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The Significance of the Homozygous *CYP*2A6 Deletion on Nicotine Metabolism: A New Genotyping Method of *CYP*2A6 Using a Single PCR–RFLP

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A convenient and specific *CYP*2A6 genotyping method was developed in this study. This method consisting of a single PCR–RFLP is capable of resolving the genotype into either *CYP*2A6*1 (wild type), *CYP*2A6*2, or *CYP*2A6*3. Among 252 Japanese persons genotyped, 241 were genotyped as the wild type, 1 as an unknown variant, and none as either *CYP*2A6*2 or *CYP*2A6*3. A homozygous deletion was found in the 10 remaining subjects. To clarify the metabolic significance of this deletion in the whole human body, urinary cotinine, the principal metabolite of nicotine, was analyzed subsequent to smoking. Cumulated urinary cotinine excretion in the homozygously *CYP*2A6-deleted individuals was about one-seventh compared to the control group (wild type). This study provides a firm experimental basis for correlating genotypic characterization of *CYP*2A6 with phenotypic expression of nicotine metabolism.

Cytochrome P450 (*CYP*) superfamily is the principal enzyme system involved in the metabolism of xenobiotics and endogenous compounds. Each *CYP* has a subfamily; such as *CYP*2A, *CYP*2B, and *CYP*2F that form gene clusters in 350 kb on human chromosome 19 (1). Genetic polymorphisms of *CYP*1A1, *CYP*2D6, and *CYP* 2E1 have been reported along with their metabolic and clinical significance (2–6). CYP2A6 is responsible for coumarin-7-hydroxylase activity in humans and is also involved in the metabolic activation of several procarcinogens including aflatoxin B1 and 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) (7, 8). In addition, nicotine and cotinine are also thought to be selective substrates of metabolism by CYP2A6 (9–11).

The genetic polymorphisms of *CYP*2A6 have been characterized, as variant 1 (*CYP*2A6*2) and variant 2 (*CYP*2A6*3) besides with the wild genome type, *CYP*2A6*1 (12). These variants are a result of amino acid exchanges and are thought to be enzymatically inactivated (13, 14). Furthermore, whole and/or partial *CYP*2A6 gene deletion also has been reported recently (15–18). *CYP*2A subfamily is composed of *CYP*2A6, *CYP*2A7, and *CYP*2A13, all of which share a high sequence homology. Moreover, two kinds of *CYP*2A7 pseudogenes have been reported (14). Due to this high degree of homology, a more specific *CYP*2A6 amplification system is required to avoid misjudgment due to coamplification of other genes in this highly conserved subfamily. Fernandez-Salguero *et al.* (14) and Oscarson *et al.* (19) had previously reported the analytical methods for detection the genetic polymorphism of *CYP*2A6 using a combination of two sets of the PCR system (nested PCR). However, Fernandez-Salguero's method required more than 7.8 kb length of DNA as a template for the first step PCR amplification and Oscarson's method targeted only *CYP*2A6*1 and *CYP*2A6*2, not *CYP*2A6*3.

In this study, a convenient and specific method for *CYP*2A6 genotyping system was developed, which used a single PCR method combined with the restriction enzyme fragment length polymorphism (RFLP) analysis for the identification of all three genotypes and deletions of *CYP*2A6. *CYP*2A6 genotype was studied in 252 healthy Japanese individuals by this original method.

Nicotine is a major constituent of tobacco, playing a crucial role in establishing and maintaining tobacco dependence. As the main metabolic pathway of nicotine, seventy to eighty percent of nicotine is converted to cotinine mainly by *CYP*2A6. Individuals who lack homozygous *CYP*2A6 alleles are suspected to have diminished or no enzymatic activity toward nicotine and would be anticipated to have markedly decreased uri-

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nary excretion of cotinine, if any other metabolic enzymes do not compensate for lack of CYP2A6. To investigate the metabolic significance of *CYP*2A6 polymorphism on nicotine metabolism in the whole human body, we examined the urinary cotinine excretion in volunteers who had undergone a standardized smoking period.

METHODS

Subjects. A genotyping analysis was carried out using healthy Japanese individuals (197 men and 55 women). Genomic DNA from peripheral blood was prepared by a DNA extractor (Applied Biosystems, Model 340A). All subjects gave their informed consent.

Genotyping of CYP2A6 by Kd1F/E3R PCR. The single PCR and RFLP methods was used to identify the *CYP*2A6 wild and variant alleles. *CYP*2A6 specific PCR was accomplished with the primer pair Kd1F (5′-CCT GAT CGA CTA GGC GTG GTA) and E3R (5′-TCG TCC TGG GTG TTT TCC TTC) yielding a single 215-base pair (bp) product. Forward primer Kd1F was set overlapping intron 2 and exon 3. For the reverse primer, we used E3R, which was designed in intron 3 by Fernandez-Salguero *et al.* (14). Each primer annealing site is shown in Fig. 1A. The reaction mixture contained approximately 100 ng genomic DNA, 2 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 U of rTaq polymerase (TOYOBO, Japan) in 1× reaction buffer (TOYOBO, Japan) in a total volume of 50 μl. Amplification was performed by denaturing at 94°C for 30 s, annealing at 56°C for 30 s and extending at 72°C for 30 s for 35 cycles using a program temp control system PC-701 (ASTEC, Japan). PCR products were identified in 4% agarose (Bio-Rad) gels stained with ethidium bromide. 8 μl each of the PCR products were then digested without further purification by the restriction enzymes, *Msp*I, *Xcm*I, and *Dde*I in a total volume of 20 μl. The digested PCR products were electrophoresed in 4% agarose gels stained with ethidium bromide. The fragment patterns determined the presence of the *CYP*2A6*1, *CYP*2A6*2 and *CYP*2A6*3 as indicated in Fig. 1B.

The PCR product was sequenced using an Applied Biosystems 373S DNA sequencer following the protocol provided by the manufacturer.

Smoking challenge. A challenge test of smoking was carried out on the six smokers, with homozygously deleted *CYP*2A6 exon 3. Five age and body weight-matched smokers who were wild type for *CYP*2A6 were used as controls. All subjects were confirmed to be in good health, and not to be taking any drugs or under medical treatment. Written consent was obtained from each subject. The study was approved by the Ethical Committee of University of Occupational and Environmental Health. Cigarette smoking by all subjects was prohibited from bedtime. All test subjects smoked the same kind of cigarettes (Mild Seven [Japan Tobacco Co.], labeled as containing 1.1 mg nicotine and 13 mg tar) from 9:30 to 10:30 at the rate of 1 cigarette/10 min. Subjects had to smoke all cigarettes down to the same length. Urine samples (total urine) were collected before and 0, 1.5, 3, 6, 9, and 12 h after the end of smoking and again the next morning.

Analysis of urinary cotinine. Urinary cotinine was analyzed by the ion pair reversed high performance liquid chromatography (HPLC) method, as described by Takeda *et al.* (20) with a minor modification. Urine (1 ml) was extracted with 3 ml of dichloromethane (CH_2Cl_2) after adding 0.3 ml of 3 N NaOH. After centrifugation (1500 rpm for 10 min), 1.5 ml of the CH_2Cl_2 layer was transferred to a 1.5-ml bottle and evaporated by nitrogen gas. The residue was dissolved in 0.5 ml of distilled water. Using an autosampler, 50 μl of the solution was applied to the HPLC (Hitachi: L-7200 Autosampler, L-7100 Intelligent Pump, L-7300 Column Oven, L-4200 UV-VIS Detector at 254 nm and L-7500 Chromato Integrator). The mobile phase was 20 mM of KH_2PO_4 (pH 4.7) in water containing 3 mM of 1-decane sulfonate and acetonitrile (85:15) at a flow rate of 1.0 ml/min on a TOSOH TSK-gel ODS-80TM column. The concentration of urinary cotinine was expressed after correction for the specific gravity of urine of 1.020.

RESULTS

Specific PCR for amplification of CYP2A6 and genotyping. A primer pair was designed to specifically amplify *CYP*2A6 with single PCR and to avoid coamplification of *CYP*2A13, *CYP*2A7 and *CYP*2A7 pseudogenes. *CYP*2A6*2 has only one base substitution at exon 3 in the coding region, and one of the best substitution sites of *CYP*2A6*3 is located at exon 3. Therefore, the region around exon 3 was selected as the PCR target. To avoid an extension on *CYP*2A7 or *CYP*2A13, the forward primer Kd1F was set to overlap intron 2 and exon 3, where 2 bases at the 3 prime end were identical to only *CYP*2A6 (Fig. 1A). The reverse primer, E3R, does not have a homology site with *CYP*2A13 intron 3.

The genotype of the Kd1F/E3R PCR product (215 bp) was determined according to RFLP. *CYP*2A6*1 has 2 digestion sites of MspI, but not of *Xcm*I nor *Dde*I within the amplified region. In contrast to *CYP*2A6*1, 2A6*2 has one *Xcm*I site and two *Msp*I sites, and 2A6*3 has one *Dde*I site and one *Msp*I site (Fig. 1B). Therefore, the three genotypes of *CYP*2A6, 2A6*1, 2A6*2 and 2A6*3, would be determined by the combination of RFLP using *Msp*I, XcmI and DdeI. Figure 1C shows an electrophotogram of the enzyme digestion pattern of the Kd1F/E3R PCR product.

*Detection of CYP2A6 exon 3 deletion and absence of genotypes CYP2A6*2 and 2A6*3.* The frequency of each *CYP*2A6 allele was studied using DNA samples from 252 healthy subjects. PCR product was detected in 242 (Table 1). No PCR 215-bp product was detected in 10 (4.0%) samples. We confirmed the deletion by the internal control PCR targeting beta globin for checking of DNA quality, using the GH20 and PC04 primer pair, which had been reported previously (21). Even when the Kd1F/E3R primers were used with the primers for beta globin as an internal control in the same reaction tube, only beta globin (285 bp) was amplified (data not shown). It is surmised that these 10 individuals were homozygous deletions, lacking the *CYP*2A6 exon 3 regions in both alleles. The frequency of the homozygous deletion was 4.1% (8/197) and 3.6% (2/55) in males and females, respectively (Table 1). This result would suggest that the rate of deletion was the same between sexes, although the sample size is too small to make any definitive statement concerning gender differences.

All 242 PCR products by Kd1F/E3R PCR except one showed only wild type (*CYP*2A6*1), the genotypes being either homozygote (*CYP*2A6*1/*1) or hemizygote (*CYP*2A6*1/-). The remaining individual showed an

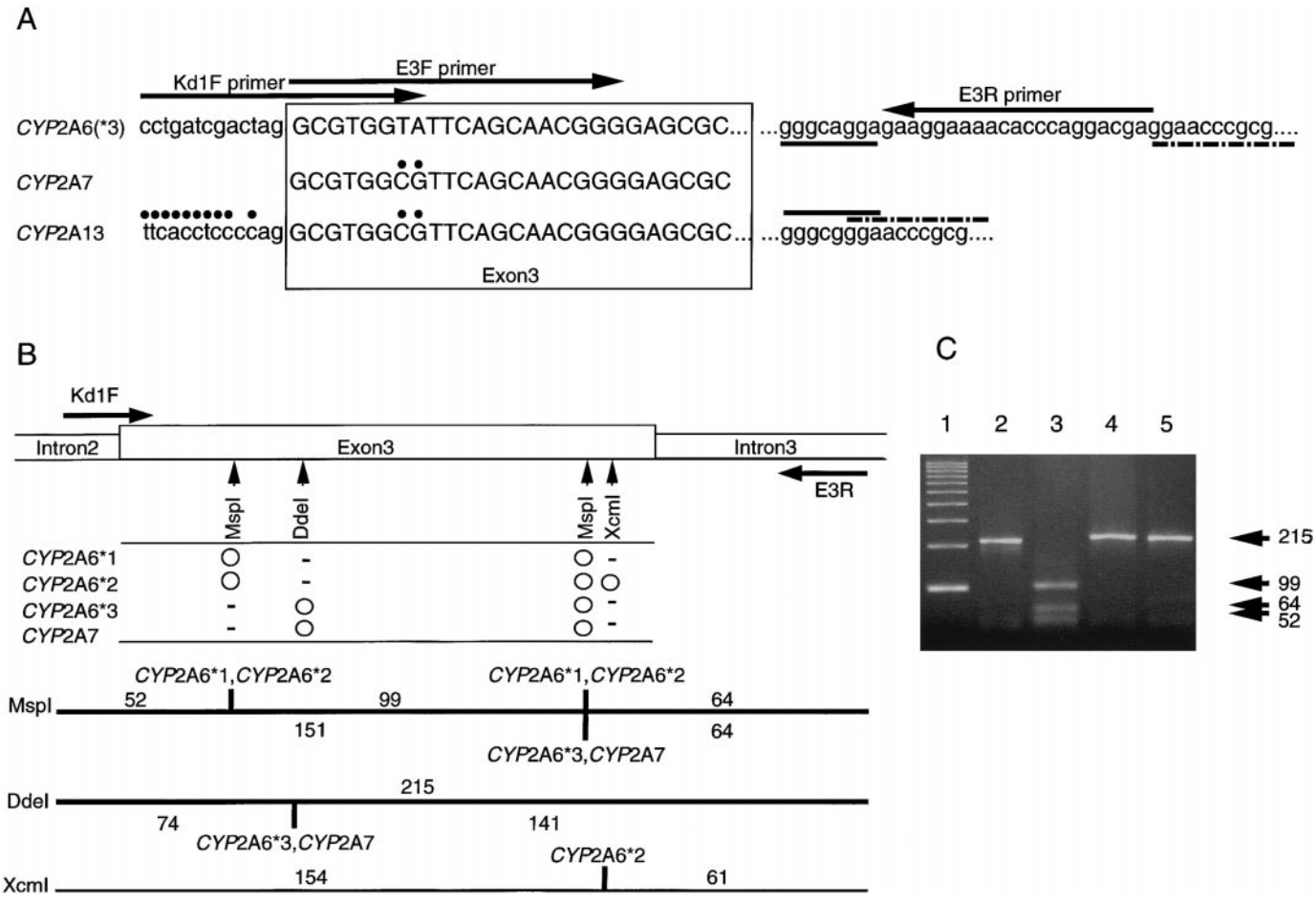

FIG. 1. Primer annealing sites in *CYP*2A6 and genotyping strategy for the *CYP*2A6 genetic polymorphism. (A) The position of the primers for Kd1F/E3R PCR and E3F/E3R PCR are indicated by horizontal arrows. Dots show the sequences that differ from the *CYP*2A6*3. Horizontal solid and dotted bars indicate the homologous sequences. *CYP*2A13 pseudogene seems to lack the E3R primer annealing site that is located between these sequences. The intron sequences of *CYP*2A6*1, *CYP*2A6*2 and *CYP*2A7 are not reported. (B) Horizontal arrows show PCR primer annealing sites. Vertical arrows and vertical bars show the restriction enzyme recognition sites in exon 3. Figures indicate the digested fragment lengths in the Kd1F/E3R PCR products. (C) Lane 1 contains a 100-bp DNA ladder size marker (GenSura); lane 2 shows the undigested PCR product (215 bp); lanes 3 to 5 show the RFLP of the PCR product, each was digested by *Msp*I, *Dde*I, and *Xcm*I, respectively.

unknown variant type heterozygously, from RFLP (data not shown). No individual with *CYP*2A6*2 or *CYP*2A6*3 was detected. The sequences of three independent cloned Kd1F/E3R PCR product were analyzed and compared with the sequences of *CYP*2A6*1, 2A6*2, 2A6*3 and *CYP*2A7. They were identical to *CYP*2A6*1 at exon 3, and included the same sequences to *CYP*2A6*3 at the area which corresponded to intron 3 (the sequence of *CYP*2A6*1 intron 3 is still unknown).

Urinary cotinine excretion after smoking. Eleven smoking subjects (6 homozygous deletions and 5 wild-type controls) were examined on nicotine metabolism. After smoking for one hour, as described under Methods, the cotinine concentrations in the urine from the subjects with *CYP*2A6*1 at 1.5 h was significantly higher than those from all the *CYP*2A6 homozygously deleted subjects. The average concentration of *CYP*2A6*1 positive subjects was 3.87 $\pm$ 1.64 μg/ml, and that of *CYP*2A6 homozygously deleted subjects was 0.40 $\pm$ 0.15 μg/ml.

The cumulative urinary excretion of cotinine for 24 h (until the next morning) are shown in Fig. 2. The average urinary cotinine excretions of the subjects with and without the *CYP*2A6 gene were 1.50 $\pm$ 0.50 vs 0.22 $\pm$ 0.08 mg for 24 h, respectively. The cotinine excretion in the homozygously *CYP*2A6 deleted subjects was about one seventh compared to the control group ($P < 0.001$).

DISCUSSION

PCR for amplification of CYP2A6. *CYP*2A6 genotyping using the single PCR method is established in this study. Attributes of this method are several. Be-

TABLE 1

*CYP*2A6 Genotyping after Kd1F/E3R PCR

Genotype	Number of subjects		
	Male	Female	Total
*CYP*2A6*1/*1 or *CYP*2A6*1/−[a]	188 (95.4%)	53 (96.4%)	241 (95.6%)
*CYP*2A6*1/*2	0	0	0
*CYP*2A6*1/*3	0	0	0
*CYP*2A6*2/*2 or *CYP*2A6*2/−	0	0	0
*CYP*2A6*2/*3	0	0	0
*CYP*2A6*3/*3 or *CYP*2A6*3/−	0	0	0
*CYP*2A6*1/unknown	1 (0.5%)	0	1 (0.4%)
Homozygous deletion	8 (4.1%)	2 (3.6%)	10 (4.0%)
Total	197 (100%)	55 (100%)	252 (100%)

[a] −, one allele of *CYP*2A6 is deleted at exon 3.

cause Kd1F/E3R PCR targets a short region (215 bp), many DNA samples from the various preparation methods may be analyzed. Single PCR yields rapid results and avoids the appearance of unexpected products, as often happens with double PCR method.

Population study of the CYP2A6 genotypes. In a previous report, the whole genome deletion of *CYP*2A6 was described as a novel variant (16). Nunoya *et al.* (15) also described the partial deletion of *CYP*2A6, which was positioned between intron 5 and exon 9. In this study, ten individuals of 252 examined showed homozygous deletion at exon 3 when their DNA was amplified using Kd1F/E3R PCR (Table 1). Further, the extent of the deletion was analyzed by two more kinds of PCR, which primers were set in exon 1 and exon 9, respectively. All ten samples were detected no expected size product by each PCR (data not shown). These results indicate that all the deletion of *CYP*2A6 detected by Kd1F/E3R PCR spread over the whole coding region.

Fernandez-Salguero *et al.* (14) reported that *CYP*2A6*2 and *CYP*2A6*3 alleles were present in 20 and 28% of Japanese, respectively. In the present paper, however, we detected neither the *CYP*2A6*2 nor the *CYP*2A6*3 allele. These results are consonant with those reported in a more recent study of the Japanese population (15). Fernandez-Salguero *et al.* (14) previously introduced the double PCR method for the amplification of *CYP*2A6, using F4/R4 primers for the first PCR and E3F/E3R for the nested PCR, in order to avoid the coamplification of *CYP*2A7. This typing method has been used often for examinations, and as the result, the frequency of *CYP*2A6*2 and *CYP*2A6*3 largely differed by the researchers (22–25). In their method, a small amount of genomic DNA, which was used as the template of the first PCR, was carried over to the nested (E3F/E3R) PCR. Although the longer genomic DNA is required for the first PCR, if the sample DNA was fragmented for any reason, it is hard to reach the efficient concentration of the target gene for the nested PCR. In this case, coamplification of *CYP*2A7, which shared sequence similarity with *CYP*2A6, could take place due to the presence of the genomic DNA. When we examined E3F/E3R PCR for

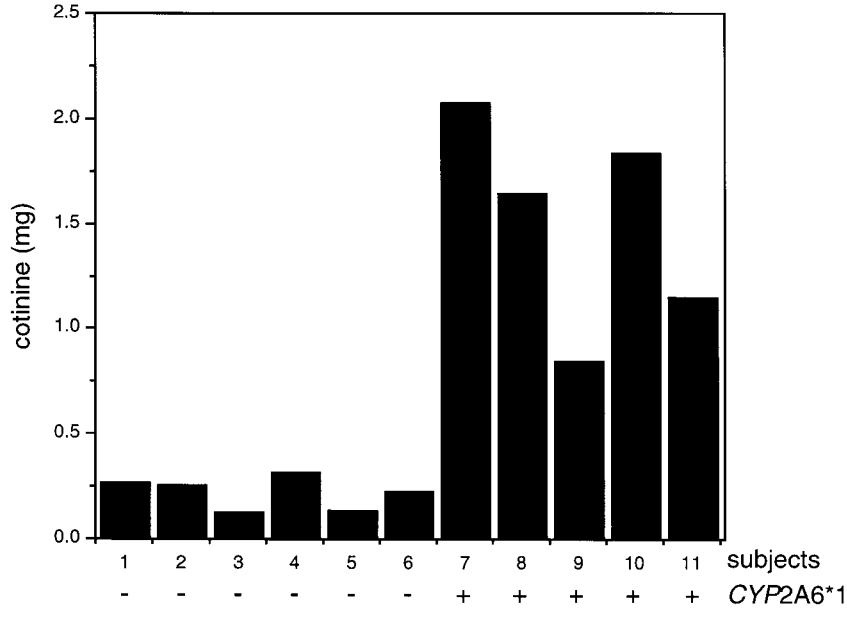

FIG. 2. Cumulative excretion of cotinine into urine. This shows urinary cotinine volume for 24 h (next morning) after smoking in each subject. *CYP*2A6*1 +; *CYP*2A6*1/*1 or *CYP*2A6*1/− *CYP*2A6*1−; *CYP*2A6 homo deletion.

10 samples which lacked *CYP*2A6 product by Kd1F/E3R PCR, it resulted in the expected size product (202 bp) from them, even if E3F/E3R PCR targeted almost the same region as Kd1F/E3R PCR (Fig. 1A). Otherwise these products included the identical sequence with *CYP*2A7 exon 3 (data not shown). The possibility of misclassification was also pointed out recently by Oscarson *et al.* (19).

We discovered one novel variant allele which did not exhibit either of the known RFLP patterns of *CYP*2A6*2 or *CYP*2A6*3, under DdeI, XcmI and MspI treatment. Further analysis of this variant, including metabolic characterization, is underway.

Urinary cotinine excretion after smoking. During cigarette smoking, nicotine is absorbed rapidly and metabolically converted to cotinine. Benowitz *et al.* (26) reported one female who was deficient in C-oxidation of nicotine. Her nicotine half time was about 3 times longer than that of the control group, and her plasma cotinine concentration was lower than the control group after intravenous infusion of nicotine. Under *in vitro* conditions, CYP2A6 has been reported as the major species catalyzing cotinine formation (9, 11). Given this metabolic scenario, it would be hypothesized that humans who lack or have no capacity to synthesize CYP2A6 enzyme would have a diminished capacity to metabolize nicotine and a longer plasma half-life of nicotine.

It has also been observed that cotinine formation from nicotine and coumarin 7-hydroxylation activity level have individual and ethnic variability, that was correlated with the level of CYP2A6 expression (27–31). These results could indicate that individuals with no or very low levels of CYP2A6 have either mutations or deletions of *CYP*2A6 gene. Furthermore, the ethnic related differences might be the cause of different distributions of genetic polymorphisms. Diminished coumarin 7-hydroxylation activity in individuals who were homozygous for *CYP*2A6*2 has been reported (19). The *in vivo* results in our study clearly show diminished cotinine excretion among subjects who were genotyped as CYP2A6 homozygous deletents.

The present study is the first report of nicotine metabolism in humans who lack *CYP*2A6 alleles homozygously and indicates that CYP2A6 has a definitive role for catalyzing cotinine formation, not only *in vitro* level, but also *in vivo.* Moreover, this study provides a firm experimental basis for correlating genotypic characterization with phenotypic expression.

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