

Individual Differences in Urinary Cotinine Levels in Japanese Smokers: Relation to Genetic Polymorphism of Drug-metabolizing Enzymes¹

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Abstract

Urinary cotinine, one of the main metabolites of nicotine, has been widely used as a biomarker for assessment of direct or passive exposure to cigarette smoke. However, there is wide variation of the cotinine level among smokers who smoke the same number of cigarettes. To use urinary cotinine as a proper exposure-biomarker for cigarette smoke, interindividual variations of cotinine formation must be considered. Therefore, we studied the effects of genetic polymorphisms in drug metabolic enzymes on urinary cotinine levels among 190 male Japanese smokers (ages 19–66 years; mean, 40.6 years). Genetic polymorphisms in cytochrome P-450s (*CYP1A1*, *CYP2A6*, *CYP2E1*), and aldehyde dehydrogenase 2 (*ALDH2*) were determined by analyzing DNA isolated from peripheral blood. Cotinine in morning spot urine was analyzed by high-performance liquid chromatography. Lifestyle, *i.e.*, smoking, alcohol consumption, and intake of coffee or tea, was examined using a questionnaire. The number of cigarettes smoked and *CYP2A6* polymorphism were significantly associated with the urinary cotinine level. Especially, the urinary cotinine levels was drastically lower in *CYP2A6*-deleted homozygous (*CYP2A6**4/*4) subjects than in *CYP2A6**1 allele-positive subjects. The polymorphism in the *CYP2E1* 5'-flanking region was related to the urinary cotinine level in intermediate smokers (who smoke 11–20 cigarettes/day; $P < 0.01$). Polymorphisms in *CYP1A1* or *ALDH2*, and consumption of alcohol, coffee, or tea were not associated with the urinary cotinine level.

Received 12/1/99; revised 3/23/01; accepted 3/29/01.

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¹ Supported in part by Grant-in-Aid 10670329 for Encouragement of Young Scientists, the Ministry of Education, Science and Culture of Japan.

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Introduction

Urinary cotinine levels have been widely used to assess direct or passive exposure to cigarette smoke, and as a motivation tool for smokers who are trying to stop smoking (1, 2). However, it is reported that there are variations in cotinine levels in smokers who smoke the same number of cigarettes (3). Thus, to use urinary cotinine as an accurate exposure- biomarker for smoking, the causes of variation in urinary cotinine levels should be determined.

One source of interindividual variation in the metabolism of xenobiotics is polymorphism of the genes that encode metabolic enzymes (4). Among the enzymes that are induced by smoking and involved in cotinine bioproduction, cytochrome P-450 *1A1* (*CYP1A1*), *CYP2A6*, *CYP2D6*, and *CYP2E1* are known to be genetically polymorphic (5–9). In addition, lifestyle factors, *e.g.*, alcohol drinking and coffee or tea consumption, may induce expression of these enzymes and affect nicotine metabolism. For example, *CYP2E1* is known to be induced by alcohol intake (10–11). Furthermore, there is an association between alcohol consumption and genetic polymorphism in aldehyde dehydrogenase 2 (*ALDH2*) in Japanese populations (12).

Kitagawa *et al.* (5) reported that urinary cotinine excretion in *CYP2A6*-deleted homozygous subjects was approximately one-seventh that of individuals who were *CYP2A6**1-positive, indicating an effect of a genetic polymorphism on nicotine metabolism. This conclusion was based on a small population, and therefore we reported here a more extensive study to investigate the effects of lifestyle and genetic polymorphisms in the above mentioned metabolic enzymes on urinary cotinine levels.

Materials and Methods

Subjects. The study subjects were 190 male students and office workers who were current smokers living in southwestern Japan during 1997–1999, and who had smoked at least one cigarette during the 24 h before urine sampling. The mean age was 40.6 years (range, 19–66 years). Peripheral blood and morning spot urine specimens were collected from each study subject. One hundred forty-nine of the subjects filled out a questionnaire concerning lifestyle, *i.e.*, smoking, alcohol consumption, coffee/tea intake, and dietary habits. All of the subjects gave their informed consent to be included in the study.

Analysis of Urinary Cotinine and Creatinine. Urinary cotinine was analyzed by the method described by Takeda *et al.* (13) with a minor modification. In brief, 1 ml of each urine sample was hydrolyzed with 0.33 ml of 3 N NaOH and extracted with 3 ml of CH_2Cl_2 . After centrifugation (1500 rpm for 5 min), 1.5 ml of the CH_2Cl_2 layer was transferred to an HPLC³

³ The abbreviation used is: HPLC, high-performance liquid chromatography.

vial and evaporated. The residue was dissolved in water and analyzed by HPLC (Hitachi L-6210 Intelligent Pump, 655A-40 Auto Sampler, 6554–52 Column Oven, L-4200 UV-VIS Detector, D-2500 Chromato-Integrator). HPLC parameters were as follows: column, TOSOH TSK-gel ODS-80TM; column temperature, 50°C; mobile phase, a mixture of acetonitrile/water (15/85) containing 20 mM potassium dihydrogen phosphate and 3 mM sodium 1-decanesulfonate (pH 4.5); flow rate, 1.0 ml/min. Absorbance was monitored at 254 nm.

Determination of Genotypes. Genomic DNA was isolated from the buffy coat fraction of each blood sample using an automated DNA extractor (Applied Biosystems Inc., Model 340A) after complete digestion with proteinase K. The genotyping of each metabolic enzyme was performed as follows. The genetic polymorphism of *CYP1A1* in exon 7, which is the substitution of codon 462 [ATT (Ile) to GTT (Val)], was analyzed by the method described by Oyama *et al.* (14). Genotypes were classified as “*Ile/Ile*” (the dominant homozygote), “*Ile/Val*,” and “*Val/Val*” (the rare homozygote).

Genotypes of *CYP2A6* were determined by the PCR-RFLP method developed by Kitagawa *et al.* (5). The alleles of *CYP2A6* were classified into *CYP2A6*1*, *CYP2A6*2*, *CYP2A6*3*, and the deletion, *CYP2A6*4*.

The *RsaI* polymorphism of *CYP2E1*, which is attributable to substitution of C to T at nucleotide 1019 of the 5' flanking region of the gene, was determined by the method of Kawamoto *et al.* (12), and classified as the predominant homozygous allele (*c1/c1*), the heterozygous allele (*c1/c2*), and the rare homozygous allele (*c2/c2*).

The genetic polymorphism of *ALDH2* in exon 12, which consists of an amino acid substitution from Glu to Lys at codon 487, was classified into *ALDH2*1*1*, *ALDH2*1*2*, and *ALDH2*2*2* according to the method of Kawamoto *et al.* (15).

Materials. Cotinine was obtained from Aldrich Chem. Co. (Milwaukee, WI). Proteinase K, Taq polymerase, dNTPs, restriction enzymes (*HincII*, *MspI*, *DdeI*, *XcmI*, *RsaI*, and *MboII*) and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Statistical Analysis. To study the correlation between urinary cotinine levels and number of cigarettes smoked, Spearman correlation analysis was used. Lifestyle and genotypes of the drug-metabolizing enzymes were quantified as follows: (a) alcohol, *i.e.*, frequency of alcohol drinking: rare, 0; 0–1 day/week, 1; 2–4 days/week, 2; >5 days/week, 3; (b) coffee and tea: not taken, 0; 0–1 cup/day, 1; 1–2 cups/day, 2; >3 cups/day, 3; (c) *CYP1A1*: *Ile/Ile*, 0; *Ile/Val* or *Val/Val*, 1; (d) *CYP2A6*: **1*1*, **1*4*, or **1/unknown variant*, 0; **4*4*, 1; (e) *CYP2E1*: *c1/c1*, 0; *c1/c2* or *c2/c2*, 1; and (f) *ALDH2*: **1*1*, 0; **1*2* or **2*2*, 1. We used Spearman rank correlation to study the significance of effects of lifestyle or genetic polymorphisms on urinary cotinine levels. The Mann-Whitney *U* test was used to compare urinary cotinine levels with the genotypes of *CYP2A6* and *CYP2E1*.

Results

Correlation between Extent of Smoking and Urinary Cotinine. Urinary cotinine was significantly correlated with the number of cigarettes smoked (Fig. 1; Spearman correlation coefficient (Rho), 0.319; $P < 0.001$). Correction for creatinine slightly improved the Rho value (r , 0.323). When we included nonsmokers who were in the same university or co-workers of the smoking subjects ($n = 248$) to the present study population, the Rho value was increased to 0.719.

The use of nicotine intake, *i.e.*, “nicotine content \times cig-

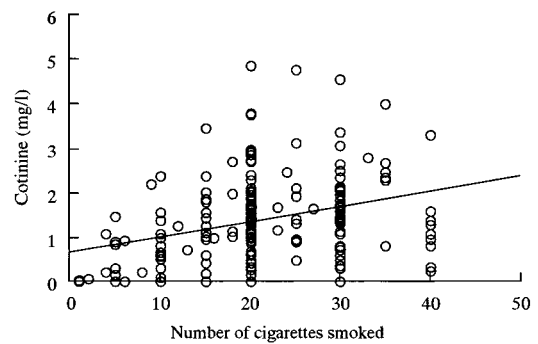


Fig. 1. Relationship between urinary cotinine level and number of cigarettes smoked during the 24 h before urine sampling. Spearman Rho, 0.319; $P < 0.001$.

arettes smoked” as a measure of nicotine exposure rather than simply the number of cigarettes smoked, produced correlations with urinary cotinine levels that were similar to those presented above (data not shown).

Genetic Characteristics of the Study Subjects. The distributions of *CYP1A1*, *CYP2A6*, *CYP2E1*, and *ALDH2* polymorphisms are shown in Table 1. The allelic frequencies of *CYP1A1*, *CYP2E1*, and *ALDH2* in the study subjects followed the Hardy-Weinberg law. The allelic frequencies of *CYP2A6* were computed by assuming that the Hardy-Weinberg law held. Allelic frequencies were in accordance with those of other Japanese populations (7, 9, 16).

One hundred eighty-one of the total subjects were found to possess the *CYP2A6*1* allele by genotyping for *CYP2A6*, and are referred to as “*CYP2A6*1*-positive.” It was not possible to distinguish homozygous *CYP2A6*1* allele (**1*1*) subjects from heterozygous *CYP2A6*1*-positive (**1*4*) subjects. A single subject was found to be heterozygous *CYP2A6*1/unknown variant*. This variant allele has been shown to have a substitution of guanine to adenine in exon 3, which results in loss of enzymatic activity (17). The three genotypes, *CYP2A6*1*1*, *CYP2A6*1*4*, and *CYP2A6*1/unknown*, were included in the *CYP2A6*1*-positive group, because they have at least one *CYP2A6*1* allele. Neither the *CYP2A6*2* nor the *CYP2A6*3* allele, which are known to be present with low frequency in Caucasian and Chinese populations (18), were detected in these study subjects.

Effects of Genetic Polymorphisms on Urinary Cotinine. Urinary cotinine levels ranged from below the detection limit (0.05 mg/liter) to 4.87 mg/liter (median, 1.36 mg/liter) in *CYP2A6*1*-positive subjects and from below the detection limit to 0.64 mg/liter (median, 0.15 mg/liter) in *CYP2A6*4*4* subjects. When subjects were grouped according to smoking status, the *CYP2A6*4*4* subjects showed a significantly lower level of urinary cotinine than the *CYP2A6*1*-positive subjects (Fig. 2). After correction for creatinine, this trend was not changed.

Among the *CYP2A6*1*-positive subjects, the *CYP2E1-c1/c2* or *c2/c2* subjects showed higher urinary cotinine levels than the *CYP2E1-c1/c1* subjects, and this difference was significant in the group of intermediate (11–20 cigarettes/day) smokers (Fig. 3). After correction for creatinine, this trend was not changed; however, the significance of this trend was slightly decreased.

On the other hand, polymorphisms of *CYP1A1* and *ALDH2* were not related to urinary cotinine levels.

Effects of Lifestyle on Urinary Cotinine. Table 2 shows the results of Spearman rank correlation analysis of the relationship

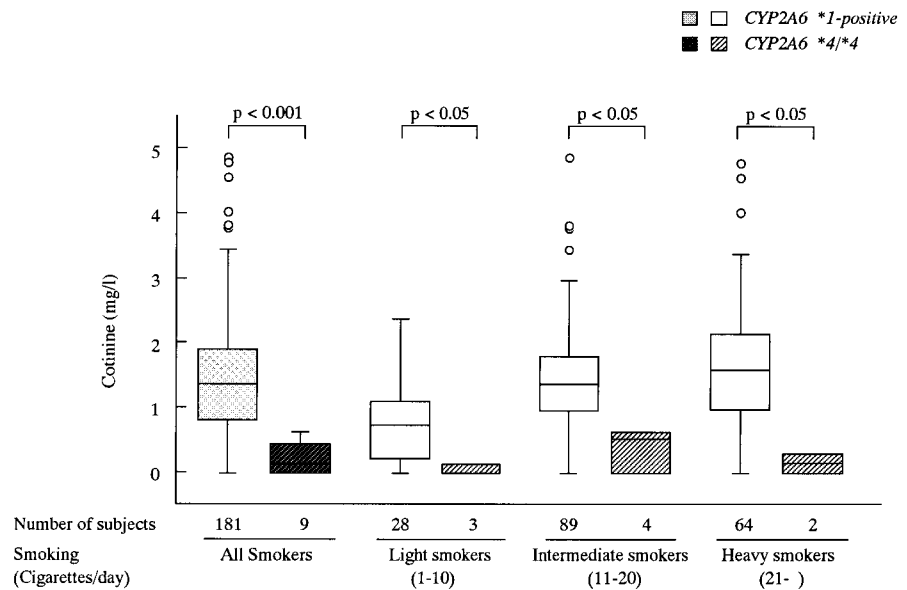
Table 1 Allele frequency of *CYP1A1*, *CYP2A6*, *CYP2E1*, and *ALDH2* in the study subjects

Gene	Total (%)	Genotype n (%)			Allele frequency		
<i>CYP1A1</i>	149 (100.0)	<i>Ile/Ile</i> 85 (57.0)	<i>Ile/Val</i> 54 (36.3)	<i>Val/Val</i> 10 (6.7)	<i>Ile</i> 0.752	<i>Val</i> 0.248	
<i>CYP2A6</i>	190 (100.0)	*1 positive ^a 181 (95.3)		*4/*4 9 (4.7)	*1 0.780	*4 0.217	Unknown 0.003 ^b
<i>CYP2E1</i>	190 (100.0)	<i>c1/c1</i> 118 (62.1)	<i>c1/c2</i> 62 (32.6)	<i>c2/c2</i> 10 (5.3)	<i>c1</i> 0.784	<i>c2</i> 0.216	
<i>ALDH2</i>	149 (100.0)	*1/*1 84 (56.4)	*1/*2 59 (39.6)	*2/*2 6 (4.0)	*1 0.762	*2 0.238	

^a Subjects who have at least one *1 allele, *1/*1 or *1/*4 (one case was a heterozygote of *1 and an unknown variant allele).

^b Allelic frequencies were computed by assuming that the Hardy-Weinberg equilibrium holds.

Fig. 2. Effects of *CYP2A6* genetic polymorphism on the urinary cotinine level according to smoking status.



between lifestyle or genetic polymorphisms and the urinary cotinine level, with adjustment for the number of cigarettes smoked. It shows that polymorphisms of *CYP2A6* and *CYP2E1* affect individual variation of the urinary cotinine level.

Coffee or tea intake was associated with the number of cigarettes smoked ($0.05 < P < 0.1$); however, it was not significantly related to the urinary cotinine level.

Because alcohol induces *CYP2E1*, we examined the effect of alcohol consumption on the relationship between *CYP2E1* polymorphism and urinary cotinine. We found that among subjects who drank 2–4 days/week, the *CYP2E1-c1/c2* or *c2/c2* type subjects had higher urinary cotinine levels than *c1/c1* type subjects ($P = 0.051$); however, this trend was not seen among drinkers with other drinking habits.

On the other hand, alcohol consumption was associated with *ALDH2* polymorphism, *i.e.*, the *ALDH2*1/*1* type subjects had higher alcohol consumption than the *ALDH2*1/*2* or *ALDH2*2/*2* type subjects ($P < 0.001$). However, alcohol consumption was not associated with the number of cigarettes smoked or with the urinary cotinine level.

Discussion

Relationship between Number of Cigarettes Smoked and Urinary Cotinine. There have been several reports in which the correlation coefficient between urinary cotinine level and

daily cigarette consumption varied from 0.39 to 0.99, and was generally greater than 0.75 (1, 19). However, one-half of the subjects in these reports were nonsmokers whose urinary cotinine levels were very low, near zero, or below the detection limit. On the other hand, a study that was restricted to smokers showed a correlation coefficient of 0.32 between the urinary cotinine level and the number of cigarettes smoked (20). This value is very similar to those determined in our study, *i.e.*, 0.319 for Spearman's Rho and 0.283 for the simple correlation coefficient (Fig. 1). When nonsmokers were included in our study, the correlation coefficient increased to 0.72, a value similar to that found in other studies in which the subjects included both smokers and nonsmokers. Thus, inclusion of nonsmokers results in a higher correlation coefficient between the number of cigarettes smoked and urinary cotinine.

These results indicate that, although urinary cotinine appears to distinguish smokers from nonsmokers, it is not a reliable quantitative measure of the extent of cigarette smoke exposure. In addition to cigarette smoking, some other factors appear to affect the level of urinary cotinine. These factors may include induction of nicotine metabolic enzymes in smokers (21). That is, heavy smokers metabolize nicotine primarily by pathways other than C-oxidation to cotinine, resulting in attenuation of the relationship between urinary cotinine and the number of cigarettes smoked.

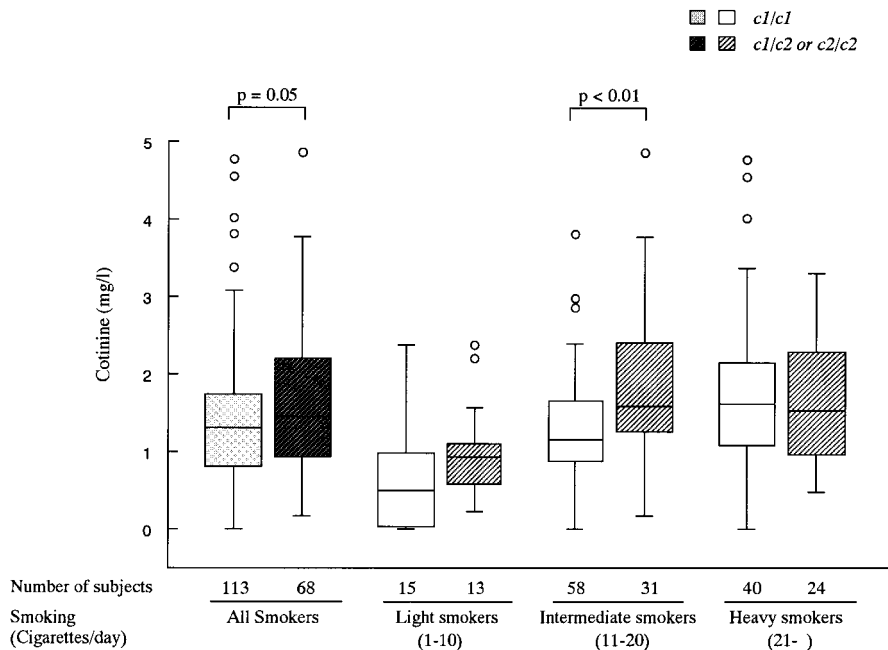


Fig. 3. Effects of *CYP2E1* genetic polymorphism on urinary cotinine levels according to smoking status among *CYP2A6**1-positive subjects.

Table 2 Relationship between lifestyle or genetic polymorphisms and urinary cotinine adjusted for cigarettes smoked per 24 hours

Lifestyle or gene	(mg/liter/cigarette)		(mg/g creatinine/cigarette)	
	Spearman's Rho	P	Spearman's Rho	P
Alcohol	0.113	0.136	0.129	0.089
Coffee/tea	0.059	0.513	0.071	0.430
<i>CYP1A1</i>	0.102	0.205	0.082	0.310
<i>CYP2A6</i>	-0.283	<0.001 ^a	-0.228	0.002 ^a
<i>CYP2E1</i>	0.166	0.022 ^b	0.132	0.068
<i>ALDH2</i>	-0.124	0.127	-0.057	0.485

^a Statistical significance, $P < 0.01$.

^b Statistical significance, $P < 0.05$.

Factors That May Cause Individual Variation in Nicotine Intake. In addition to the number of cigarettes smoked, the time between the last cigarette and urine collection, the nicotine contents of different brands of cigarettes, the depth of inhalation of tobacco smoke and other details of smoking behavior may affect the nicotine intake. However, the use of "number of cigarettes smoked" rather than "(nicotine content) \times (cigarettes smoked)" as a measure of nicotine exposure did not appear to be a confounding factor in this study. Although the presence of nicotine in foods such as eggplants, potatoes, and some tea plants has been reported (1, 22–23) and could pose a potential source of nicotine, these dietary sources of nicotine were not considered in this study as the levels are too small to significantly affect urinary cotinine levels in active smokers.

Rao *et al.* (21) reported that the individuals whose genotypes were *CYP2A6**1/*2, *CYP2A6**2/*2, or *CYP2A6**1/*4 smoked fewer cigarettes than the *CYP2A6**1/*1 type individuals but had the same intensity of smoking as *CYP2A6**1/*1 individuals. They found individuals with *CYP2A6* duplication, *CYP2A6**1/*1 plus duplication, had an increase in smoking intensity. However, *CYP2A6* polymorphisms did not affect smoking status in the present study, *i.e.*, subjects smoked 21.5 ± 8.9 cigarette/day in the *CYP2A6**1-positive group versus 16.2 ± 9.1 cigarette/day in the *CYP2A6**4/*4 group.

Individual variability in the kinetics of nicotine metabolism may also contribute to individual differences in nicotine addiction or stimulation (24). However, lifestyle factors that induce metabolic enzymes, *e.g.*, consumption of coffee, tea, or alcohol, did not affect the urinary cotinine level (Table 2). Thus, those lifestyle factors may not induce variation in cotinine bioformation.

Effect of Genetic Polymorphisms on Variation of Urinary Cotinine. Among the P450 enzymes, *CYP2A6* was reported to have the highest nicotine *C*-oxidation activity at low nicotine concentration (25). In addition, we previously reported a remarkable decrease of urinary cotinine excretion in homozygously *CYP2A6*-deleted subjects (5). Moreover, the present study showed that the *CYP2A6* genetic polymorphism is related to the urinary cotinine level adjusted for the number of cigarettes smoked (Table 2), and, thus, *CYP2A6* genetic polymorphism is an indispensable biomarker if urinary cotinine is to be used for monitoring cigarette smoking.

CYP2E1, 2C19, 1A2, 2C8, 3A4, 2C9, and 1A1 are also known to catalyze nicotine *C*-oxidation but only at high nicotine concentration (25). A study done with chloramphenicol acetyltransferase (CAT) reporter assays showed that the *CYP2E1*-c2 allele exhibited higher expression than the c1 allele

(26). Similarly, individuals with the *c2/c2* allele showed higher expression of CYP2E1 mRNA than *c1/c1* type subjects (9). Moreover, our previous epidemiological study showed that *CYP2E1-c1/c2* or *c2/c2* subjects have higher naphthol bioformation than *c1/c1* type subjects (2). Thus, the *c2* allele can be considered as a predictor of "rapid" *CYP2E1*-mediated oxidation activity, probably because of increased transcription of the gene. In the present study, in intermediate smokers, higher urinary cotinine levels were found in *CYP2E1-c1/c2* or *c2/c2* subjects than in *c1/c1* subjects (Fig. 3). When alcohol consumption, an inducer of CYP2E1, was considered, there were also higher urinary cotinine levels in *CYP2E1-c1/c2* or *c2/c2* subjects than in *c1/c1* subjects among intermediate (2–4 days/week) drinkers. These results are the first demonstration that genetic polymorphism in the *CYP2E1* 5'-flanking region affects nicotine metabolism; however, this effect was not seen among nondrinkers, light (0–1 day/week), or heavy (>5 days/week) drinkers. Thus, to clarify the effects of *CYP2E1* polymorphism on cotinine biotransformation, additional studies would be required in a larger number of subjects.

On the other hand, there was no relationship between polymorphism of *CYP1A1* or *ALDH2* and urinary cotinine level. Thus, *CYP1A1*, and *ALDH2* are not likely to play important roles in cotinine bioproduction.

Recently, genetic polymorphisms in the dopamine transporter (*SLC6A3*) and D2 dopamine receptor (*DRD2*) have been reported to influence the initiation of smoking and nicotine dependence (27). In addition to these two polymorphisms, *CYP2A6* and *CYP2E1* polymorphisms may induce individual variability in smoking cessation or nicotine addiction, because they affect cotinine biotransformation.

In addition to genetic polymorphisms, there may be several other factors that cause interindividual variability in nicotine metabolism. For example, metabolism of cotinine to 3-hydroxycotinine (23), enzyme induction by diet or smoking itself, and variation in urinary pH could affect urinary cotinine biotransformation. These are potential variables that should be addressed in future studies.

In summary, the effects of lifestyle and genetic polymorphisms of drug metabolic enzymes on the urinary cotinine level were studied in male Japanese smokers. The number of cigarettes smoked and the *CYP2A6* polymorphism were significantly associated with the urinary cotinine level. Polymorphism in the *CYP2E1* 5'-flanking region also affected the urinary cotinine level, particularly in intermediate smokers who smoked 11–20 cigarettes/day. Thus, interindividual variability in nicotine metabolism appears to be an important factor that limits the quantitative application of urinary cotinine as a biomarker of tobacco smoke exposure.

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