INTRODUCTION

Lung cancer remains one of the major causes of mortality in many developed countries. 1) Although cigarette smoking is the main cause of lung cancer, not all smokers develop the disease, and only a fraction of lifelong non-smokers will die from it. Genetic host factors have recently been implicated to account for some of the observed differences in susceptibility. In this respect, drug metabolizing enzymes are the most studied biotransforming enzymes. The polymorphic expression of these enzymes has been associated with individual cancer susceptibility.2–4) The CYP2A subfamily has been shown to exhibit high activity toward tobacco-specific N-nitrosamines (TSNAs). Some kinds of TSNAs induce lung adenocarcinoma in laboratory animals. Thus, polymorphisms of CYP2A6 and CYP2A13 genes may be important as genetic factors of lung cancer, particularly lung adenocarcinoma. We genotyped 179 patients with lung cancer and 183 controls for CYP2A6 gene deletion-type and CYP2A13 C3375T polymorphisms by polymerase chain reaction-based techniques on DNA prepared from peripheral blood. In addition, a questionnaire was used to collect demographic, lifestyle and other information from each subject. Unconditional logistic regression was used to compute the odds ratios (ORs) and their 95% confidence intervals (95% CIs), with adjustments for several covariates found to be associated with risk. Compared with one or more copies of the T allele, the CYP2A13 CC genotype was associated with a substantially increased risk for lung adenocarcinoma (OR = 2.41, 95% CI = 1.03–6.28). Smokers with the CYP2A13 CC genotype showed significantly higher risk (OR = 4.88, 95% CI = 1.35–26.53) compared to non-smokers with possession at least one copy of the CYP2A13 T allele. Although our results indicate that the CYP2A13 gene may play a role in the development of lung adenocarcinoma, the risk for those individuals with single at-risk genotype of CYP2A13 C3375T polymorphism may not be so large. In future studies, analyses based on haplotypes are recommended to confirm the role of CYP2A13 gene in the development of lung adenocarcinoma.

Key words —— lung adenocarcinoma, CYP2A6, CYP2A13, tobacco specific N-nitrosamine

Both cytochrome P450 (CYP) 2A6 and CYP2A13 are involved in the metabolic activation of tobacco-specific N-nitrosamines (TSNAs). Some kinds of TSNAs induce lung adenocarcinoma in laboratory animals. Thus, polymorphisms of CYP2A6 and CYP2A13 genes may be important as genetic factors of lung cancer, particularly lung adenocarcinoma. We genotyped 179 patients with lung cancer and 183 controls for CYP2A6 gene deletion-type and CYP2A13 C3375T polymorphisms by polymerase chain reaction-based techniques on DNA prepared from peripheral blood. In addition, a questionnaire was used to collect demographic, lifestyle and other information from each subject. Unconditional logistic regression was used to compute the odds ratios (ORs) and their 95% confidence intervals (95% CIs), with adjustments for several covariates found to be associated with risk. Compared with one or more copies of the T allele, the CYP2A13 CC genotype was associated with a substantially increased risk for lung adenocarcinoma (OR = 2.41, 95% CI = 1.03–6.28). Smokers with the CYP2A13 CC genotype showed significantly higher risk (OR = 4.88, 95% CI = 1.35–26.53) compared to non-smokers with possession at least one copy of the CYP2A13 T allele. Although our results indicate that the CYP2A13 gene may play a role in the development of lung adenocarcinoma, the risk for those individuals with single at-risk genotype of CYP2A13 C3375T polymorphism may not be so large. In future studies, analyses based on haplotypes are recommended to confirm the role of CYP2A13 gene in the development of lung adenocarcinoma.

Key words —— lung adenocarcinoma, CYP2A6, CYP2A13, tobacco specific N-nitrosamine
among Japanese populations.\textsuperscript{13-16} In contrast to those findings among Japanese, Chinese individuals with at least one \textit{CYP2A6*4} deletion allele were at a 2-fold (95\% CI = 1.2–3.2) increased risk of developing lung cancer compared with those without any \textit{CYP2A6*4} allele.\textsuperscript{17} CYP2A13 has been found predominantly in the respiratory tract.\textsuperscript{18} The catalytic activity of CYP2A13 toward NNK is more efficient than that of CYP2A6.\textsuperscript{18} Zhang \textit{et al}.\textsuperscript{19,20} and Fujieda \textit{et al}.\textsuperscript{21} have analyzed structural variations in the \textit{CYP2A13} gene. A C to T transition in exon 5 of the \textit{CYP2A13} (\textit{CYP2A13*7} allele, C3375T, Arg257Cys) has been shown to lead to a significant reduction in enzymatic activity (half the activity of the wild-type enzyme).\textsuperscript{19} This missense mutation occurs with varying frequency, ranging from 2\% in a white population to 14\% in a black population.\textsuperscript{19} Wang \textit{et al}. reported that the variant \textit{CYP2A13} allele is associated with a reduced risk of lung adenocarcinoma in a Chinese population.\textsuperscript{22} Another nonsense mutation polymorphism, C301T (\textit{CYP2A13*7} allele, Arg101Stop), which was detected in a Chinese population, is likely to be functionally important since it entails a severely truncated protein lacking enzymatic activity.\textsuperscript{20} Zhang \textit{et al}. reported that this variant allele also exhibits a protective effect against procarcinogens in the respiratory tract.\textsuperscript{20} This \textit{CYP2A13} C301T polymorphism has been associated with the risk of small cell lung cancer.\textsuperscript{23}

To more fully evaluate the relationship between lung adenocarcinoma and smoking, we conducted a case-control study of lung cancer in a Japanese population with special reference to the interaction between smoking and either the \textit{CYP2A6} deletion-type or \textit{CYP2A13} C3375T polymorphism.

### MATERIALS AND METHODS

#### Study Subjects and Data Collection
This case-control study involved 179 male patients with lung cancer and 183 healthy male controls who were employees of the Fukuoka Prefectural Government. Details are shown elsewhere.\textsuperscript{24} Subjects with histologically confirmed primary lung cancer were recruited from September 1996 to September 1998 at the Kyushu University Hospital (Research Institute for Diseases of the Chest, Kyushu University). All subjects were unrelated ethnic Japanese. Information on smoking and drinking habits, family history of cancer, possible occupational exposure and medications was gathered from both patients and controls. Table 1 summarizes characteristics with respect to age, Brinkman index (number of cigarettes smoked per day multiplied by number of years of smoking), prevalence of smokers, and distribution of \textit{CYP2A6} deletion-type and the \textit{CYP2A13} C3375T polymorphisms among lung cancer patients and healthy controls.

The study protocol was approved by our institutional review board, and all participants provided written informed consent.

#### Genetic Analyses
Genomic DNA was extracted from buffy coat stored at –80°C using the QIAamp blood kit (QIAGEN, Inc., Santa Clarita, CA, U.S.A.) and genotyped by polymerase chain reaction based-techniques. Genotyping was conducted with blinding to case/control status. \textit{CYP2A6} genotypes were analyzed for the wild-types (\textit{CYP2A6*1A} and \textit{CYP2A6*1B}), and the deletion-type (\textit{CYP2A6*4}) by the method established by Ariyoshi \textit{et al}.\textsuperscript{25} The \textit{CYP2A6*4} allele in this study is the \textit{CYP2A6*4A}, which is equivalent to \textit{CYP2A6*4C}, a major causal allele in Japanese lacking \textit{CYP2A6} activity.\textsuperscript{26,27} Therefore, the

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean age (range)</th>
<th>Brinkman index (median)</th>
<th>Prevalence of smokers (%)</th>
<th>Frequency of genotypes, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>\textit{CYP2A6} deletion-type polymorphism</td>
</tr>
<tr>
<td>Lung cancer patients</td>
<td>179</td>
<td>64.3 (35–86)</td>
<td>700</td>
<td>69.8</td>
<td>44 (24.6)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>88</td>
<td>61.5 (35–84)</td>
<td>65</td>
<td>50</td>
<td>19 (21.5)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>55</td>
<td>67.8 (36–80)</td>
<td>1000</td>
<td>85.5</td>
<td>14 (25.5)</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>27</td>
<td>66.4 (52–78)</td>
<td>1000</td>
<td>100</td>
<td>6 (22.2)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>9</td>
<td>63.3 (52–86)</td>
<td>900</td>
<td>77.8</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Control individuals</td>
<td>183</td>
<td>57.7 (32–86)</td>
<td>500</td>
<td>42.1</td>
<td>34 (18.6)</td>
</tr>
</tbody>
</table>
CYP2A6 genotypes were categorized into *1/*1, *1/*4, and *4/*4 (the deletion type). The genotypes of CYP2A13 C3375T polymorphism were analyzed by the method by Zhang et al. The wild-type C allele had a HhaI restriction site that resulted in two bands (217 and 58 bp) and the T allele lacked the restriction site. For quality control, both assays were repeated on a random 5% of all samples, and the replicates were 100% concordant.

**Statistical Analysis** — We used \( \chi^2 \) statistics for homogeneity to test for case-control differences in the distribution of genotypes or other parameters under study. Unconditional logistic regression was used to compute the ORs and their 95% CIs, with adjustments for several covariates found to be associated with risk (age, sex, smoking status, alcohol intake, and family history of lung cancer). Subjects were considered current-smokers who had smoked before either the date of diagnosis for lung cancer or the date of filling out the questionnaires. Non-smokers were defined as those who had smoked fewer than 365 cigarettes in their lifetime. Since very few subjects were ex-smokers, they were included in the current-smokers category. To test for statistical interactions between genotypes and smoking status, we entered interaction terms reflecting the product of genotype and smoking status into the logistic models.

All statistical analyses were performed using the computer program STATA Version 8 (STATA Corporation, College Station, TX, U.S.A.). All P values were two-sided, with those less than 0.05 considered statistically significant.

### RESULTS

Our analysis included 179 lung cancer patients (88 with adenocarcinoma, 55 with squamous cell carcinoma, 27 with small cell carcinoma, and 9 with large cell carcinoma). The distributions of selected characteristics among subjects are summarized in Table 1. There were no significant differences between cases and controls in terms of age distribution (data not shown). As for the median of the Brinkman index, fewer cigarettes were smoked by subjects with adenocarcinoma compared to those with other cell types (\( p < 0.001 \)). The median Brinkman index was lower in cases with adenocarcinoma than in controls, although the prevalence of smokers was similar between them. Regarding the CYP2A6 deletion-type polymorphism, the distribution of *1/*1, *1/*4, and *4/*4 genotypes among controls was 81.4, 17.5, and 1.0%, respectively. As for the CYP2A13 C3375T polymorphism, the frequency of the three genotypes CC, CT, and TT among controls was 82.5, 16.4 and 1.1%, respectively. The allelic frequencies of the CYP2A6 deletion-type or CYP2A13 C3375T polymorphism in controls were calculated to be Hardy-Weinberg linkage equilibrium: 0.902 and 0.098 for the *1 and *4 allele; 0.907 and 0.093 for the C and T allele, respectively. Distribution of the *4/*4 and *1/*4 genotypes combined or the TT and CT genotypes combined did not differ among different histological types. Genotype distributions of CYP2A6 and CYP2A13 were similar between cases and controls. Since both the CYP2A6 *4/*4 and CYP2A13 TT genotypes were extremely rare in our Japanese population, they were combined with the CYP2A6 *1/*4, respectively, or CYP2A13 CT for subsequent analysis.

Table 2 represents adjusted ORs and 95\% CIs for all lung cancer and specific cell types by CYP2A6.
or CYP2A13 genotype. The presence of at least one copy of the CYP2A6*4 allele was not associated with the risk of overall lung cancer or adenocarcinoma. According to histological type, the CYP2A13 CC genotype was significantly associated with an increased risk of adenocarcinoma (OR = 2.41, 95% CI = 1.03–2.37) compared to one or more copies of the T allele. The joint effects of CYP2A6 and CYP2A13 genotypes were assessed separately in adenocarcinoma and other cell types combined. Lung cancer risk was compared between individuals with variant allele(s) and those with non-variant allele. No clear association was found in the joint effects for adenocarcinoma or other cell types combined (data not shown).

Table 3 shows the modifying effect of the CYP2A6 genotypes on the association of smoking with lung cancer risk. Smokers with the *1/*1 genotype presented a higher risk of lung cancer (other than adenocarcinoma) than those with at least one variant allele (OR = 3.24, 95% CI = 1.23–9.50) as compared to non-smokers with at least one variant allele. The *1/*1 genotype was not associated with an increased risk of adenocarcinoma. Smoking was associated with an increased risk of lung cancer other than adenocarcinoma (OR = 11.15, 95% CI = 5.37–25.50) (data not shown). The interactions between genotypes and smoking were far from significant (Pinteraction = 0.64, 0.62 and 0.91), and smoking was not strongly associated with adenocarcinoma (OR = 1.38, 95% CI = 0.80–2.37) (data not shown).

Table 4 shows the modifying effect of CYP2A13 genotypes on the association of smoking with lung cancer. The adjusted OR for lung cancer was significantly high in smokers with the CC genotype (OR = 3.45, 95% CI = 1.55–7.97) compared to non-smokers with the variant genotype (the CT and TT genotypes combined) while the corresponding value of smokers with the variant genotype was not significant (OR = 2.56, 95% CI = 0.73–8.99). A gene-environment interaction among adenocarcinoma patients was suggested, with a combination of the CYP2A13 CC genotype with smoking conferring a significantly higher risk (OR = 4.88, 95% CI = 1.35–26.53) compared to a combination of at least one copy of the CYP2A13 T allele with non-smoking.

Table 3. Risk of Lung Cancer Associated with CYP2A6 Genotypes by Histological Types

<table>
<thead>
<tr>
<th>Genotype + Smoking</th>
<th>All cases</th>
<th>Cases with adenocarcinoma</th>
<th>Cases with other cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*1 + smoking (+)</td>
<td>1.76 (0.85–3.66)</td>
<td>1.02 (0.43–2.46)</td>
<td>3.24 (1.23–9.50)</td>
</tr>
<tr>
<td>variant + smoking (+)</td>
<td>1.43 (0.69–2.98)</td>
<td>0.97 (0.25–3.71)</td>
<td>2.48 (0.26–6.72)</td>
</tr>
<tr>
<td>*1/*1 + smoking (−)</td>
<td>0.80 (0.18–3.52)</td>
<td>0.66 (0.28–1.61)</td>
<td>1.14 (0.26–5.09)</td>
</tr>
<tr>
<td>variant + smoking (−)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Pinteraction</td>
<td>0.64</td>
<td>0.62</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Other cell types (n = 91) include squamous cell carcinoma (n = 55), small cell carcinoma (n = 27) and large cell carcinoma (n = 9). Adjusted for age, sex, alcohol intake and family history of lung cancer. Variant indicates combined genotype, which has at least one variant allele (*1/*4 + *4/*4).

Table 4. Risk of Lung Cancer Associated with CYP2A13 Genotypes by Histological Types

<table>
<thead>
<tr>
<th>Genotype + Smoking</th>
<th>All cases</th>
<th>Cases with adenocarcinoma</th>
<th>Cases with other cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC + smoking (+)</td>
<td>3.45 (1.55–7.97)</td>
<td>4.88 (1.35–26.53)</td>
<td>2.38 (0.49–11.63)</td>
</tr>
<tr>
<td>variant + smoking (+)</td>
<td>2.56 (0.73–8.99)</td>
<td>4.76 (0.61–38.86)</td>
<td>0.92 (0.37–2.24)</td>
</tr>
<tr>
<td>CC + smoking (−)</td>
<td>1.14 (0.49–2.74)</td>
<td>2.27 (0.91–24.08)</td>
<td>0.80 (0.18–3.52)</td>
</tr>
<tr>
<td>variant + smoking (−)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Pinteraction</td>
<td>0.84</td>
<td>0.48</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Other cell types (n = 91) include squamous cell carcinoma (n = 55), small cell carcinoma (n = 27) and large cell carcinoma (n = 9). Adjusted for age, sex, alcohol intake and family history of lung cancer. Variant indicates combined genotype, which has at least one variant allele (CT + TT).
DISCUSSION

Principal histological types of lung cancer are squamous cell carcinoma, large cell carcinoma, small cell carcinoma and adenocarcinoma, and the former three are strongly associated with smoking. In recent decades, the frequency of adenocarcinoma has risen and that of squamous cell carcinoma has declined in a number of developed countries. The increase in incidence of adenocarcinoma could be partly explained by an increase in filtered cigarette smoking. Filter cigarettes with low-tar and low-nicotine have replaced nonfilter cigarettes. One key characteristic of such changes over time has been an increased nitrate content of the tobacco blends from about 0.5 to 1.3%. TSNAs are formed by the N-nitrosation of nicotine and other minor alkaloids during tobacco processing and while smoking. Since nitrate is the major precursor of nitrogen oxides, increased nitrate content leads to higher yields of NNK in the smoke. To satisfy a craving for nicotine, a smoker of low-yield nicotine filtered cigarettes may tend to compensate by increasing the number and depth of puffs. Therefore, the peripheral lung, where adenocarcinoma generally arises, is exposed to higher amounts of smaller particles such as NNK. Indeed, NNK is a systemic carcinogen that has been shown to induce lung carcinoma in laboratory animals independent of the site of application, whereas the intratracheal inhalation of benzo[a]pyrene and other polycyclic aromatic hydrocarbons has preferentially induced squamous cell carcinoma.

Adenocarcinoma is the most frequent type of lung cancer that is observed in the population among non-smokers. Passive smoking, involuntary smoking and environmental tobacco smoke (ETS) are often used synonymously to describe the involuntary exposure of non-smokers. ETS is comprised of diluted sidestream smoke from the smoldering of the cigarette between puffs and mainstream smoke exhaled by the smokers. Today ETS is judged to be a cause of lung cancer in non-smokers by IARC. ETS contains over 40 known carcinogens, including polycyclic aromatic hydrocarbons, nitrosamines, and TSNAs, nitrosamines from nicotine. The most abundant constituent in ETS is NNK, which is metabolized by both CYP2A6 and CYP2A13, causes adenocarcinoma in humans, low activity of both CYP2A6 and CYP2A13 may be associated with a decreased risk of lung adenocarcinoma.

In this study, we observed an allelic frequency of 9.8% for the \textit{CYP2A6}^*4 allele among controls. Frequency of the \textit{CYP2A6}^*4 allele in our study is one-half of that reported by other Japanese studies (18.4–20.4%). Frequencies of the deletion allele among two Chinese populations were 8.6 and 8.0%. The reasons remains unclear for the discrepancy in the frequency of the \textit{CYP2A6}^*4 allele between our study and those of other Japanese as well as for the agreement between the two Chinese populations and ours. In this connection, the \textit{CYP2A6}^*4 alleles were found to be fairly common in Chinese and Japanese but rare in Caucasians (0.5–3.0%). An allelic frequency of 9.3% for the \textit{CYP2A13} allele shown among controls in the present study is similar to that among Chinese (9%). The Chinese study is the only study to investigate the effect of \textit{CYP2A13} polymorphism on susceptibility to lung cancer.

As lung adenocarcinoma is likely to be linked to NNK, a protective effect of the variant genotypes (decreased metabolic activation activity) on cancer risk has been assumed. In the present study, we could find no significant association between the \textit{CYP2A6} deletion-type polymorphism and the risk of adenocarcinoma. Nor was such an association observed in another study. However, the whole \textit{CYP2A6} gene deletion was associated with a significantly decreased risk of lung cancer among Japanese populations, showing that the variant genotypes were at a 2-fold (95% CI = 1.2–3.2) greater risk of lung cancer (unspecified cell types) compared with those without the \textit{CYP2A6}^*4 allele. Furthermore, the authors concluded that the effect of \textit{CYP2A6}^*4 on lung cancer risk was mainly limited to squamous cell carcinoma. The relationship between the \textit{CYP2A6} gene and lung cancer remains questionable.

Among several genetic polymorphisms of the \textit{CYP2A13} gene, a single nucleotide polymorphism has been detected in exon 5, leading to an Arg257Cys amino acid change (C3375T). Since the 257Cys allele has been shown to contribute to decreased catalytic efficiency, it might provide some protec-
tion against TSNAs in lung cancer. However, compared with CYP2A6, little is known about the possible association of this polymorphism with lung cancer risk. In this study, compared with individuals with one or more copies of the T allele, those with the CYP2A13 CC genotype presented the strongest association with adenocarcinoma (OR = 2.41, 95% CI = 1.03–6.28). A significant reduction in the risk of adenocarcinoma among patients with the CYP2A13 TT genotype (OR = 0.41, 95% CI = 0.23–0.71) was observed in a Chinese population.22) On the other hand, a significantly increased risk of adenocarcinoma was seen among subjects with the CYP2A13 CC genotype (OR = 2.44, 95% CI = 1.41–4.35).22) Again, our findings coincided with those from Chinese subjects. In contrast, no association was found between this CYP2A13 C3375T polymorphism and the risk of squamous cell carcinoma.22) Cazuffo et al.23) also did not detect the CYP2A13 C3375T polymorphism in a French population although they did find another polymorphism, CYP2A13 C301T, that produced an elevated risk of small cell lung cancer in subjects with the CT genotype (OR = 9.9, 95% CI = 1.9–52.2).23) However, the TT genotype of the CYP2A13 C301T polymorphism was not detected in this French population. Speculation that an adenocarcinoma risk may be linked to the CYP2A13 C3375T polymorphism is biologically plausible for a variety of reasons. The amino acid sequence of CYP2A13 shares a 95.4% identity with that of CYP2A6.8) Although the latter may play an important role in the activation of TSNAs, its expression in the lung, if any, appears to be relatively low. CYP2A13 has been found to be expressed predominantly in the human lung.19) This enzyme is highly involved in the activation of NNK and some other carcinogens, with a catalytic efficiency much greater than that of CYP2A6.18)

As for adenocarcinoma, smokers with the CC genotype of the CYP2A13 C3375T polymorphism showed a significantly higher risk (OR = 5.88, 95% CI = 1.35–26.53) than non-smokers with at least one copy of the CYP2A13 T allele. Wang et al.22) reported that the risk of adenocarcinoma for the CC genotype of the CYP2A13 C3375T polymorphism among smokers (OR = 2.94, 95% CI = 1.45–5.88) was higher than that among non-smokers (OR = 1.67, 95% CI = 0.99–2.86). Furthermore, they indicated that the effect of the CC genotype was evident at low dose of smoking. Genetic susceptibility may be more important even among infrequent smokers.44) From a metabolic point of view, this might be explained by the fact that at high levels the relevant enzyme is saturated irrespective of the genotype, while this does not occur at low levels.

In conclusion, our results do not support a major role for the CYP2A6 gene deletion-type polymorphism as a risk factor for lung adenocarcinoma in our Japanese population. Although the functional C3375T polymorphism in the CYP2A13 gene may be associated with adenocarcinoma, our current limited sample size makes it difficult to establish. Furthermore, the etiology of lung cancer cannot be explained by allelic variability at a single locus. The associations between other functional CYP2A13 polymorphisms and lung cancer risk will require further studies. Therefore, it is likely that the defining feature of future epidemiologic studies will be the simultaneous analysis of large samples of cases and controls.45,46) Advances in identification of new variants and in high-throughput genotyping techniques will facilitate analysis of multiple polymorphisms within the genes with the same pathway.47) The major burden of lung cancer in the population probably results from complex interaction between many genetic and environmental factors over time. The effects of polymorphisms are best represented by their haplotypes. In future association studies on lung cancer, haplotype-based methods will facilitate the evaluation of haplotype effects, either for selected polymorphisms physically close to each other or for multiple genes within the same drug-metabolism pathway.

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