

Evaluation of *CYP2A6* genetic polymorphisms as determinants of smoking behavior and tobacco-related lung cancer risk in male Japanese smokers

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We reported previously that subjects homozygous for the *cytochrome P450 2A6 (CYP2A6)* *4 have a lower risk of lung cancer. The purpose of this study was to clarify whether or not the alterations of smoking behavior and risk for lung cancer could be found in subjects possessing novel *CYP2A6* variants discovered recently. An epidemiological study was performed with 1094 cases and 611 controls in male Japanese smokers. It was found that the amounts of daily cigarette consumption in subjects who harbored *CYP2A6**4/*7, *4/*10, *7/*7, *7/*9 and *4/*4 genotypes were significantly less than those in subjects carrying the *1/*1 genotype ($P < 0.01$). Even after adjustment with cigarette consumption, the adjusted odds ratios (ORs) for lung cancer were significantly lower in subjects who harbored *CYP2A6**1/*4, *1/*7, *1/*9, *1/*10, *4/*4, *4/*7, *4/*9, *7/*7 and *7/*9 genotypes than those who possessed the *1/*1 genotype ($P < 0.05$). When participants were classified into four groups according to the *CYP2A6* genotypes, group 1 (*1/*1), group 2 (heterozygotes for the *1 and a variant allele), group 3 (heterozygotes and homozygotes for variant alleles except for *4/*4) and group 4 (*4/*4), lung cancer risk was found to be less in subjects with the variant of *CYP2A6* alleles {group 2, OR of 0.59 [95% confidence interval (CI), 0.44–0.79]; group 3, OR of 0.52 (95% CI, 0.37–0.72); group 4, OR of 0.30 (95% CI, 0.16–0.57)}. The reduced risk for lung cancer was seen more clearly in heavy smokers than in light smokers. Additional stratification analysis showed that the ORs for squamous cell carcinoma (OR of 0.07) and small cell carcinoma (OR of 0.39) in group 4. These results suggest that the *CYP2A6* is one of the principal determinants affecting not only smoking behavior but also susceptibility to tobacco-related lung cancer.

Introduction

Cytochrome P450 2A6 (*CYP2A6*) is known as an enzyme responsible for the metabolism of chemicals and drugs such as coumarin (1), nicotine (2), (+)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride (SM-12502) (3), tegafur (4), fadrozole (5), methoxyflurane (6) and valproic acid (7). The enzyme can also metabolically activate a number of carcinogens including tobacco-specific *N*-nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (8,9).

The existence of a genetic polymorphism of *CYP2A6* was first suggested by evidence that there was a large inter-individual difference in the capacity of coumarin 7-hydroxylation (10,11). In fact, various variants of the *CYP2A6* gene have been found in recent years (1,12–18). Analyzing the genes of subjects who showed a poor metabolizer phenotype toward SM-12502, we found two novel deletion-type variants of the *CYP2A6* gene, *CYP2A6**4B and *CYP2A6**4C (19,20); the *CYP2A6**4C was one of the major variants in Japanese. Following the discovery of *CYP2A6**4C, we discovered two additional alleles, *CYP2A6**7 and *CYP2A6**11, showing a decrease in enzymatic activity (21,22). A novel variant, the *CYP2A6**9, has a –48T to G nucleotide substitution in the TATA box of the 5'-flanking region of the *CYP2A6*, which reduced the expression levels of *CYP2A6* mRNA and protein in human livers (23). The *CYP2A6**10 allele possessing two simultaneous amino acid substitutions seen in the *CYP2A6**7 and *CYP2A6**8 also shows decreased enzymatic activity (24).

Most cancers are caused by chemical carcinogens present in our environment (25,26). These chemical carcinogens exert their genotoxicity after undergoing metabolic activation by enzymes present in our bodies. Thus, the capacity of enzymes to activate chemical carcinogens has been recognized as one of the factors determining the risk of cancer. Genetic polymorphism of the genes for such enzymes has been expected to be the most typical factor altering the activity and the amounts of the enzymes. Thus, it has been hypothesized that the genetic polymorphism alters the risk of chemical carcinogenesis. However, no conclusive evidence for the association between the genetic polymorphism of carcinogen-activating enzyme and the lung cancer risk has been reported as yet. Recently, several reports, including our group, have demonstrated the role of *CYP2A6* genetic polymorphisms in lung cancer risk with some conflicting results in several populations from different ethnicities (27–32). In our previous paper (31), we reported a clear relationship between *CYP2A6* genetic polymorphisms and lung cancer risk in smokers. To our knowledge, our results were not supported by other investigators, who reported that no clear association between *CYP2A6* genetic polymorphisms and lung cancer risk could be seen (27,29,30,32). The reason for this discrepancy is not known as yet. However, the most possible explanation for this discrepancy is that they analyzed the genes from smokers and non-smokers. Our epidemiology has indicated that the

Abbreviations: Ad, adenocarcinoma; CI, confidence interval; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; OR, odds ratio; SCC, small cell carcinoma; SqCC, squamous cell carcinoma.

significant difference can be seen only in smokers, but not in non-smokers. To support our previous results, we needed to clarify further the effects of the *CYP2A6* genetic polymorphism on the inter-individual differences in the risk of lung cancer especially in smokers. Thus, we performed a large-scale epidemiological study to investigate the relationship between a variety of genetic polymorphisms of *CYP2A6* as well as *CYP2A6*4C* and tobacco-related lung cancer risk in male Japanese smokers. The present results clearly provide evidence that the variants examined in this study decrease the risk of lung cancer in male Japanese smokers. Based on the results presented in this paper and the results reported quite recently that 8-methoxypsoralen, a specific inhibitor of CYP2As, completely inhibited the occurrence of adenoma caused by treatment of mice with NNK (33), we propose that genetic polymorphism and the inhibition of *CYP2A6* reducing the capacity of *CYP2A6* activity result in the reduction of the risk of lung cancer caused by tobacco smoking.

Materials and methods

Subjects

All subjects employed in this study were unrelated male Japanese smokers. Smokers included current and ex-smokers with a minimum smoking history of 0.5 pack/day for at least 1 year. The patient group consisted of a total of 1094 males with a mean age (\pm SD) of 62.4 ± 9.4 years. The control group consisted of 611 unrelated healthy males with a mean age (\pm SD) of 53.0 ± 11.2 years. The control subjects did not have any history of cancer. The subjects in case and control groups smoked 53.9 ± 31.9 and 38.3 ± 24.0 pack-years (\pm SD), respectively. Pack-years smoked was shown to indicate cumulative cigarette dose. Light and heavy smokers were categorized by the 50th percentile pack-years value among controls, and defined as light smokers (<38.3 pack-years) and heavy smokers (≥ 38.3 pack-years). Most cases were recruited from 1997 to 2003 in the National Cancer Center Hospital, Tokyo, Japan. Incidental cases were ~95% of the total population, with the remaining 5% of prevalent cases. Control subjects recruited in this study composed of healthy volunteers, who visited one of the hospitals that took part in the research within the same time period described above for a health check-up. This procedure provided a natural balance between cases and controls regarding possible confounding factors such as birthplace, since the cancer patients and controls that visited the National Cancer Center Hospital and Maruyama Clinic, respectively, were from all regions of Japan. The age of the lung cancer patients was defined when the lung cancer was first diagnosed pathologically. According to the criteria described in the literature pathological classification of lung cancer was determined by more than three pathologists (34). Participants were interviewed using a structured questionnaire, which included queries about the city of residence, birthplace, occupational history, smoking status and previous family (parents and siblings) history of cancer. Each patient in this study was required to sign a consent form. This study was approved by the ethics committee of the National Cancer Center and Hokkaido University.

Genotyping

Genomic DNA was prepared from the peripheral lymphocytes of the patients according to the method of phenol-chloroform extraction followed by ethanol precipitation (35).

Genotyping of the *CYP2A6*1A*, *CYP2A6*1B*, *CYP2A6*4C*, *CYP2A6*7* and *CYP2A6*9* alleles was carried out by the method developed in our laboratory (21,23,36). Genotyping of the *CYP2A6*10* allele was carried out by the method reported by Xu *et al.* (24). The genotyping method, based on the PCR-restriction enzyme fragment length polymorphism (RFLP) for the *CYP2A6*4B* allele, was newly developed in this study. The reaction mixture (25 μ l) for PCR contained LA PCR buffer II, 2.5 mM MgCl₂, 2.0 mM dNTPs, 2A6JDG sense (5'-GCA CAA TAG GGT GAA TGT AGT TAA CA-3') and 2A6JDG AS6 (5'-GGA ATA ACT GAA TTT CCT TAA GG-3') primers (0.2 μ M), 1.0 U of LA *Taq* DNA polymerase (Takara, Kyoto, Japan) and ~50 ng of the genomic DNA. PCR was carried out under the following conditions: initial denaturation at 94°C for 5 min followed by 25 cycles of reactions composed of cycle denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. After amplification of a PCR fragment (1080 bp), restriction digestion using two restriction enzymes, *Bsp*HI and *Cla*I, was carried out. The resulting fragments were analyzed on a 1.5% agarose gel. *CYP2A6*1*4B* yielded 1080, 885, 698, 382 and 195 bp fragments, while

*CYP2A6*1*1* or *CYP2A6*1*4C* yielded 1080, 698 and 382 bp fragments. *CYP2A6*4B*4B*, *CYP2A6*4B*4C* and *CYP2A6*4C*4C* yielded 885 and 195 bp fragments, 885, 698, 382 and 195 bp fragments, and 698 and 382 bp fragments, respectively.

A new genotyping method based on the PCR-RFLP for the *CYP2A6*11* allele was developed in this study. The reaction mixture (25 μ l) for PCR consisted of PCR buffer II, 2.0 mM MgCl₂, 2.4 mM dNTPs, 2A6-int4-new sense primer (5'-GCT CCA ATC CAG CCT CGT TTA A-3') and 2A6-int5R-new AS primer (5'-TCC AGC CCT TGC AGC AAC TG-3') (0.4 μ M), 2.0 U of AmpliTaq DNA polymerase and ~50 ng of the genomic DNA. PCR was carried out under the following conditions: initial denaturation at 94°C for 1.5 min followed by 30 cycles of reactions composed of cycle denaturation at 94°C for 20 s, annealing at 63°C for 30 s and extension at 72°C for 45 s. After amplification of a 344 bp fragment, restriction digestion using the restriction enzyme, *Mbo*II, was carried out. The digested fragments were analyzed on a 1.5% agarose gel. *CYP2A6*11*11* yielded 300, 228, 72 and 44 bp fragments, and *CYP2A6*11*11* yielded 300 and 44 bp fragments, while *CYP2A6*11*1* yielded 228, 72 and 44 bp fragments.

To examine the relationships between the enzymatic function of *CYP2A6* predicted by the genotypes and smoking behavior or lung cancer risk, we have defined the following groups: group 1 contains individuals with two copies of wild-type alleles (*CYP2A6*1*1*). Group 2 contains subjects carrying at least one wild-type allele (*CYP2A6*1*4*, **1*7*, **1*9*, **1*10* or **1*11*). Group 3 consists of subjects heterozygous or homozygous for variant alleles except for those homozygous for the *CYP2A6*4* allele (*CYP2A6*4*7*, **4*9*, **4*10*, **4*11*, **7*7*, **7*9*, **7*10*, **9*9*, **9*10*, **9*11* or **10*10*). Subjects in the group 4 have two copies of the deletion alleles (*CYP2A6*4*4*).

Statistical analysis

Differences in age and the amount of cigarette smoking between lung cancer patients and control subjects were tested using unpaired *t*-test with Welch correction. A relationship between the amount of cigarette smoking and each *CYP2A6* genotype was evaluated by one-way ANOVA followed by Games-Howell test as a post hoc comparison. To determine if an association existed between the *CYP2A6* genotypes and lung cancer risk, the significance of the difference in the case-control distribution was calculated by χ^2 test and shown by *P* value (two-sided). A *P* value <0.05 was considered to be statistically significant. Compliance with the Hardy-Weinberg equation was tested by χ^2 test. The association between the genotype distribution and lung cancer risk was assessed by odds ratio (OR) and 95% confidence interval (CI) and were calculated by unconditional logistic regression to adjust for age and cigarette smoking. All statistical computations were carried out using the statistical software SAS, version 5.1 (SAS Institute, Cary, NC).

Results

CYP2A6 genotypes and smoking behavior

Genomic DNA samples from a total of 1705 subjects (1094 lung cancer patients and 611 controls) were analyzed for each *CYP2A6* genotype, using the genotyping methods shown in Table I. The relationship between *CYP2A6* genotypes and the amounts of daily cigarette consumption in all subjects employed in the present study is shown in Figure 1A. It was found that the amounts of daily cigarette consumption of the subjects, who harbored *CYP2A6*4*7*, *CYP2A6*4*10*, *CYP2A6*7*7*, *CYP2A6*7*9* and *CYP2A6*4*4*, were significantly less than that of the subjects who carried *CYP2A6*1*1* ($P < 0.01$). As mentioned in the Introduction section, the variant *CYP2A6* alleles were assumed to generate transcripts possessing lower or no enzymatic activity. Thus, all subjects employed in this study were classified into four groups according to the *CYP2A6* genotypes, group 1 (homozygotes for wild-type *CYP2A6*1* allele), group 2 (heterozygotes for the *CYP2A6*1* allele and a variant allele), group 3 (heterozygotes and homozygotes for variant alleles except for homozygotes for the *CYP2A6*4* allele) and group 4 (homozygotes for the *CYP2A6*4* allele), to examine the relationship between each genotypic group of *CYP2A6* and the amounts of daily cigarette consumption (Figure 1B), expecting that the *CYP2A6* enzymatic function predicted by *CYP2A6* genotypes will be related

Table I. Summary of detection methods for *CYP2A6* alleles

Allele	Type of mutation	Detection of mutation (ref.)
<i>CYP2A6*1A</i>	Wild	<i>AccII</i> -RFLP, <i>Eco8II</i> -RFLP (36)
<i>CYP2A6*1B</i>	Gene conversion with <i>CYP2A7</i> in the 3'-untranslated region	<i>AccII</i> -RFLP, <i>Eco8II</i> -RFLP (36)
<i>CYP2A6*4B</i>	Whole deletion	<i>BspHI</i> and <i>ClaI</i> -RFLP
<i>CYP2A6*4C</i>	Whole deletion	<i>AccII</i> -RFLP, <i>Eco8II</i> -RFLP (36)
<i>CYP2A6*7</i>	Point mutation in exon 9 (1412T>C, I471T) and gene conversion with <i>CYP2A7</i> in the 3'-untranslated region	Two-step allele-specific PCR (21)
<i>CYP2A6*9</i>	TATA box (-48T>G)	Hybridization probe (23)
<i>CYP2A6*10</i>	Point mutations in exon 9 (1412T>C, I471T and 1454G>T, R485L) and gene conversion with <i>CYP2A7</i> in the 3'-untranslated region	Two step allele-specific PCR (24)
<i>CYP2A6*11</i>	Point mutation in exon 5 (670T>C, S224P)	<i>MboII</i> -RFLP

to smoking behavior. It was found that the amounts of daily cigarette consumption in the subjects significantly decreased in the order from group 1 to group 4.

CYP2A6 genotypes and tobacco-related lung cancer risk

The results of analysis on the association between *CYP2A6* genotypes and tobacco-related lung cancer risk are shown in Table II. The *CYP2A6*1A* and *CYP2A6*1B* alleles were classified as *CYP2A6*1*, because the functions of both alleles were thought to be the same. Also, *CYP2A6*4B* and *CYP2A6*4C* alleles were classified into *CYP2A6*4* (Tables II–V), since both alleles caused the same consequence on the enzyme expression. The distribution of *CYP2A6* genotypes in both controls and cases were not different from that expected from the Hardy–Weinberg equilibrium ($\chi^2 = 10.1$ and 8.6 , $P = 0.90$ and 0.95 , respectively) (Table II). In contrast, the distribution of the *CYP2A6* genotypes in cases was significantly different from that in controls ($\chi^2 = 42.6$, $P = 0.0005$). Even after adjustment with cigarette consumption and age by logistic regression analysis, the adjusted ORs for the risk of lung cancer were still significantly lower in subjects who harbored *CYP2A6*1/4*, *CYP2A6*1/7*, *CYP2A6*1/9*, *CYP2A6*1/10*, *CYP2A6*4/4*, *CYP2A6*4/7*, *CYP2A6*4/9*, *CYP2A6*7/7* and *CYP2A6*7/9* genotypes as compared with those who possessed the *CYP2A6*1/1* genotype ($P < 0.05$) (Table II).

According to the same criteria as Figure 1B, all subjects employed in the present study were classified into groups 1–4 (Table III). The adjusted ORs of groups 2–4 in overall cases decreased to 0.59 (95% CI of 0.44–0.79), 0.52 (95% CI of 0.37–0.72) and 0.30 (95% CI of 0.16–0.57), respectively. Furthermore, when smokers were stratified by pack-years smoked, the reduced risk of lung cancer was seen more clearly in heavy smokers (smoked ≥ 38.3 pack-years) according to the genotypes; group 2 (adjusted OR, 0.42; 95% CI, 0.28–0.62), group 3 (adjusted OR, 0.39; 95% CI, 0.25–0.63) and group 4 (adjusted OR, 0.19; 95% CI, 0.05–0.65), than in light smokers (smoked < 38.3 pack-years); group 2 (adjusted OR, 0.95; 95% CI, 0.61–1.49), group 3 (adjusted OR, 0.73; 95% CI, 0.46–1.18) and group 4 (adjusted OR, 0.48; 95% CI, 0.22–1.04).

Allele-based analysis on the risk for tobacco-related lung cancer was also performed (Table IV). The distribution of the *CYP2A6* alleles between cases and controls was also significantly different ($\chi^2 = 35.7$, $P < 0.0001$). The ORs were found to be significantly low for the *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9* and *CYP2A6*10* alleles ($P < 0.05$), supporting the idea that capacity of *CYP2A6* activity is one of the determinants affecting susceptibility to tobacco-related lung cancer.

To further examine the impact of the *CYP2A6* genetic polymorphism on tobacco-related lung cancer risk, lung cancer patients were divided into three groups, squamous cell carcinoma (SqCC), small cell carcinoma (SCC) and adenocarcinoma (Ad), according to a pathological classification (Table V). SqCC and SCC have been major types of lung cancer caused by smoking, whereas Ad had not been recognized as a common histological type of lung cancer in smokers until recent years, when it was demonstrated that Ad could be increased by smoking (37,38). Significant differences in the distribution of the four *CYP2A6* groups between controls and cases suffering from SqCC ($\chi^2 = 20.8$, $P = 0.0001$), SCC ($\chi^2 = 15.8$, $P < 0.01$) and Ad ($\chi^2 = 15.6$, $P < 0.01$) were found. Among overall cases, the adjusted ORs for SqCC (adjusted OR, 0.07; 95% CI, 0.01–0.33) and SCC (adjusted OR, 0.10; 95% CI, 0.01–0.78) were lower than that of Ad (adjusted OR, 0.39; 95% CI, 0.20–0.77) in group 4 (*CYP2A6*4/4*). Additional analysis with stratification of histological cancer subtypes revealed that this protection effect for lung cancer was mainly due to markedly reduced risk among heavy smokers. In contrast, among light smokers, no significant association between *CYP2A6* groups and the risk for each subtype of lung cancer was found.

Discussion

One of the most important issues to clarify was that the lower tobacco-related lung cancer risk seen in smokers possessing the *CYP2A6*4C* allele appeared in association with the activity of *CYP2A6*. Thus, we performed an additional epidemiological study to confirm this possibility. In this context, we analyzed the frequency of novel *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10* and *CYP2A6*11*, in addition to *CYP2A6*4C* to know if the frequency of these alleles associated with susceptibility to tobacco-related lung cancer. We found that there was a clear relationship between the various *CYP2A6* genotypes and tobacco-related lung cancer risk in male Japanese smokers in the present study.

In the Japanese population used in this study, allele frequencies of *CYP2A6*1*, *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10* and *CYP2A6*11* in healthy controls were essentially the same as compared with previous studies from our and other laboratories (13,22–24,28,36,39), except for a few reports showing the allele frequencies of *CYP2A6*4* in a Chinese (30,32) and *CYP2A6*7* and *CYP2A6*10* in the Japanese population (40) being 8, 7 and 1%, respectively. These allele frequencies are much lower than that reported by us. The discrepancy of allele frequencies between their

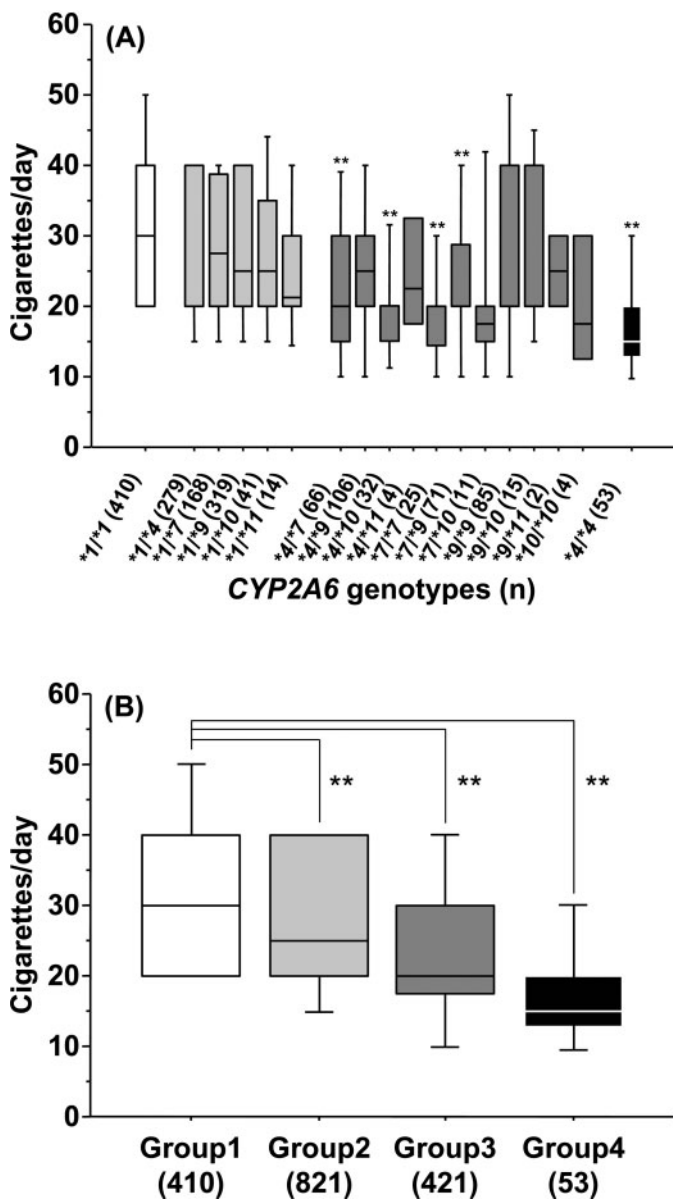


Fig. 1. Relationship between the *CYP2A6* genotypes and the amounts of daily cigarette consumption (A), and between *CYP2A6* groups classified by *CYP2A6* genotypes and daily cigarette consumption (B). Cigarette consumption was investigated with all subjects employed in this study. *CYP2A6**1 consists of *CYP2A6**1A and *1B alleles. *CYP2A6**4 consists of *CYP2A6**4B and *4C alleles. The number of subjects is shown under each genotypic group in brackets. Subjects were classified into four groups, according to the *CYP2A6* genotypes. Group 1 includes the subjects carrying *CYP2A6**1/*1 (wild-type). Group 2 contains subjects heterozygous for wild-type allele (*CYP2A6**1/*4, *CYP2A6**1/*7, *CYP2A6**1/*9, *CYP2A6**1/*10, *CYP2A6**1/*11). Group 3 consists of subjects carrying *CYP2A6**4/*7, *CYP2A6**4/*9, *CYP2A6**4/*10, *CYP2A6**4/*11, *CYP2A6**7/*7, *CYP2A6**7/*9, *CYP2A6**7/*10, *CYP2A6**9/*9, *CYP2A6**9/*10, *CYP2A6**9/*11 or *CYP2A6**10/*10. Group 4 contains subjects homozygous for *CYP2A6* deletion allele (*CYP2A6**4/*4). Genotypes are shown in numerical order. Horizontal lines mean medians. Boxes show 25th and 75th percentile of the observed values. Bars mean 10th and 90th percentiles. The amount of daily cigarette consumption was significantly less than those carrying the *CYP2A6**1/*1 genotype (***P* < 0.01).

study and the present one may be explained by several possibilities. First, the sample size was small in the study reported by Yoshida *et al.* (40). They analyzed only 92 Japanese individuals, whereas we analyzed 611 controls. Secondly, the

Table II. Distribution of *CYP2A6* genotypes in lung cancer patients

<i>CYP2A6</i> ^a	Cases (%) (n = 1094)	Controls (%) (n = 611)	Crude OR (95% CI)	Adjusted OR (95% CI) ^b
*1/*1	300 (27.4)	110 (18.0)	1.00 ^c	1.00 ^c
*1/*4	185 (16.9)	94 (15.4)	0.72 (0.52–1.00)	0.68 (0.47–0.98) ^d
*1/*7	106 (9.7)	62 (10.1)	0.63 (0.43–0.92) ^d	0.55 (0.36–0.84) ^d
*1/*9	207 (18.9)	112 (18.3)	0.68 (0.49–0.93) ^d	0.59 (0.41–0.84) ^d
*1/*10	21 (1.9)	20 (3.3)	0.39 (0.20–0.74) ^d	0.30 (0.14–0.61) ^d
*1/*11	9 (0.8)	5 (0.8)	0.66 (0.22–2.01)	1.05 (0.29–3.78)
*4/*4	25 (2.3)	28 (4.6)	0.33 (0.18–0.59) ^d	0.29 (0.15–0.56) ^d
*4/*7	36 (3.3)	30 (4.9)	0.44 (0.26–0.75) ^d	0.45 (0.25–0.82) ^d
*4/*9	62 (5.7)	44 (7.2)	0.52 (0.33–0.81) ^d	0.51 (0.31–0.83) ^d
*4/*10	16 (1.5)	16 (2.6)	0.37 (0.18–0.76) ^d	0.55 (0.24–1.28)
*4/*11	2 (0.2)	2 (0.3)	0.37 (0.05–2.64)	0.52 (0.05–5.62)
*7/*7	12 (1.1)	13 (2.1)	0.34 (0.15–0.76) ^d	0.28 (0.11–0.71) ^d
*7/*9	39 (3.6)	32 (5.2)	0.45 (0.27–0.75) ^d	0.44 (0.25–0.79) ^d
*7/*10	7 (0.6)	4 (0.7)	0.64 (0.18–2.24)	0.98 (0.25–3.92)
*9/*9	55 (5.0)	30 (4.9)	0.67 (0.41–1.10) ^d	0.71 (0.41–1.23)
*9/*10	11 (1.0)	4 (0.7)	1.01 (0.31–3.23)	0.98 (0.28–3.41)
*9/*11	1 (0.1)	1 (0.2)	0.37 (0.02–5.91)	0.36 (0.02–5.81)
*10/*10	0 (0.0)	4 (0.7)	NA ^e	NA ^e

Significant difference in the distribution of *CYP2A6* genotypes was found between lung cancer cases and control subjects (χ^2 value 42.6, *P* = 0.0005).

^a*CYP2A6**1 consists of *CYP2A6**1A and *1B alleles. *CYP2A6**4 consists of *CYP2A6**4B and *4C alleles.

^bTo adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.

^cReference category.

^dSignificant decrease of OR is indicated by 95% CI.

^eNot applicable.

ethnic difference of the *CYP2A6**4 allele frequency might exist between Japanese and Chinese populations. In fact, the frequencies of *CYP2A6**4 in controls in their two different studies were almost the same (30,32), although the frequencies in lung cancer cases were largely different.

We assessed the impact of *CYP2A6* genetic polymorphisms on the number of cigarettes smoked per day and the risk of lung cancer. Regarding the basis for the classification of the *CYP2A6* genotypes into groups 1–4, we recently analyzed that the relationship between the *in vivo* catalytic activity of *CYP2A6* towards nicotine and the polymorphism of the *CYP2A6* gene in healthy Thai volunteers (unpublished data). The levels of plasma cotinine concentration in subjects genotyped as *CYP2A6**1/*4, *CYP2A6**1/*7, *CYP2A6**1/*9, *CYP2A6**1/*10, *CYP2A6**4/*7, *CYP2A6**4/*9, *CYP2A6**7/*7 and *CYP2A6**9/*9 showed 53.9, 61.4, 72.2, 63.4, 11.7, 35.8, 20.4 and 58.9% of the plasma cotinine concentration of subjects carrying *CYP2A6**1/*1, respectively, suggesting that the catalytic activity of *CYP2A6* is lower in the subjects homozygous for either *CYP2A6**7 or *CYP2A6**9, or heterozygous within the *CYP2A6**4, *CYP2A6**7, *CYP2A6**9 and *CYP2A6**10 variants. Additionally, we also found the *CYP2A6**4/*11 genotype from a patient who also showed a poor metabolic phenotype in the metabolism of tegafur to yield 5-fluorouracil (22), suggesting that the enzyme encoded by *CYP2A6**11 had a lower metabolic capacity. In fact, we clarified that the recombinant *CYP2A6*.11 had a lower capacity to metabolize tegafur (41% of *CYP2A6*.1) and coumarin (59%) (22). Furthermore, analyzing the plasma concentration of nicotine, Xu and colleagues (24) have reported that individuals who possessed the *CYP2A6**7/*7, *CYP2A6**4/*7 and *CYP2A6**4/*10 genotypes showed apparently intermediate and poor metabolic phenotype, probably indicating that the *CYP2A6**7 and *CYP2A6**10 are among the causative alleles

Table III. Relationship between the *CYP2A6* groups and lung cancer risk

Group ^a		1	2	3	4
All cases	Cases (%) / controls (%)	300 (27.4) / 110 (18.0)	528 (48.3) / 293 (47.9)	241 (22.0) / 180 (29.5)	25 (2.3) / 28 (4.6)
	OR (95% CI) ^b	1.00 ^c	0.59 (0.44–0.79) ^d	0.52 (0.37–0.72) ^d	0.30 (0.16–0.57) ^d
< 38.3 pack-years	Cases (%) / controls (%)	66 (19.0) / 63 (18.1)	161 (46.4) / 146 (42.0)	102 (29.4) / 117 (33.6)	18 (5.2) / 22 (6.3)
	OR (95% CI) ^b	1.00 ^c	0.95 (0.61–1.49)	0.73 (0.46–1.18)	0.48 (0.22–1.04)
≥ 38.3 pack-years	Cases (%) / controls (%)	234 (31.3) / 47 (17.9)	367 (49.1) / 147 (55.9)	139 (18.6) / 63 (24.0)	7 (1.0) / 6 (2.2)
	OR (95% CI) ^b	1.00 ^c	0.42 (0.28–0.62) ^d	0.39 (0.25–0.63) ^d	0.19 (0.05–0.65) ^d

Significant difference in the distribution of *CYP2A6* genotypic groups was found between lung cancer cases and control subjects (χ^2 value 24.2, $P < 0.0001$).

^aGroups 1, 2, 3 and 4 were classified according to the *CYP2A6* genotypes. Group 1 includes the subjects carrying *CYP2A6**1/*1 (wild-type). Group 2 contains subjects heterozygous for wild-type allele (*CYP2A6**1/*4, *CYP2A6**1/*7, *CYP2A6**1/*9, *CYP2A6**1/*10, *CYP2A6**1/*11). Group 3 consists of subjects carrying *CYP2A6**4/*7, *CYP2A6**4/*9, *CYP2A6**4/*10, *CYP2A6**4/*11, *CYP2A6**7/*7, *CYP2A6**7/*9, *CYP2A6**7/*10, *CYP2A6**9/*9, *CYP2A6**9/*10, *CYP2A6**9/*11 or *CYP2A6**10/*10. Group 4 contains subjects homozygous for *CYP2A6* deletion allele (*CYP2A6**4/*4).

^bTo adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.

^cReference category.

^dSignificant decrease of OR is indicated by 95% CI.

Table IV. Allele frequency of *CYP2A6* in lung cancer patients

Allele ^a	Cases (%) ($n = 2188$)	Controls (%) ($n = 1222$)	OR (95% CI) ^b
<i>CYP2A6</i> *1	1128 (51.6)	513 (42.0)	1.00 ^c
<i>CYP2A6</i> *4	351 (16.0)	242 (19.8)	0.66 (0.54–0.80) ^d
<i>CYP2A6</i> *7	212 (9.7)	154 (12.6)	0.63 (0.50–0.79) ^d
<i>CYP2A6</i> *9	430 (19.7)	253 (20.7)	0.77 (0.64–0.93) ^d
<i>CYP2A6</i> *10	55 (2.5)	52 (4.3)	0.48 (0.32–0.71) ^d
<i>CYP2A6</i> *11	12 (0.5)	8 (0.7)	0.68 (0.28–1.68)

Significant difference in the distribution of the six alleles between lung cancer cases and control subjects was found (χ^2 value 35.7, $P < 0.0001$).

^a*CYP2A6**1 consists of *CYP2A6**1A and *1B alleles. *CYP2A6**4 consists of *CYP2A6**4B and *4C allele.

^bCrude OR.

^cReference category.

^dSignificant decrease of OR is indicated by 95% CI.

reducing enzymatic activity of *CYP2A6*. These lines of evidence support our idea that not only the amounts of daily cigarette consumption but also the risk for tobacco-related lung cancer decrease in association with the impaired function of *CYP2A6* (Figure 1B and Table III). Results reported by Tyndale and coworkers (41–44) on the association between the *CYP2A6* genetic polymorphism with smoking behavior are in agreement with our results. The present study also clearly indicates that the predicted capacity of *CYP2A6* correlates well with the tobacco-related lung cancer risk, suggesting that the inhibition of this enzyme by some inhibitors of this enzyme results in the prevention of the occurrence of tobacco-related lung cancer. Supporting this idea, our recent study showed that treatment of A/J mice with NNK together with 8-methoxypsoralen, a specific inhibitor of *CYP2As*, completely abolished the occurrence of NNK-induced adenoma (33).

SqCC and SCC have been recognized as major types of lung cancer caused by smoking, whereas Ad has not been regarded as a common histological type of lung cancer caused by smoking until recent years, when it was demonstrated that Ad could be increased by smoking (37,38). Thus, it is of interest to note that in the present study the decreased ORs are seen in SqCC and SCC rather than in Ad, which was in agreement with a previous concept that SqCC and SCC appeared highly related to tobacco smoking.

Conflicting results have been reported on the association of *CYP2A6* genetic polymorphisms and lung cancer risk (27–32).

These contradictory results seem to be caused by several factors. First, the original genotyping method (45), which was employed in the previous two reports (27,46) is rather non-specific, which caused a misclassification of *CYP2A6* genotypes. Secondly, the frequencies of the inactive alleles such as *CYP2A6**2 and *CYP2A6**4 in their studies were too small to detect a potential relationship with sufficient statistical power (29,30). A larger population is needed to confirm their findings. Thirdly, they analyzed the genes of combined groups of smokers and non-smokers (30,32) as pointed out in the Introduction section. As reported in this and a previous paper (31), we found that the association between the genotype of *CYP2A6* and the lung cancer risk could be seen only in smokers. In our preliminary results, ORs of subjects heterozygous for the *CYP2A6**1 and *CYP2A6**4 allele and homozygous for the *CYP2A6**4 allele were 0.79 (95% CI of 0.59–1.07) and 1.48 (95% CI of 0.80–2.76) among 331 healthy controls and 743 cases in Japanese non-smokers, respectively (data not shown). In contrast, Tan *et al.* (30) recently reported that Chinese individuals carrying at least one *CYP2A6**4 allele were at a 2-fold increased risk of lung cancer compared with those without a *CYP2A6**4 allele. However, this effect was limited mainly to non-smokers in their study (30). In their more recent report, they reported again that no association was observed between the *CYP2A6* genotype and the risk of lung cancer (32). In this study, they analyzed the gene from subjects of smokers and non-smokers (OR = 0.97, 95% CI of 0.72–1.31). Careful analyses using only smokers will be needed to elucidate the impact of *CYP2A6**4 for lung cancer risk in their studies (30,32), since *CYP2A6* is one of the key enzymes in the metabolic activation of NNK and other *N*-nitrosamines in tobacco smoke (9) and in the metabolism of nicotine (2). The importance of categorization by the number of cigarettes smoked was first proposed by analyzing data between the genetic polymorphisms of *CYP2D6* and lung cancer risk (47). In our study, it is of interest to note that a reduced risk for lung cancer associated with the *CYP2A6* genetic polymorphisms was seen more clearly in heavy smokers (≥ 38.3 pack-years). The 50th percentile pack-years value (38.3 pack-years) in the Japanese population was higher than in the Chinese population (30,32). The reason for this is not known at present. It is also of interest to perform another sub-analysis separating subjects by current and ex-smokers. However, we could not analyze according to this classification, because of the small number of ex-smokers in the present study.

Table V. Relationship between the *CYP2A6* groups and tobacco-related lung cancer risk according to the histological types of lung cancer

Group ^a		1	2	3	4
SqCC	Cases (%) / controls (%)	80 (27.0) / 110 (18.0)	152 (51.4) / 293 (47.9)	60 (20.9) / 180 (29.5)	2 (0.7) / 28 (4.6)
	OR (95% CI) ^b	1.00 ^c	0.62 (0.41–0.93) ^d	0.52 (0.32–0.84) ^d	0.07 (0.01–0.33) ^d
< 38.3 pack-years	Cases (%) / controls (%)	13 (19.4) / 63 (18.1)	33 (49.3) / 146 (42.0)	20 (29.8) / 117 (33.6)	1 (1.5) / 22 (6.3)
	OR (95% CI) ^b	1.00 ^c	0.93 (0.44–1.98)	0.75 (0.34–1.70)	0.12 (0.01–1.02)
≥ 38.3 pack-years	Cases (%) / controls (%)	67 (29.3) / 47 (17.9)	119 (52.0) / 147 (55.9)	42 (18.3) / 63 (24.0)	1 (0.4) / 6 (2.2)
	OR (95% CI) ^b	1.00 ^c	0.46 (0.28–0.74) ^d	0.41 (0.23–0.73) ^d	0.09 (0.01–0.84) ^d
SCC	Cases (%) / controls (%)	45 (33.6) / 110 (18.0)	65 (48.5) / 293 (47.9)	23 (17.2) / 180 (29.5)	1 (0.7) / 28 (4.6)
	OR (95% CI) ^b	1.00 ^c	0.46 (0.28–0.77) ^d	0.39 (0.21–0.72) ^d	0.10 (0.01–0.78) ^d
< 38.3 pack-years	Cases (%) / controls (%)	5 (23.8) / 63 (18.1)	11 (52.4) / 146 (42.0)	5 (23.8) / 117 (33.6)	0 (0.0) / 22 (6.3)
	OR (95% CI) ^b	1.00 ^c	0.81 (0.26–2.51)	0.51 (0.14–1.90)	NA ^e
≥ 38.3 pack-years	Cases (%) / controls (%)	40 (35.4) / 47 (17.9)	54 (47.8) / 147 (55.9)	18 (15.9) / 63 (24.0)	1 (0.9) / 6 (2.2)
	OR (95% CI) ^b	1.00 ^c	0.36 (0.21–0.63) ^d	0.31 (0.15–0.62) ^d	0.17 (0.02–1.60)
Ad	Cases (%) / controls (%)	143 (25.7) / 110 (18.0)	256 (46.0) / 293 (47.9)	138 (24.8) / 180 (29.5)	20 (3.6) / 28 (4.6)
	OR (95% CI) ^b	1.00 ^c	0.59 (0.42–0.81) ^d	0.54 (0.37–0.78) ^d	0.39 (0.20–0.77) ^d
< 38.3 pack-years	Cases (%) / controls (%)	41 (18.0) / 63 (18.1)	100 (43.9) / 146 (42.0)	71 (31.1) / 117 (33.6)	16 (7.0) / 22 (6.3)
	OR (95% CI) ^b	1.00 ^c	0.95 (0.58–1.57)	0.82 (0.48–1.38)	0.67 (0.30–1.51)
≥ 38.3 pack-years	Cases (%) / controls (%)	102 (31.0) / 47 (17.9)	156 (47.4) / 147 (55.9)	67 (20.4) / 63 (24.0)	4 (1.2) / 6 (2.2)
	OR (95% CI) ^b	1.00 ^c	0.42 (0.27–0.64) ^d	0.43 (0.26–0.72) ^d	0.23 (0.06–0.92) ^d

Significant association between the *CYP2A6* groups and lung cancer risk with SqCC, SCC and Ad seen as χ^2 value 20.8 ($P = 0.0001$), χ^2 value 15.8 ($P < 0.01$) and χ^2 value 15.6 ($P < 0.01$), respectively.

^aGroups 1, 2, 3 and 4 were classified according to the *CYP2A6* genotypes. See Table III for details.

^bTo adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.

^cReference category.

^dSignificant decrease of OR is indicated by 95% CI.

^eNot applicable.

Tobacco smoke contains a number of tobacco-specific *N*-nitrosamines, such as *N*-nitrosodiethylamine, NNK and *N*-nitrosornicotine (48). In addition to *CYP2A6*, *CYP1A1* and *CYP2A13* are able to activate NNK (8,9,49,50). Thus, it can be expected that the genetic polymorphism of the *CYP1A1* and *CYP2A13* genes affect the tobacco-related cancer risk. Recently, we found 14 novel *CYP2A13* haplotypes including the Arg257Cys variant, which was named as *CYP2A13**2 (51). Wang *et al.* (32) have reported recently that the frequency of the *CYP2A13* variant associated with the reduced risk of lung Ad in light smokers. However, analyzing our data using the same subjects employed in the present study, we found no clear association between the lung cancer risk and the *CYP2A13**2 allele (data not shown). The reason for this discrepancy is unknown at present. Furthermore, the contribution to cancer risk of other carcinogens such as polycyclic aromatic hydrocarbons and aromatic amines in tobacco smoke could not be ruled out. In fact, the enzymes belonging to the *CYP1* gene family play central roles in the metabolic activation of these compounds present in tobacco smoke (52,53). However, we were unable to find out any clear relationships between genetic polymorphism of *CYP1A1* and tobacco-related lung cancer risk with the same population employed in the previous epidemiological study (31), probably suggesting that the metabolic activation by *CYP2A6* of nitrosamines or carcinogens other than polycyclic aromatic hydrocarbons is the key step determining the tobacco-related lung cancer risk.

In conclusion, our results suggest strongly that the genetic polymorphism of *CYP2A6* is one of the principal determinants affecting not only smoking behavior but also tobacco-related lung cancer risk in the Japanese population.

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