cDNA fragments (GenBank accession no. AF209774.1) were obtained from GenScript (USA). Q3 *CYP2A13* cDNA fragments from plasmid DNA were PCR-amplified using a forward primer (5′-CACCATGCTGGCCTCAGGGCTGCTTC-3′) and a reverse primer (5′-TCAGCGGGGCAGGAAGCTCATGGTGTAG-3′) and *PfuUltra* High-Fidelity DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA), the underlined sequence in the forward primer were introduced for directional TOPO cloning. Following amplification, the wild-type *CYP2A13* fragments were subcloned into the pENTR/D-TOPO vector (ThermoFisher Scientific, Waltham, MA). A plasmid containing *CYP2A13*1* cDNA was used as a template to generate various *CYP2A13* allelic variant constructs (*CYP2A13*2*, *CYP2A13*4*–*CYP2A13*6*, *CYP2A13*8*, and *CYP2A13*9*), using the primer set for site-directed mutagenesis (Supplemental Table 1) and the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. Other *CYP2A13* constructs were generated from plasmids carrying other cDNA templates: *CYP2A13*3* from *CYP2A13*8* cDNA, and *CYP2A13*10* from *CYP2A13*2* cDNA. All wild-type and variant cDNAs prepared were confirmed by Sanger sequencing. Wild-type and CYP2A13 variant cDNAs were subcloned into the mammalian expression vector pcDNA3.4 (ThermoFisher Scientific).

2.3. Expression of CYP2A13 variants in 293FT cells

293FT cells, purchased from ThermoFisher Scientific, were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque) containing 10% fetal bovine serum at 37 °C under 5% CO_2. Cells were transfected with a plasmid (5 μg) encoding *CYP2A13* cDNA, using

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