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A novel *CYP2A6*20* allele found in African-American population produces a truncated protein lacking enzymatic activity

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Abstract

Human CYP2A6 is a cytochrome P450 (CYP) isoform responsible for the metabolism of nicotine, coumarin, tegafur, and valproic acid, and metabolic activation of nitrosamines. Genetic polymorphisms of the *CYP2A6* gene are a major causal factor of the large interindividual differences in nicotine metabolism. In the present study, we identified a novel allele, termed *CYP2A6*20*, in an African-American population. The allele possesses the deletion of two nucleotides in exon 4 resulting in a frame-shift from codon 196 and an early stop codon at 220 (exon 5) as well as three synonymous SNPs of G51A (G51A in cDNA), T5684C (T1191C), and C6692G (C1546G, 3′-untranslated region). The allele frequency in the African-American population ($n = 96$) was 1.6% (95% confidence interval, 0.6–4.5%). In contrast, the *CYP2A6*20* allele was not found in Caucasians (European-American) ($n = 185$), Japanese ($n = 184$) and Korean ($n = 209$) populations. To investigate the effects of the polymorphism on the enzymatic activities, we expressed a wild type or variant (deletion of two nucleotides) CYP2A6 together with NADPH-CYP reductase in *Escherichia coli*. SDS-PAGE and immunoblot analyses demonstrated that truncated CYP2A6 protein was produced from the variant allele, although detected mRNA was the predicted size by reverse transcriptional-polymerase chain reaction. Coumarin 7-hydroxylation and nicotine *C*-oxidation, which are typical CYP2A6 activities, were completely abolished in the *E. coli* membrane expressing the variant allele. In vivo nicotine metabolism was evaluated using the cotinine/nicotine ratio 2 h after the chewing of one piece of nicotine gum. Two *CYP2A6*1*/*CYP2A6*20* heterozygotes and a single *CYP2A6*17*/*CYP2A6*20* heterozygote revealed lower cotinine/nicotine ratios compared with *CYP2A6*1*/*CYP2A6*1* subjects (1.6 and 4.5, and 1.8 versus 9.5 ± 5.4, $n = 52$, respectively). We found a novel *CYP2A6*20* allele in African-American subjects which codes a truncated protein lacking enzymatic activity.

Keywords: Cytochrome P450; Genetic polymorphism; Interindividual variability; Racial difference; Coumarin 7-hydroxylation; Nicotine *C*-oxidation

1. Introduction

Cytochrome P450 (CYP) is a superfamily of hemoproteins, many of which can metabolize xenobiotics such as clinically used drugs, procarcinogens, and environmental pollutants. CYP2A6 is a major hepatic member of the CYP family in humans that metabolizes pharmaceutical agents such as coumarin, methoxyflurane, halothane, losigamone, letrozole, valproic acid, and disulfiram, and activates some procarcinogens such as 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone and *N*-nitrosodiethylamine [1]. Moreover, CYP2A6 is a major enzyme involved in nicotine *C*-oxidation to cotinine [2]. There are genetic polymorphisms in the *CYP2A6* gene. To date, over 30 alleles have been reported for the *CYP2A6* gene (http://www.imm.ki.se/CYPalleles/cyp2a6.htm). Concerning the alleles leading to the lack of or decreased activity, *CYP2A6*4* and *CYP2A6*9* are found in Asian populations with relatively high frequency, but rarely found in Caucasians [1]. *CYP2A6*7*, *CYP2A6*10*, and *CYP2A6*11* alleles are specifically found in Asians, whereas *CYP2A6*2* and *CYP2A6*12* are found in Caucasians [1,3] and *CYP2A6*17* are found in only African-Americans [4]. Thus, there are racial differences in the allele frequencies.

In our previous studies, we have reported the relationships between interindividual differences in nicotine meta-

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bolism and genetic polymorphisms of the *CYP2A6* gene in Japanese and Korean subjects [5–7]. Recently, we found a *CYP2A6*17* allele which is specific for African-Americans [4] and leads to decreased enzymatic activity. Subsequently, an individual was identified that was genotyped as *CYP2A6*1A*/*CYP2A6*1G* and phenotyped as having a low cotinine/nicotine ratio similar to that observed in a *CYP2A6*17* homozygote. The previously described *CYP2A6*1G* allele was predicted to have normal functional [8]. In the present study, further DNA sequence analyses of this individual revealed a novel allele, *CYP2A6*20*, which results in a frame-shift and early stop codon. We have determined the effects of this new *CYP2A6* polymorphism on enzymatic activity in vitro and in vivo.

2. Materials and methods

2.1. Chemicals and regents

Taq polymerase and Ex Taq polymerase were obtained from Greiner Japan (Tokyo, Japan) and Takara (Shiga, Japan), respectively. Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Restriction enzymes were purchased from Takara, Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA) and Fermentas (Hanover, MD). Recombinant CYP2A6 expressed in baculovirus-infected insect cells was from BD Gentest (Worburn, MA) and polyclonal rabbit anti-human CYP2A6 antibody was from Nosan (Yokohama, Japan). Nicotine, cotinine, coumarin, and 7-hydroxycoumarin were from Sigma–Aldrich (St. Louis, MO). Nicorette® (nicotine gum containing 2 mg of nicotine) was obtained from Pfizer Japan (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available.

2.2. Genomic DNA

This study was approved by the Human Studies Committee of Washington University School of Medicine (St. Louis, MO) and the Ethics Committee of Kanazawa University (Kanazawa, Japan) and Soonchunhyang University Hospital (Chonan, Korea). Written informed consent was obtained from all subjects. Blood samples were collected from 185 Caucasian (European-American), 96 African-American, 184 Japanese, and 209 Korean subjects. Genomic DNA was extracted from peripheral lymphocytes using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

2.3. In vivo phenotyping of nicotine metabolism

Written informed consent was obtained from 96 healthy African-American subjects. All subjects were non-smokers. The phenotyping of in vivo nicotine metabolism was performed according to the method established in our previous study [5]. Briefly, the subjects chewed one piece of nicotine gum for 30 min, chewing for 10 s per 30 s. Blood samples were collected from a cubital vein just before and 2 h after the start of chewing. The concentrations of nicotine and cotinine in the plasma samples were determined by HPLC as described previously [9]. The cotinine/nicotine ratio of the plasma concentration was calculated as an index of nicotine metabolism.

2.4. Genotyping of CYP2A6 alleles

The genotyping of *CYP2A6*1B* [8], *CYP2A6*1F* [8], *CYP2A6*1G* [8], *CYP2A6*1X2* [7], *CYP2A6*2* [10], *CYP2A6*3* [10], *CYP2A6*4A* [8], *CYP2A6*4D* [8], *CYP2A6*5* [5], *CYP2A6*6* [7], *CYP2A6*7* [7], *CYP2A6*8* [7], *CYP2A6*9* [11], *CYP2A6*10* [7], *CYP2A6*11* [7], *CYP2A6*12* [8], *CYP2A6*13* [12], *CYP2A6*14* [12], *CYP2A6*15* [12], *CYP2A6*16* [12], and *CYP2A6*17* [4] were performed as described previously.

2.5. Sequence analyses of all exons and exon–intron junctions of CYP2A6 gene

To examine the nucleotide sequences of the *CYP2A6* gene in a subject with a low cotinine/nicotine ratio (1.6) and *CYP2A6*1A/CYP2A6*1G* genotype, we performed direct sequence analyses as described previously [4]. The PCR product with the primers of 2A6int1 and 2A6int4R [4] were subcloned into pT7Blue T-Vector (Novagen, Madison, WI). The plasmid DNA was purified by a QIAGEN Plasmid Midi kit (QIAGEN, Valencia, CA) and submitted to DNA sequencing using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) with T7F and T7R (Amersham Pharmacia Biotech). DNA sequences were analyzed on a Long-Read Tower DNA sequencer (Amersham Pharmacia Biotech).

2.6. Genotyping of CYP2A6*20 allele

An allele specific-polymerase chain reaction (AS-PCR) method was developed for the genotyping of *CYP2A6*20* allele. Sense primers were 2A6*20-wt and 2A6*20-mut which are specific to *CYP2A6*1* and *CYP2A6*20*, respectively, and the antisense primer was 2A6int4R2 (Table 1). The reaction mixture contained genomic DNA (0.5 μg), 1× PCR buffer [2 mM Tris–HCl (pH 8.0), 10 mM KCl, 0.01 mM EDTA, 0.1 mM DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, 2 mM $MgCl_2$], 0.25 mM dNTPs, 0.4 μM each primer, and 1 U of Ex Taq DNA polymerase (Takara) in a final volume of 25 μl. After an initial denaturation at 94 °C for 3 min, the amplification was performed by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 25 s for 31 cycles, followed by a final extension at 72 °C for 5 min. An aliquot (10 μl) of the PCR product was analyzed by

Table 1
Primers used in the present study

Primer	Sequence	Location
2A6ex1	5′-GCTGAACACAGAGCAGATGTACA-3′	Exon 1
2A6*20-wt	5′-CGCTTTGACTATAAGGACAA-3′	Exon 4
2A6*20-mut	5′-CGCTTTGACTATAAGGACAG-3′	Exon 4
2A6*20-SDM-S	5′-GGACCGCTTTGACTATAAGGACAGAGTTCCTGTCACTGTTGC-3′	Exon 4
2A6*20-SDM-AS	5′-GCAACAGTGACAGGAACTCTGTCCTTATAGTCAAAGCGGTCC-3′	Exon 4
2A6int4R2[a]	5′-CTTGGAGACAGGGTATTGGA-3′	Intron 4
2Aex9AS[b]	5′-TGGTGAAGAAGAGAAAGAG-3′	Exon 9

[a] From [12].
[b] From [4].

electrophoresis with 2% agarose gel. The *CYP2A6*1* allele was amplified with the primer set of 2A6*20-wt and 2A6int4R2 (274 bp) and the *CYP2A6*20* allele was amplified with the primer set of 2A6*20-mut and 2A6int4R2 (272 bp). Using positive controls of which the sequences were analyzed, we confirmed that the primers 2A6*20-wt and 2A6*20-mut can specifically anneal to the wild type and mutant type, respectively.

2.7. Construction of expression plasmids

A bicistronic construct consisting of the coding sequence of CYP2A6 followed by that of NADPH-cytochrome P450 reductase (NPR) was previously constructed in the pCW expression vector [4]. To construct the expression vector of *CYP2A6*20*, two deoxyadenosines (586 and 587) were deleted from the wild-type CYP2A6 cDNA by site-directed mutagenesis with a QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used for the site-directed mutagenesis were 2A6*20-SDM-S and 2A6*20-SDM-AS (Table 1). The deleted two deoxyadenosines were between the underlined nucleotides. The nucleotide sequences of the constructed CYP2A6*20 cDNA were confirmed by DNA sequence analysis. The wild-type or variant plasmids were transformed to *Escherichia coli* JM109.

2.8. Detection of CYP2A6 mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

E. coli JM109 cells transformed with plasmid DNA were grown overnight at 37 °C with shaking at 170 rpm in LB medium containing 25 μg/ml ampicillin. The culture was diluted 1:100 into TB medium containing 100 μg/ml ampicillin and additives (0.5 mM δ-aminolevulinic acid, 1.0 mM isopropyl β-D-thiogalactoside, trace salts, and 1.0 mM thiamine). The expression cultures (100 ml) were grown at 30 °C with shaking at 120 rpm for 30 h in 500 ml triple-baffled flasks. Total RNA was extracted from the *E. coli* using ISOGEN (Nippon Gene, Tokyo, Japan) according to the protocol supplied by the manufacturer. The RNA concentration and its purity were determined by ultraviolet spectroscopy. Total RNA (4 μg) was added to a reaction mixture containing 150 ng of random hexamer, 5 U of Moloney murine leukemia virus-reverse transcriptase, 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM dNTPs in a final volume of 40 μl. The reaction mixture was incubated at 37 °C for 1 h and heated at 70 °C for 10 min to inactivate the enzyme. A 1 μl portion of the RT mixture was added to a PCR mixture containing 1× PCR buffer [67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, and 0.02% gelatin], 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 μM of each primer, and 1 U of Taq DNA polymerase (Greiner) in a final volume of 25 μl. The primers were 2A6ex1 and 2Aex9AS [4] (Table 1). After an initial denaturation at 94 °C for 3 min, the amplification was performed by denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min 15 s for 28 cycles. The expected size of the PCR products (exons 1–9) was 1216 bp (wild type) or 1214 bp (variant). An aliquot (5 μl) of the PCR product was analyzed by electrophoresis with 1.2% agarose gel.

2.9. E. coli membrane preparations and enzyme assay

E. coli membranes expressing wild or variant CYP2A6/NPR were prepared as described previously [4]. The CYP content and protein concentration were determined according to a method described previously [13,14]. NADPH-cytochrome *c* reductase activity was determined as described previously [15,16] using $\Delta\varepsilon_{550} = 21.1\ mM^{-1}\ cm^{-1}$ and the content was calculated using a specific activity of 3.0 μmol reduced cytochrome *c*/min/nmol NPR based on purified rabbit NPR preparations [17]. Coumarin 7-hydroxylase [18], nicotine *C*-oxidase [2] activities were determined as described previously. The concentrations of coumarin and nicotine were 5 and 200 μM, respectively, which were approximately five-fold higher than the K_m values. Data were expressed as the mean ± S.D. of three independent determinations in duplicate. The reproducibility of the data was confirmed using three independent membrane preparations.

2.10. SDS-PAGE and immunoblot analyses of CYP2A6

SDS-polyacrylamide gel electrophoresis and immunoblot analysis for the *E. coli* membranes expressing

CYP2A6 protein were performed according to Laemmli [19]. The *E. coli* membranes were separated on 15% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Recombinant CYP2A6 expressed in baculovirus-infected insect cells was applied as a positive control. Polyclonal rabbit anti-human CYP2A6 antibody was used. Biotinylated anti-rabbit IgG and VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) were used for diaminobenzidine staining. The other of the duplicate polyacrylamide gel was stained with coomassie brilliant blue.

3. Results

3.1. Identification of novel alleles of CYP2A6 gene

With the sequence analyses in a subject (*CYP2A6*1A/CYP2A6*1G*) with low potency of nicotine metabolism, we found a novel *CYP2A6* allele with two deleted deoxyadenosines in exon 4 (2140 and 2141 in genomic DNA; 586 and 587 in cDNA). This allele also possessed three SNPs with no amino acid change of G51A (G51A, V17V), T5684C (T1191C, S397S), and C6692G (C1546G, 3′-

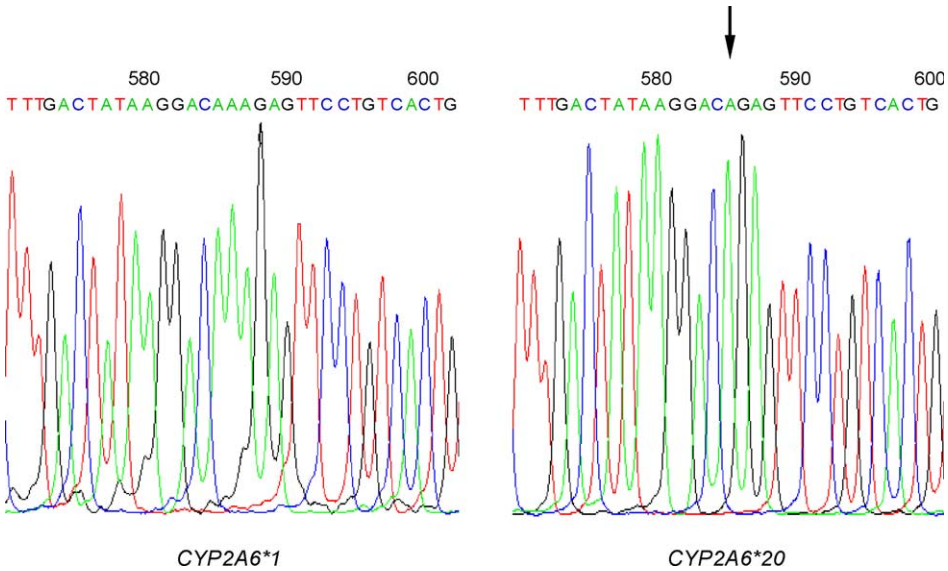

Fig. 1. Electrophoregrams of the sequence of exon 4 in the *CYP2A6*1* and *CYP2A6*20* alleles. Arrow indicates the location of the deletion of two deoxyadenosines in *CYP2A6*20* allele. The nucleotide numbering refers to the ATG in translation starting with A as 1 with the reference cDNA sequence of NM000762.

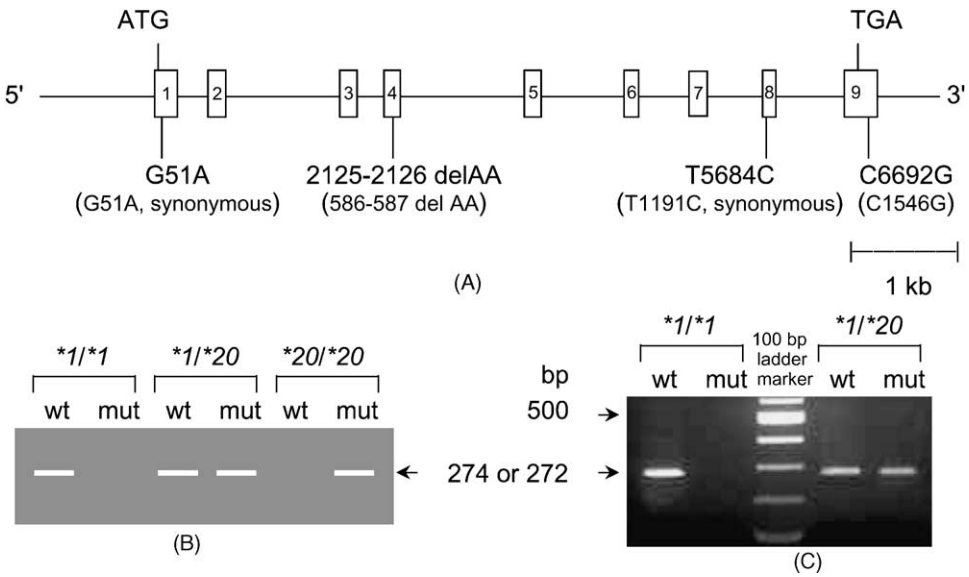

Fig. 2. Characteristics of *CYP2A6*20* allele. (A) Schematic structure of *CYP2A6*20* allele. Boxes represent exons and lines represent introns. The nucleotide numbers on exons refer to the genomic sequence of NG000008. The corresponding numbers on cDNA (NM000762) are shown in parentheses. The nucleotide numbering refers to the ATG in translation starting with A as 1. (B) Schematic AS-PCR patterns for genotyping of *CYP2A6*20* allele. PCR products with the wild- and mutant-specific primers are 274 and 272 bp, respectively. (C) Representative photograph of AS-PCR patterns for different genotypes.

untranslated region) (Fig. 1). The allele was termed *CYP2A6*20* by the Human CYP Allele Nomenclature Committee. The schematic structure of the *CYP2A6*20* allele is shown in Fig. 2. The deletion of two nucleotides causes a frame-shift from codon 196 and an early stop codon at 220 (exon 5).

3.2. Allele frequencies of the CYP2A6*20 allele

We performed the genotyping analysis for the *CYP2A6*20* allele in 185 Caucasians, 96 African-Americans, 184 Japanese, and 209 Koreans. The *CYP2A6*20* allele was not found in Caucasians, Japanese, and Koreans. In 96 African-Americans, three subjects were heterozygotes of the *CYP2A6*20* allele, resulting in an allele frequency of 1.6% (95% confidence interval, 0.6–4.5%). The sequences in the three subjects were confirmed as the *CYP2A6*20* allele with DNA direct sequencing analyses.

3.3. Expression of wild or variant CYP2A6 in E. coli

To confirm the expression of CYP2A6 mRNA in *E. coli* cells transformed with the wild type or variant CYP2A6/NPR plasmid, we performed RT-PCR analyses to amplify from exons 1 to 9. As a result, the expressions of both wild type and variant CYP2A6 mRNA were observed (Fig. 3A).

When the *E. coli* membrane preparations were examined by SDS-PAGE, the preparation from the CYP2A6.1 bac-

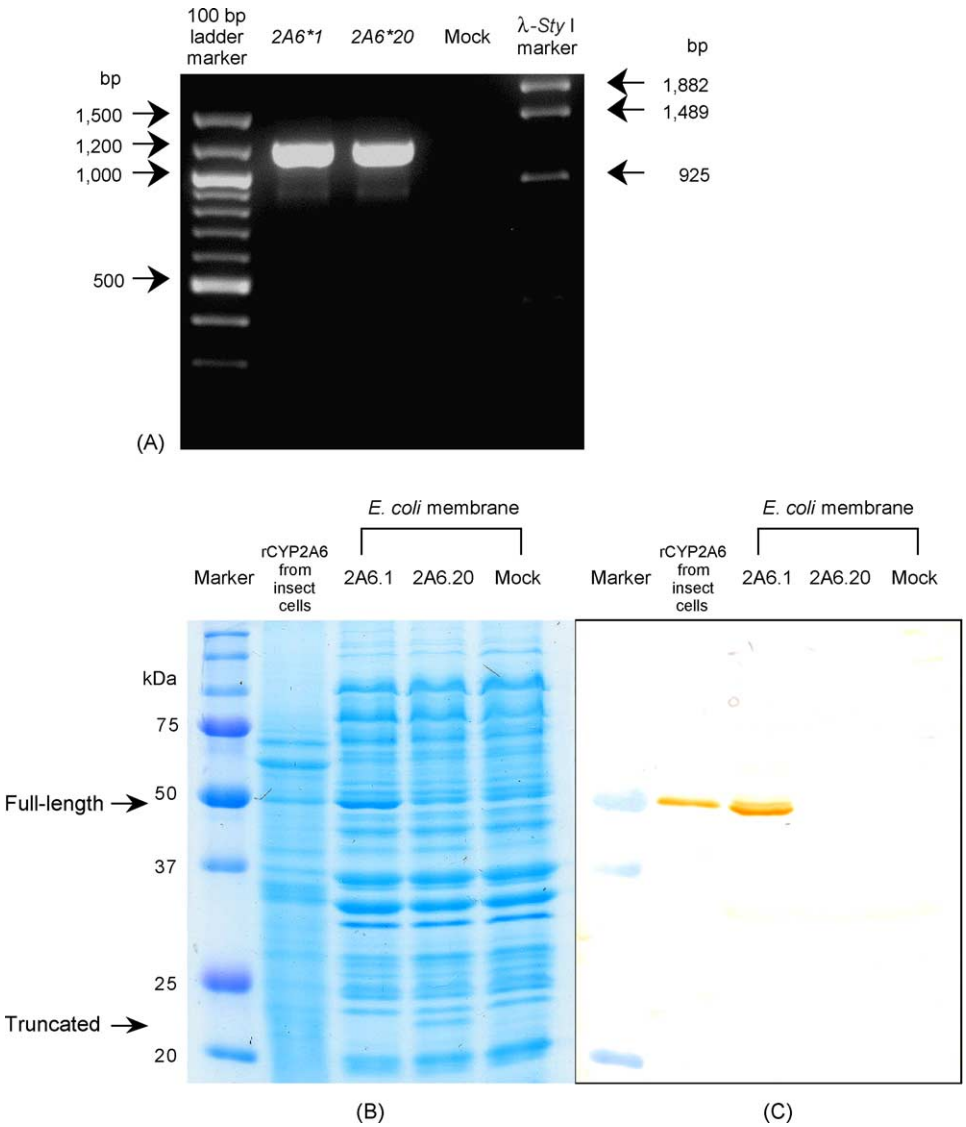

Fig. 3. Expression of CYP2A6.1 and CYP2A6.20 in transformed *E. coli*. (A) RT-PCR analysis using the primer set of 2A6ex1 and 2Aex9AS. *E. coli* transformed with wild or variant CYP2A6/NPR showed an obvious band at 1216 bp. The band was not observed in mock (pCW vector transformed) *E. coli*. The markers used are a 100 bp ladder and λ-*Sty* I. (B) SDS-PAGE analysis of *E. coli* membrane expressing CYP2A6. Recombinant CYP2A6 from baculovirus-infected insect cells (1.6 pmol), *E. coli* membrane (40 μg) of CYP2A6.1, CYP2A6.20, and mock were loaded on 15% acrylamide gel and visualized by staining with Coomassie brilliant blue. Upper arrow indicates full-length CYP2A6.1 protein of 49 kDa, and lower arrow indicates truncated CYP2A6 (CYP2A6.20) of 22 kDa. (C) Immunoblot analysis using rabbit anti-human CYP2A6 antibody. *E. coli* membrane expressing wild CYP2A6.1 showed a distinct band with the same mobility as the recombinant CYP2A6 from baculovirus-infected insect cells.

teria exhibited a band not observed in the mock transformed cells and which had the same mobility as the 49 kDa recombinant CYP2A6 protein from baculovirus-infected cells from Gentest (Fig. 3B). Both proteins also reacted with the anti-human CYP2A6 antibody (Fig. 3C). In contrast, a 49 kDa band was not observed in the *E. coli* membrane preparation from cells expressing CYP2A6.20 nor was any immunoreactive product detected (Fig. 3B and C). However, a band at 22 kDa corresponding the predicted, 219 amino acid truncated peptide, was observed by Coomassie Brilliant Blue staining (Fig. 3B).

3.4. Enzymatic activities of the recombinant CYP2A6.20

The *E. coli* membrane expressing CYP2A6.1 showed a typical CO-difference spectrum (Fig. 4). The CYP content was 26.1 pmol/mg protein. In contrast, no peak at 450 nm was observed with the *E. coli* membranes expressing CYP2A6.20 or mock. The activities of NPR in the *E. coli* membrane expressing CYP2A6.1 or CYP2A6.20 were 0.76 and 0.57 reduced cytochrome *c*/min/mg protein, respectively. The NPR activity could not be detected with the mock membrane. According to the specific activity of 3.0 μmol reduced cytochrome *c*/min/nmol NPR, the molar ratio of the NPR to CYP in the membrane expressing CYP2A6.1 was calculated to be approximately 10. Reproducibility of the data was confirmed with three independent membrane preparations.

The *E. coli* membrane preparations were used for the determination of the catalytic activities for coumarin 7-hydroxylation and nicotine *C*-oxidation. In the recombinant CYP2A6.1, the coumarin 7-hydroxylase (5 μM coumarin) and nicotine *C*-oxidase (200 μM nicotine) activities were 3.9 ± 0.1 pmol/min/pmol CYP and 13.6 ± 3.4 pmol/min/pmol CYP, respectively ($n = 3$). In contrast, the recombinant CYP2A6.20 did not show detectable activities.

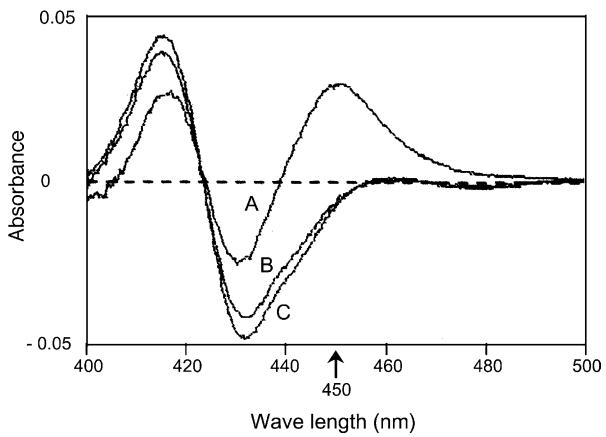

Fig. 4. CO-difference spectra of *E. coli* membrane expressing CYP2A6.1 (A), CYP2A6.20 (B), and mock (C). *E. coli* membrane expressing CYP2A6.1 showed a typical peak at 450 nm, whereas no peak was observed in *E. coli* membranes of CYP2A6.20 and mock.

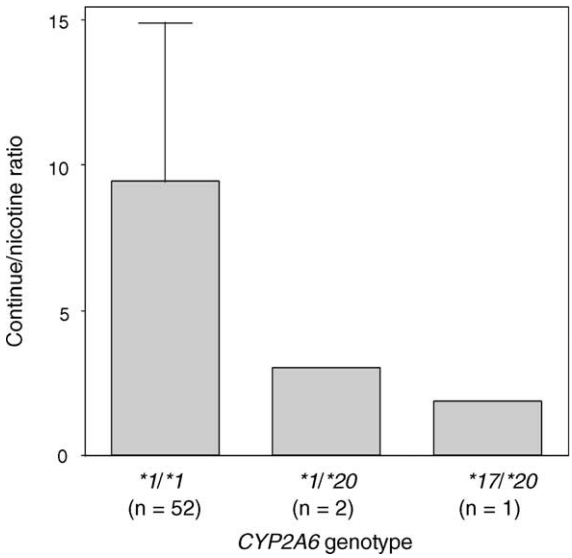

Fig. 5. Cotinine/nicotine ratios in plasma 2 h after chewing one piece of nicotine gum in 55 African-American subjects. All subjects were non-smokers. The numbers of subjects are shown in parentheses. Data are expressed as mean ± S.D.

3.5. In vivo nicotine metabolism in the subjects possessing the CYP2A6*20 allele

The cotinine/nicotine ratios in plasma were calculated as an index of nicotine metabolism in the subjects of the different *CYP2A6* genotype groups (Fig. 5). Two *CYP2A6*1*/*CYP2A6*20* heterozygotes and a single *CYP2A6*17*/*CYP2A6*20* heterozygote revealed lower cotinine/nicotine ratios compared with *CYP2A6*1*/*CYP2A6*1* subjects (1.6 and 4.5, and 1.8 versus 9.5 ± 5.4, $n = 52$, respectively). These results suggested that the decreased in vivo nicotine metabolism resulted from the *CYP2A6*20* allele.

4. Discussion

Until now, about 30 alleles have been reported for *CYP2A6*. Most alleles possess a single nucleotide substitution (such as *CYP2A6*2*, *CYP2A6*5*, and *CYP2A6*7*). Others are the alleles produced by cross-over with *CYP2A7* (such as *CYP2A6*1B*, *CYP2A6*1X2*, *CYP2A6*4*, and *CYP2A6*12*). In the present study, we first found a novel allele with two nucleotide deletions. The *CYP2A6*20* allele has a deletion of two nucleotides (586–587delAA) in exon 4 as well as three silent SNPs. The deletion of two nucleotides causes a frame-shift and an early stop codon at 220 in exon 5. With the SDS-PAGE analysis of recombinant CYP2A6, we confirmed that the *CYP2A6*20* allele produces a truncated protein. Unexpectedly, in the immunoblot analysis, the truncated protein did not react with anti-human CYP2A6 antibody, despite it was polyclonal. Although there is no conclusive explanation, it appeared

possible that the epitopes of the antibody would be after codon 196. A cystein residue, which binds to heme, is located at codon 439 in CYP2A6 protein. Thus, the recombinant CYP2A6.20 did not show the detectable CO-difference spectrum. The enzymatic activities toward coumarin 7-hydroxylation and nicotine *C*-oxidation were completely abrogated in the recombinant CYP2A6.20 with the substrate concentrations (5 and 200 μM, respectively) that were approximately five-fold higher than the K_m values [2,20]. Furthermore, no activity was detected with higher substrate concentrations (25 μM coumarin and 500 μM nicotine). Thus, we found that the *CYP2A6*20* allele encodes a truncated protein lacking enzymatic activity.

According to the findings of the *CYP2A6*20* allele, the subject with a low cotinine/nicotine ratio (1.6) with *CYP2A6*1A/CYP2A6*1G* were re-genotyped as *CYP2A6*1G/CYP2A6*20*. Because of the low allele frequency of the *CYP2A6*20* allele (1.6%), a homozygote of the *CYP2A6*20* allele was not found in 96 African-American subjects in the present study. Therefore, it was difficult to investigate the effects of the *CYP2A6*20* allele on the in vivo nicotine metabolism. However, two heterozygotes of *CYP2A6*1/CYP2A6*20* showed lower cotinine/nicotine ratios compared with the homozygotes of the wild type, suggesting that nicotine metabolism was decreased with the *CYP2A6*20* allele. In addition, a subject with *CYP2A6*17/CYP2A6*20* also showed a low cotinine/nicotine ratio. Previously, we have reported in an in vitro study that CYP2A6.17 protein shows significantly decreased enzymatic activity (40–60% decreases) compared with CYP2A6.1 protein [4]. Thus, the low cotinine/nicotine ratio in the subject with *CYP2A6*17/CYP2A6*20* would be due to CYP2A6.17 as well as CYP2A6.20.

The *CYP2A6*20* allele was found in African-Americans but not found in Caucasians, Japanese, and Koreans. The phenomenon is reminiscent of the *CYP2A6*17* allele [4]. In addition to the *CYP2A6*17* allele (with relatively high frequency: 9.4%) which leads to decreased enzymatic activity, the *CYP2A6*20* allele may also contribute to the interindividual difference in nicotine metabolism in African-Americans.

In conclusion, we found a novel *CYP2A6*20* allele in African-American subjects. This is the first report of a *CYP2A6* allele leading to a truncated protein by the deletion of nucleotides. The in vitro study revealed that the protein coded by *CYP2A6*20* had completely abrogated enzymatic activity. In vivo nicotine metabolism was also diminished with the *CYP2A6*20* allele.

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References

[1] Nakajima M, Kuroiwa Y, Yokoi T. Interindividual differences in nicotine metabolism and genetic polymorphisms of human CYP2A6. Drug Metab Rev 2002;34:865–77.
[2] Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, et al. Role of human cytochrome P4502A6 in *C*-oxidation of nicotine. Drug Metab Dispos 1996;24:1212–7.
[3] Oscarson M, McLellan RA, Asp V, Ledesma M, Ruiz MLB, Sinues B, et al. Characterization of a novel *CYP2A7/CYP2A6* hybrid allele (*CYP2A6*12*) that causes reduced CYP2A6 activity. Hum Mutat 2002;20:275–83.
[4] Fukami T, Nakajima M, Yoshida R, Tsuchiya Y, Fujiki Y, Katoh M, et al. A novel polymorphism of human *CYP2A6* gene *CYP2A6*17* has an amino acid substitution (V365M) that decreases enzymatic activity in vitro and in vivo. Clin Pharmacol Ther 2004;76:519–27.
[5] Nakajima M, Kwon J-T, Tanaka N, Zenta T, Yamamoto Y, Yamamoto H, et al. Relationship between interindividual differences in nicotine metabolism and *CYP2A6* genetic polymorphism in humans. Clin Pharmacol Ther 2001;68:72–8.
[6] Kwon JT, Nakajima M, Chai S, Yom YK, Kim HK, Yamazaki H, et al. Nicotine metabolism and CYP2A6 allele frequencies in Koreans. Pharmacogenetics 2001;11:317–23.
[7] Yoshida R, Nakajima M, Watanabe Y, Kwon J-T, Yokoi T. Genetic polymorphisms in human *CYP2A6* gene causing impaired nicotine metabolism. Br J Clin Pharmacol 2002;54:511–7.
[8] Nakajima M, Yoshida R, Fukami T, McLeod HL, Yokoi T. Novel human *CYP2A6* alleles confound gene deletion analysis. FEBS Lett 2004;569:75–81.
[9] Nakajima M, Yamamoto T, Kuroiwa Y, Yokoi T. Improved highly sensitive method for determination of nicotine and cotinine in human plasma by high-performance liquid chromatography. J Chromatogr B 2000;742:211–5.
[10] Nakajima M, Yamagishi S, Yamamoto H, Yamamoto T, Kuroiwa Y, Yokoi T. Deficient cotinine formation from nicotine is attributed to the whole deletion of the CYP2A6 gene in humans. Clin Pharmacol Ther 2000;67:57–69.
[11] Yoshida R, Nakajima M, Nishimura K, Tokudome S, Kwon J-T, Yokoi T. Effects of polymorphism in promoter region of human *CYP2A6* gene (*CYP2A6*9*) on expression level of messenger ribonucleic acid and enzymatic activity in vivo and in vitro. Clin Pharmacol Ther 2003;74:69–76.
[12] Fukami T, Nakajima M, Higashi E, Yamanaka H, Sakai H, McLeod HL, et al. Characterization of novel *CYP2A6* polymorphic alleles (*CYP2A6*18* and *CYP2A6*19*) that affect enzymatic activity. Drug Metab Dispos, in press.
[13] Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. J Biol Chem 1964;239:2370–8.
[14] Bradford A. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[15] Williams Jr CH, Kamin H. Microsomal triphosphopyridine nucleotide-cytochrome *c* reductase of liver. J Biol Chem 1962;237:587–95.
[16] Yasukochi Y, Masters BS. Some properties of detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. J Biol Chem 1976;251:5337–44.

[17] Parikh A, Gillam EM, Guengerich FP. Drug metabolism by *Escherichia coli* expressing human cytochromes P450. Nat Biotechnol 1997;15:784–8.

[18] Ohyama K, Nakajima M, Suzuki M, Shimada N, Yamazaki H, Yokoi T. Inhibitory effects of amiodarone and its *N*-deethylated metabolite on human cytochrome P450 activities: prediction of in vivo drug interactions. Br J Clin Pharmacol 2000;49:244–53.

[19] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[20] Daigo S, Takahashi Y, Fujieda M, Ariyoshi N, Yamazaki H, Koizumi W, et al. A novel mutant allele of the *CYP2A6* gene (*CYP2A6*11*) found in a cancer patient who showed poor metabolic phenotype towards tegafur. Pharmacogenetics 2002;12: 299–306.