

Table 1 Continued

<i>Patients</i>	<i>NS223</i>	<i>NS231</i>	<i>NS239</i>	<i>NS248</i>	<i>NS254</i>	<i>NS263</i>	<i>NS299</i>	<i>NS318</i>	<i>NS324</i>	<i>Total</i>
Gender	F	F	M	M	F	M	F	F	F	
Age	6 months	5 months	18 years	5 years	2 months	1 month	3 years	1 month	1 year 6 months	
Paternal age at birth (years)	34	27	27	NA	37	35	34y	33	33	
Maternal age at birth (years)	36	27	26	30	34	36	35y	32	33	
<i>Growth and development</i>										
Postnatal failure to thrive	+	+	+	+	+	+	+	+	+	21/21
Mental retardation	+	+	+	+	NA	+	+	+	+	20/20
<i>Craniofacial characteristics</i>										
Relative macrocephaly	-	+	+	-	+	+	-	-	+	17/21
Coarse facial appearance	+	+	+	+	+	+	+	+	+	21/21
<i>Musculoskeletal characteristics</i>										
Short neck	-	+	NA	NA	+	+	+	-	-	14/19
Hyperextensive fingers	-	+	-	+	+	-	-	+	+	13/21
Tight Achilles tendon	+	NA	-	+	-	-	-	+	+	10/20
Abnormal foot position	-	-	NA	NA	NA	-	-	+	+	9/16
<i>Skin characteristics</i>										
Curly, sparse hair	+	Curly	Curly	+	+	+	Curly	+	Curly	21/21
Soft, loose skin	-	+	+	+	+	+	-	+	+	18/21
Deep palmer/planter creases	+	-	+	+	+	+	+	+	+	20/21
<i>Cardiac defect</i>										
Hypertrophic cardiomyopathy	+	-	+	+	+	+	+	+	+	14/20
Other	PAC	PVC	-	-	-	-	-	PAC	PAC	
<i>Neoplasia</i>										
Papillomata	+	-	+	-	-	-	-	-	-	6/20
Other tumors										
<i>Others</i>										
	Prabastatin administration	Laryngomalasia, hydrocephallus	GH deficiency, Arnold Chiari, scoliosis	Empty sella, GH deficiency, hypothyroidism, hypogonadism, syringomyelia		Hyperinsulinemia		Laryngomalasia seizure	Laryngomalasia	
<i>HRAS mutation</i>										
Nucleotide substitution	c.34G>T	c.35G>A	c.34G>A	c.34G>A	c.34G>A	c.35G>C	c.34G>A	c.35G>C	c.34G>A	
Amino acid substitution	p.G12C	p.G12D	p.G12S	p.G12S	p.G12S	p.G12A	p.G12S	p.G12A	p.G12S	

Abbreviations: -, absent; +, present; ASD, atrial septal defect; F, female; GER, gastroesophageal reflux; GH, growth hormone; GSDIII, glycogen storage disease III; M, male; NA, not available; PAC, premature atrial contraction; PS, pulmonic stenosis; PSVT, paroxysmal supraventricular tachycardia; PVC, premature ventricular contraction; VSD, ventricular septal defect.



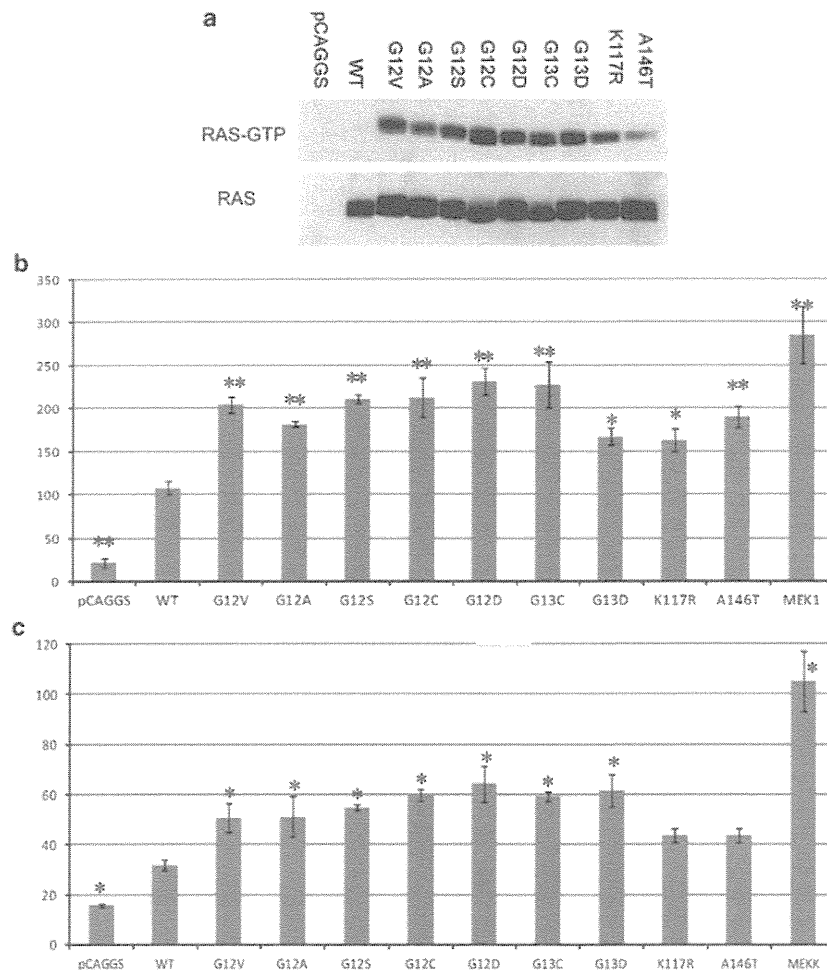


Figure 1 Functional characterization of HRAS mutants. (a) Ras-guanosine triphosphate (GTP) in NIH 3T3 cells transfected with wild-type or mutant HRAS constructs. HRAS protein levels were similar in NIH3T3 cells expressing each protein and were subsequently used as a loading control. (b, c) Stimulation of ELK (b) and c-Jun (c) transcription by HRAS mutants. The ELK- and c-Jun-GAL4 vectors and GAL4-luciferase trans-reporter vector were transiently co-transfected with various HRAS constructs into unstimulated NIH 3T3 cells. Relative luciferase activity (RLA) was normalized to the activity of a co-transfected control vector (pRLnull-luc) expressing *Renilla reniformis* luciferase. The results are expressed as the means and s.d. from triplicate samples. MEK1 and MEKK were used as positive controls. WT, wild type. * $P < 0.05$; ** $P < 0.01$ compared with WT.

associated β -galactosidase staining confirmed that these cells showed cellular senescence.

Two downstream signaling pathways, p53 and Rb-p16, are activated during cellular senescence. To examine oncogene induced cellular senescence at the molecular level, we assessed senescence markers, including phosphorylated extracellular signal-regulated kinase, phosphorylated p53 and p16, in cells expressing HRAS mutant proteins (Figure 2b). As expected, phosphorylated p53 (Ser15) and p16 levels, as well as phospho-extracellular signal-regulated kinase levels, were significantly increased in the cells transfected with HRAS mutants relative to cells transfected with mock vector or wild-type HRAS. These results demonstrate that not only p.G12V, but also the other eight CS-related HRAS mutants, can cause OIS.

DISCUSSION

In this study, we identified four HRAS mutations in 21 patients with CS and evaluated their detailed clinical manifestations of the disease in these patients. Biochemical analyses, including a GTP binding assay

and luciferase assays to detect ELK and c-Jun trans-activation, showed that there were no significant differences among the analyzed mutations in codon 12/13. The p.A146T mutant demonstrated the weakest Raf binding activity, and the p.K117R and p.A146T mutants had weaker effects on downstream c-Jun N-terminal kinase signaling than mutants in codon 12 or 13. Our results indicated that all HRAS mutants detected in CS patients were able to cause OIS.

Our study is the first to demonstrate that HRAS mutants other than p.G12V can induce senescence when they are overexpressed in human fibroblasts. The symptoms of CS seem to be caused by either hyperproliferation or hypoproliferation, coupled with growth factor resistance, which may be ascribable to DNA damage response or OIS. Postnatal cerebellar tonsillar herniation, Chiari 1 malformation,⁴² deep palmar and plantar creases and papillomata may all be caused by hyperproliferation. In contrast, the poor weight gain, short stature and endocrine dysfunction observed in CS patients^{43–45} might be caused by hypoproliferation. Adult brain and heart progenitor cells in a zebrafish CS model with a homozygous HRAS p.G12V mutation

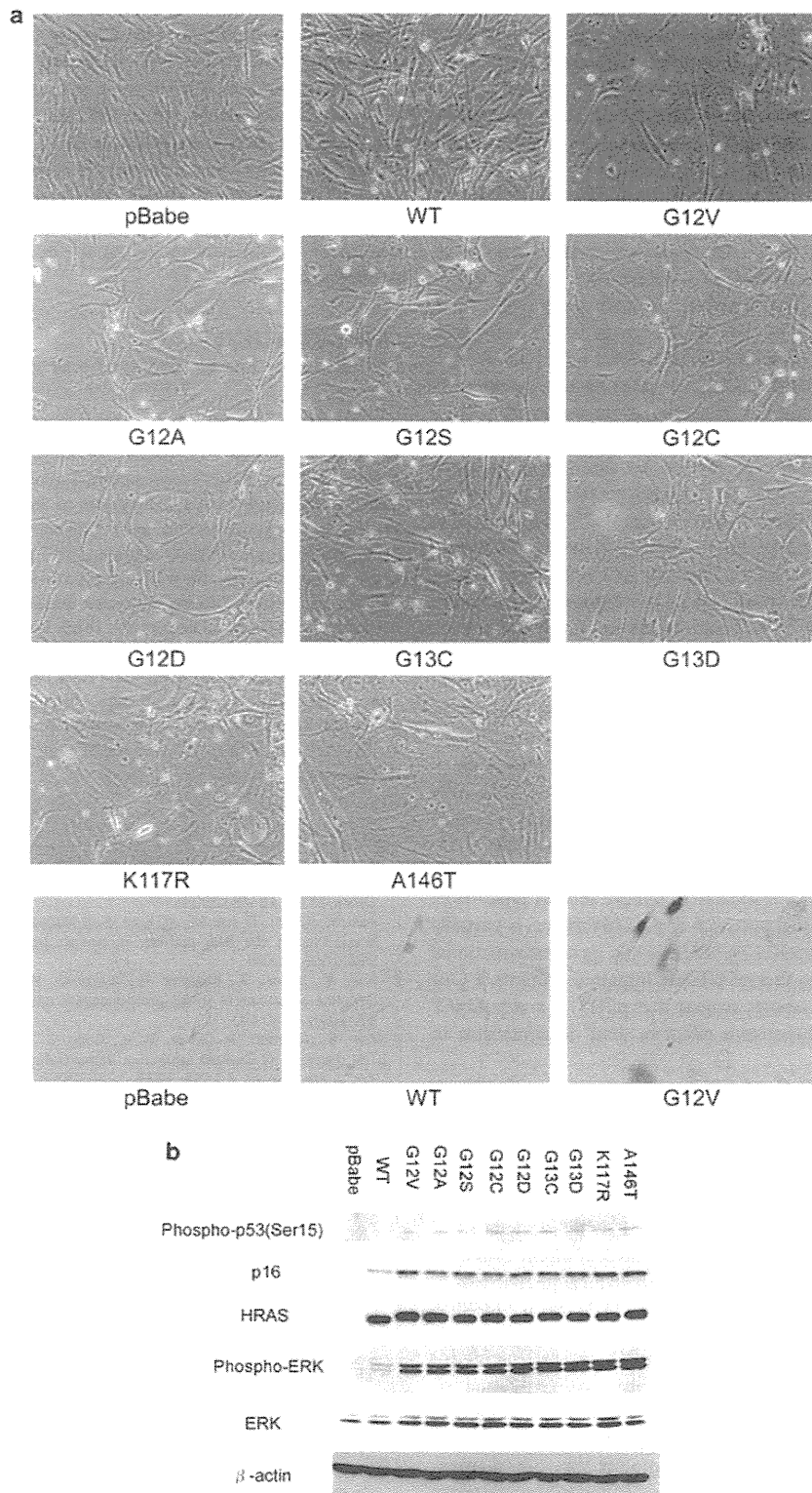


Figure 2 Effect of Costello syndrome (CS)-associated HRAS mutants on primary fibroblasts. (a) BJ cells transduced with retroviruses expressing wild-type or mutant HRAS. Images in the lowest tier show senescence-associated β -galactosidase staining. (b) Immunoblots of cellular lysates from BJ cells transduced with empty vector (pBabe) or with wild-type or mutant HRAS retroviruses.

exhibited cellular senescence, suggesting that the age-related worsening of the Costello phenotype⁴⁶ might occur, because the replicative capability of adult progenitor cells is exhausted. Osteoporosis has frequently been found in adult patients with CS,⁴⁷ suggesting that cellular senescence affects osteogenesis. However, further studies will be needed to determine whether OIS indeed contributes to the pathogenesis in patients with CS.

It has been suggested that clinical symptoms vary among patients with mutations in codon 12 or 13. In previous studies, a total of 19 CS patients have been reported to die from severe cardiomyopathy, cardiac arrhythmia, rhabdomyosarcoma, respiratory failure, multi-organ failure or sepsis. The number of fatal cases was 5/138 patients with p.G12S, 4/6 with p.G12C, 3/17 with p.G12A, 3/4 with p.G12D, 2/2 with p.G12V, 1/1 with p.G12E and 1/1 with p.E63K.^{3,5–23} The mortality of patients with p.G12C or p.G12D was significantly higher than that of the patients with the more common p.G12S ($P=0.026$ by Fisher's exact test). Previous studies have shown that the p.G12V substitution has the highest transformative potential (p.G12V > p.G12A, p.G12S, p.G12C, p.G12D > p.G13D) and is the most frequently found mutation in human tumors.^{48,49} However, our Ras activity assays and luciferase assays did not show any differences among HRAS codon 12/13 mutants. This may be due to the extremely high expression level of HRAS protein in our transient transfection study, which could make it difficult to detect subtle differences between mutants. Further studies will be necessary to clarify whether the high mortality in patients with p.G12C or p.G12D is due to functional differences in these mutants or due to bias because of our small sample size of patients.

Mutations at codons 117 and 146 are rare in CS and somatic cancers. Meanwhile, mutations at codons G12, G13 and Q61 have been shown to impair intrinsic and GTPase activating protein-mediated GTP hydrolysis, leading to elevated levels of cellular RAS-GTP. It has been reported that the nucleotide exchange rate of both p.K117R and p.A146V HRAS is increased, relative to wild type.^{13,27,28} However, the transformational potential of p.A146V HRAS is partially activated,²⁷ whereas that of p.K117R-HRAS is not; its transformational activity is instead similar to that of GTPase impaired mutants.²⁸ Our results and those of other reports suggest that p.K117R and p.A146T have milder effects on downstream effectors than do mutations in codon 12/13.

The clinical manifestations of CS in patients with p.K117R or p.A146V mutations suggest that these alleles have distinct effects, compared with mutations in codon 12/13. Of two CS patients with a p.K117R mutation, one patient had an atypical phenotype such as microretrognathism and slightly less-pronounced palmar and palmar creases.⁷ The other patient had mild craniofacial manifestations of CS.¹³ One patient with the p.A146V mutation showed a mildly coarse face and did not have deep palmar creases.⁶ These atypical phenotypes might be attributed to the mild effects of p.K117R or p.A146V compared with codon 12/13 mutants.

Inhibitors of the RAS/MAPK pathway could provide benefits for patients with RAS/MAPK syndromes. Statins are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors that result in decreased isoprenylation of RAS⁵⁰ and are now widely used for the treatment of hyperlipidemia. Statins have been used to modify the clinical manifestation of neurofibromatosis type I, which is caused by a genetic defect in a negative regulator of the RAS/MAPK pathway. Studies using mouse models of NF1 (Nf1 mice) have shown that treatment with a statin reverses the cognitive deficits of these mice.⁵¹ A randomized control trial for neurofibromatosis type I treatment with simvastatin had a negative outcome.⁵² Furthermore, statins have

displayed antitumor activity in experimental tumor models, though clinical antitumor effects of statins have not been established.⁵³ Well-designed clinical studies will be needed to determine the effects of statins or other RAS inhibitors on manifestations of CS.

In conclusion, we identified HRAS mutations in 21 patients and examined the clinical manifestations of mutation-positive patients. Functional analysis revealed that CS-causing mutant HRAS proteins caused OIS in human fibroblasts. These findings may help enable more accurate prognoses for patients with HRAS mutations and contribute to our understanding of the mechanism underlying CS pathogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Regular Article

Association between Cancer Risk and Drug-metabolizing Enzyme Gene (CYP2A6, CYP2A13, CYP4B1, SULT1A1, GSTM1, and GSTT1) Polymorphisms in Cases of Lung Cancer in Japan

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Full text of this paper is available at <http://www.jstage.jst.go.jp/browse/dmpk>

Summary: Genetic polymorphisms of enzymes involved in the metabolism of carcinogens are suggested to modify an individual's susceptibility to lung cancer. The purpose of this study was to investigate the relationship between lung cancer cases in Japan and variant alleles of cytochrome P450 (CYP) 2A6 (CYP2A6*4), CYP2A13 (CYP2A13*1-10), CYP4B1 (CYP4B1*1-7), sulfotransferase 1A1 (SULT1A1*2), glutathione S-transferase M1 (GSTM1 null), and glutathione S-transferase T1 (GSTT1 null). We investigated the distribution of these polymorphisms in 192 lung cancer patients and in 203 age- and sex-matched cancer-free controls. The polymorphisms were analyzed using various techniques including allele-specific PCR, hybridization probe assay, multiplex PCR, denaturing high-performance liquid chromatography (DHPLC), and direct sequencing. We also investigated allele and genotype frequencies and their association with lung cancer risk, demographic factors, and smoking status. The prevalence of the CYP2A6*4/*4 genotype in lung cancer cases was 3.6%, compared with 9.4% in the controls (adjusted OR = 0.36, 95% CI = 0.15–0.88, $P = 0.025$). In contrast, there was no association between the known CYP2A13, CYP4B1, SULT1A1, GSTM1, and GSTT1 polymorphisms and lung cancer. These data indicate that CYP2A6 deletions may be associated with lung cancer in the Japanese population studied.

Keywords: lung cancer; CYP2A6; CYP2A13; CYP4B1; SULT1A1; GSTM1; GSTT1; genetic polymorphism

Introduction

Lung cancer is a major cause of cancer-related death worldwide.¹⁾ In Japan, lung cancer is the most common cause of death in the male population and the second most common cause of death in the female population, after colon cancer. Identifying the risk factors for lung cancer development is essential to prevent this deadly disease. Environmental

exposure to tobacco smoke is the primary risk factor for lung cancer.^{2,3)} Tobacco smoke contains hundreds of known and probable carcinogens that are either activated or detoxified by xenobiotic metabolizing enzymes. In general, the metabolism of xenobiotics consists of phases I and II. Phase I enzymes, mainly cytochrome P450 (CYP), are typically involved in metabolic pathways involving activation of carcinogens, whereas phase II enzymes play a central role in detoxification.

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Human CYP2A6 and CYP2A13 are important phase I enzymes involved in metabolizing nicotine and the metabolic activation of tobacco-specific nitrosamines such as 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK).^{4,5)} CYP2A6 is mainly expressed in the human liver, whereas CYP2A13 is selectively expressed in the human respiratory tract.^{5,6)} To date, a large number of CYP2A6 and CYP2A13 genetic polymorphisms and alleles have been identified (<http://www.cypalleles.ki.se/>). These alleles are derived from single nucleotide polymorphisms (SNPs) in regulatory and coding regions, deletions, insertions, and conversions. CYP2A6*4 is a major mutant allele associated with decreased metabolic activity.⁷⁾ Several studies have elucidated the role of the CYP2A6*4 allele in tobacco dependence and lung cancer risk.⁸⁻¹⁰⁾ Similarly, the CYP2A13 polymorphism 3375C>T has been correlated with a reduced risk of lung adenocarcinoma in a Chinese population.¹¹⁾

CYP4B1 is primarily an extrahepatic form of P450. CYP4B1 mRNA has been detected in the human lung and bladder.^{12,13)} In animals, CYP4B1 is involved in the metabolism of several xenobiotics such as 2-aminofluorene, 2-naphthylamine, and benzidine.^{14,15)} To date, seven variant alleles of CYP4B1 have been identified in French Caucasian and Japanese individuals. We previously reported that the alleles CYP4B1*2 (AT881-882del, 993G>A, 1018C>T, and 1123C>T) and CYP4B1*3 (517C>T) are common in the Japanese population.¹⁶⁾ In particular, premature termination of protein synthesis by the double nucleotide deletion AT881-882del has been speculated to render the CYP4B1*2 allele non-functional. CYP4B1 genotypes may have an effect on the risk of bladder cancer;¹⁷⁾ however, it is unclear whether CYP4B1 polymorphisms are associated with lung cancer susceptibility.

Sulfotransferases (SULTs) appear to play an important role in phase II metabolism of xenobiotics, small endogenous compounds, and procarcinogenic agents.^{18,19)} Some studies have shown that genetic polymorphisms of SULT1A1 are associated with susceptibility to lung cancer.^{20,21)}

Glutathione S-transferases (GSTs) are phase II enzymes that catalyze the conjugation of reactive intermediates to soluble glutathione. Some GSTs are polymorphic, and some genetic variants, such as GSTM1 null and GSTT1 null, may be associated with increased susceptibility to lung cancer.^{22,23)} Homozygous deletions of the GSTM1 and GSTT1 genes are common and result in complete loss of enzyme activity.

We conducted a case-control study to examine the association between the risk of lung cancer in Japanese individuals and P450s CYP2A6 (CYP2A6*4), CYP2A13 (CYP2A13*1-10), CYP4B1 (CYP4B1*1-7), sulfotransferase 1A1 (SULT1A1*2), glutathione S-transferase M1 (GSTM1 null), and glutathione S-transferase T1 (GSTT1 null) polymorphisms. In addition, we investigated the effect of smoking status and genetic combinations on the association between lung cancer risk and genetic polymorphisms.

Materials and Methods

Subject selection: From February 1995 to July 2003, 1,536 autopsies were performed at the Department of Pathology, Tokyo Metropolitan Geriatric Medical Center, Tokyo, Japan. DNA samples from 395 of these autopsies were analyzed in this case-control study; 192 lung cancer cases and 203 cancer-free controls were sex- and age-matched. The smoking status of the individuals was retrospectively determined by reviewing medical records, and subjects were classified as smokers (including current smokers and ex-smokers) and non-smokers (individuals who have never smoked in their lifetime). Research protocols were approved by the Ethics Committees of Tokyo Metropolitan Geriatric Hospital and the Graduate School of Pharmaceutical Sciences, Tohoku University.

Genetic analysis: The presence of CYP2A6*4 (whole gene deletion) was determined by the two-step allele-specific PCR assay described by Oscarson *et al.*²⁴⁾ The first step involved amplification of a region from exon 7 to approximately 420 bp downstream of exon 9 of CYP2A6 or the CYP2A6/CYP2A7 hybrid from all individuals with or without the deleted CYP2A6 gene. The reaction mixture contained approximately 30 ng genomic DNA, 0.5 μM of each primer (2AE7F, 5'-GGCCAAGATGCCCTACATG-3'; 2A6R1, 5'-GCACTTATGTTTTGTGAGACATCAGAGACAA-3'), 0.25 mM dNTPs, LA Taq polymerase (TaKaRa, Otsu, Japan), and 2× GC Buffer I (TaKaRa) in a total reaction volume of 16 μL. The thermal cycling conditions were as follows: 95°C for 1 min; followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, extension at 72°C for 3 min; and a final extension at 72°C for 7 min. The PCR product was then used as a template in the second step, in which the deleted CYP2A6 gene was detected. PCR amplification was performed with 0.5 μL of the first PCR product, 0.25 μM forward primer (2A6E8F, 5'-CACTTCCTGAATGAG-3', or 2A7E8F, 5'-CATTTCCTGGATTGAC-3'), 0.25 μM reverse primer (2A6R2, 5'-AAAATGGGCATGAACGCC-3'), and 2× Amplitaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL. Thermal cycling conditions involved an initial denaturation at 95°C for 10 min; followed by 16 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 2 min; and a final extension at 72°C for 7 min. Amplified products were analyzed by electrophoresis in 1% agarose gel. The presence of a CYP2A-specific 1181-bp product amplified with the 2A6E8F/2A6R2 primer pair indicated the presence of wild-type CYP2A6 (defined as CYP2A6 non*4 allele in this study). The product amplified from the primer pair 2A7E8F/2A6R2 indicated a CYP2A6 deletion (CYP2A6*4), and the presence of the product in both reactions from one individual indicated heterozygosity.

CYP2A13 genotypes were determined by our previously described assay.²⁵⁾ Long PCR was performed in the first

round to amplify exons 1–5 and 6–9 of the *CYP2A13* gene using 10–50 ng of genomic DNA and LA-Taq DNA polymerase (TaKaRa). The thermal cycling consisted of an initial denaturation at 95°C for 1 min; followed by 25 cycles of denaturation at 95°C for 15 s, annealing at 68°C or 65°C for 20 s, and extension at 72°C for 5 min; and then a final extension at 72°C for 7 min. First-round PCR products were diluted 1:500 and used as templates for the second round of amplification for all *CYP2A13* exons. The amplicons for each exon were generated using AmpliTaq Gold PCR Master Mix (Applied Biosystems). Second-round PCR comprised an initial denaturation at 95°C for 10 min; followed by 25 cycles at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and then a final extension at 72°C for 7 min. Heteroduplexes were generated by thermal cycling under the following conditions: 95°C for 1 min, followed by 45 temperature decrements of 1.5°C/min. PCR products were analyzed with the DHPLC system WAVE (Transgenomic Inc., Omaha, NE, USA). This involved separating PCR products (5 µL) on a heated C18 reverse-phase column using 0.1 M triethylammonium acetate (TEAA) in water and 0.1 M TEAA in 25% acetonitrile at a flow rate of 0.9 mL/min. The temperature for heteroduplex separation of a heterozygous *CYP2A13* fragment was determined with the WAVE software, and the linear acetonitrile gradient was adjusted so that the retention time of the DNA peak was 3–5 min. The resultant chromatograms were compared with those of wild-type DNA, and both DNA strands were sequenced for samples in which variants were detected.

*CYP4B1**1 (wild-type), *CYP4B1**2 (AT881-882del, 993G>A, 1018C>T, and 1123C>T), *CYP4B1**3 (517C>T), *CYP4B1**5 (993G>A), *CYP4B1**6 (517C>T and 1033G>A), and *CYP4B1**7 (AT881-882del, 993G>A, and 1018C>T) were also genotyped by the hybridization probe assay described by Sasaki *et al.*¹⁷⁾ Analysis of the distribution of the five polymorphisms (517C>T, AT881-882del, 993G>A, 1033G>A, and 1123C>T) allowed the characterization of six different *CYP4B1* alleles.

*SULT1A1**1 (wild-type) and *SULT1A1**2 (638G>A) were genotyped by a hybridization probe assay. The PCR mixtures contained 3 mM MgCl₂, 0.5 µM each of the PCR primers, 0.4 µM of LC Red 640-labeled hybridization probes, 0.2 µM fluorescein isothiocyanate (FITC)-labeled hybridization probes, 1 µM LightCycler DNA Master Hybridization Mix (Roche Diagnostics Inc., Mannheim, Germany), and approximately 30 ng of genomic DNA in a final volume of 10 µL. The thermal profile consisted of 30 s of initial denaturation at 95°C, followed by 45 cycles at 95°C for 1 s, annealing at 50°C for 5 s, and extension at 72°C for 5 s. The analytical melting program involved melting the PCR products at 95°C for 30 s and at 40°C for 30 s, followed by increasing the temperature to 80°C at a ramp rate of 0.2°C/s, with continuous fluorescence data collection.

GSTM1 and *GSTT1* null (whole gene deletion) were identified by a multiplex PCR assay described by Abdel-Rahman *et al.*²⁶⁾ Genomic DNA (10–50 ng) was amplified in a 20-µL reaction mixture containing 0.25 µM of each of the *GSTM1* and *GSTT1* primers. Exon 7 of the *CYP1A1* gene was co-amplified as an internal control. Thermal cycling conditions comprised denaturation at 95°C for 10 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. PCR products from co-amplification of the *GSTM1*, *GSTT1*, and *CYP1A1* genes were analyzed by electrophoresis in 2% agarose gel. The *GSTM1* and *GSTT1* genes were detected by the presence or absence of a 215-bp (corresponding to *GSTM1*) or a 480-bp band (corresponding to *GSTT1*).

Statistical analysis: We evaluated the frequency distribution of patient characteristics, including sex, age, and smoking status, between the lung cancer cases and controls with the chi-squared and unpaired *t*-tests. Hardy-Weinberg equilibrium (HWE) was tested separately for each genotype in the cases and controls. The crude odds ratio (crude OR) and 95% confidence interval (95% CI) were used as estimates of the relative risk. The adjusted OR was calculated using binomial logistic regression to control for sex, age, and smoking status. A two-tailed *P* value < 0.05 indicated statistical significance. All statistical analyses were performed with Dr. SPSS software (Ver. 11.0.1).

Results

Cases and controls were classified according to sex, age, smoking status, and histological types of lung cancer (Table 1). The mean ages at the time of death were 80.3 ± 7.9 years in lung cancer patients and 80.3 ± 7.7 years in the cancer-free controls (*P* = 0.991). There were no statistically significant differences in sex distribution between the two groups (67.2% male cancer patients versus 69.0% male control patients, *P* = 0.705). More smokers were present in the cancer group compared with the control group (70.3% smokers among the cancer patients versus 51.7% smokers among the controls, *P* < 0.001). Of the 192 cancer patients, 41.7% had adenocarcinoma (AC), 24.5% had squamous cell carcinoma (SQCC), 21.4% had small-cell carcinoma (SCC), and 12.4% had other types of lung cancer.

The genotype distributions of *CYP2A6*, *CYP2A13*, *CYP4B1*, *SULT1A1*, *GSTM1*, and *GSTT1* in lung cancer cases and controls are summarized in Table 2. The frequency of *CYP2A6**4/*4 in cancer cases was significantly lower than that in the controls (adjusted OR = 0.36, 95% CI = 0.15–0.88, *P* = 0.025). Although the distribution of *CYP2A13**1/*2 (9.4%) and *1/*3 (3.1%) in cancer cases was lower than that in the controls (12.3% and 3.9%, respectively), there was no significant association with lung cancer risk. In addition, the *CYP4B1*, *SULT1A1*, *GSTM1*, and *GSTT1* genotypes were not associated with lung cancer risk.

Table 1. The characteristics of subjects who had lung cancer (cases) and those not having cancer (controls)

Characteristic	Cases (n = 192)	Controls (n = 203)	P-value
Gender, n (%)			
Male	129 (67.2)	140 (69.0)	0.705 ^a
Female	63 (32.8)	63 (31.0)	
Mean age, n (SD)	80.3 (7.9)	80.3 (7.7)	0.991 ^b
Smoking status, n (%)			
Non-smokers	39 (20.3)	78 (38.4)	<0.001 ^a
Smokers	135 (70.3)	105 (51.7)	
No information	18 (9.4)	20 (9.9)	
Histological type			
AC	80 (41.7)		
SQCC	47 (24.5)		
SCC	41 (21.4)		
ASQC	4 (2.1)		
LCC	1 (0.5)		
Unknown	7 (3.6)		
AC + SQCC + SCC	1 (0.5)		
SQCC + SCC + ASQC	1 (0.5)		
AC + SQCC	4 (2.1)		
AC + SCC	1 (0.5)		
AC + Unknown	2 (1.0)		
SQCC + SCC	3 (1.6)		

AC, adenocarcinoma; SQCC, squamous cell carcinoma; SCC, small-cell carcinoma; ASQC, adenosquamous cell carcinoma; LCC, large cell carcinoma; SD, Standard deviation.

^aChi-squared test, ^bunpaired *t* test.

Table 3 summarizes the relationship between the *CYP2A6* and *CYP2A13* genotypes among lung cancer cases and controls, stratified by smoking status. There was a statistically significant association between smokers carrying *CYP2A6**4/*4 and lung cancer risk (OR = 0.32, 95% CI = 0.10–0.99, *P* = 0.049). ORs were not calculated for non-smokers carrying *CYP2A13**1/*2 and *1/*3, as the frequency was 0 in cancer cases. There was no statistically significant association between the *CYP4B1*, *SULT1A1*, *GSTM1*, and *GSTT1* genotypes stratified by smoking status and lung cancer risk (data not shown). When stratifying by histological type, no significant association was found for any of the analyzed polymorphisms and lung cancer risk (data not shown).

Discussion

In this study, we observed that polymorphism of the *CYP2A6* gene, but not of the *CYP2A13*, *CYP4B1*, *SULT1A1*, *GSTM1*, or *GSTT1* genes, was associated with decreased risk of lung cancer in the Japanese population studied.

Defective *CYP2A6* alleles have been associated with both increased and decreased risks of lung cancer in different ethnic groups. A Chinese lung cancer study suggested that

the presence of the defective allele *CYP2A6**4 increases lung cancer risk.¹⁰⁾ In contrast, a Japanese lung cancer study suggested that the presence of the *CYP2A6**4 allele decreases the risk of lung cancer.⁹⁾ The results of our study were consistent with the latter study. *CYP2A6* is responsible for the metabolic activation of NNK, one of the components of tobacco smoke. Reducing the production of ultimate carcinogens may lead to decreased DNA damage and reduced cancer development. This hypothesis was supported by a statistically significant association between smokers carrying *CYP2A6**4/*4 and lung cancer risk.

To the best of our knowledge, this is the first case-control study evaluating the relationship between *CYP2A13* genetic polymorphism and lung cancer risk in the Japanese population. *CYP2A13* is highly active in the metabolic activation of several carcinogens. Thus, we speculated that reduction in enzymatic activity observed in the *CYP2A13**2 (74G>A and 3375C>T) allelic variant could provide some protection against xenobiotic toxicity. Wang *et al.* reported that Chinese individuals carrying the variant *CYP2A13* allele (3375CT or TT) have a reduced risk of lung adenocarcinoma in relation to light tobacco smoking, but protection against lung squamous cell carcinoma was not observed.¹¹⁾ Timofeeva *et al.* found no significant association between *CYP2A13* polymorphisms and lung cancer risk in Caucasian patients.²⁷⁾ We also did not find significant association between the variant *CYP2A13* allele and lung cancer. Interestingly, none of the non-smokers with lung cancer had the *CYP2A13**2 or *3 alleles (Table 3). In non-smoking individuals with low carcinogenic activity caused by *CYP2A13* polymorphisms, the risk of lung cancer in the population may be much lower than that in smokers with *CYP2A13* wild-type genotypes.

The effect of *CYP4B1* polymorphisms on susceptibility of lung cancer has not previously been investigated. Our study is the first to provide evidence that *CYP4B1* polymorphisms may not be associated with lung cancer risk. Our previous study indicated that *CYP4B1* genotypes may affect bladder cancer risk.¹⁷⁾ Thus, the effect of the *CYP4B1* polymorphism may differ among human organs with respect to cancer development.

Since *SULT1A1* catalyzes the sulfation of numerous carcinogenic and mutagenic compounds such as heterocyclic and aromatic amines and polycyclic aromatic hydrocarbons, it was suggested that the reduction in enzymatic activity observed in *SULT1A1**2 could affect the risk of lung cancer; however, the association between *SULT1A1**2 and lung cancer risk was not statistically significant in this study. Nevertheless, Liang *et al.* conducted a study on 805 individuals with cancer and 809 control subjects in China, and demonstrated that the lung cancer risk was elevated among individuals with the *SULT1A1**2 allele.²¹⁾ The reason for the discrepancy between our results and Liang's is unknown but may be related to differences in ethnicity, sample size, or environmental carcinogen exposure.

Table 2. Genotype and allele frequencies of CYP2A6, CYP2A13, CYP4B1, SULT1A1, GSTM1, and GSTT1 polymorphisms among cases and controls and their association with lung cancer

Genotypes	Cases, n (%)	Controls, n (%)	Crude OR ^a (95% CI)	Adjusted OR ^b (95% CI)
<i>CYP2A6</i>				
<i>non*4/non*4</i>	122 (63.5)	118 (58.1)	1.00	1.00
<i>non*4/*4</i>	63 (32.8)	66 (32.5)	0.92 (0.60–1.42)	0.93 (0.61–1.43)
<i>*4/*4</i>	7 (3.6)	19 (9.4)	0.36 (0.14–0.88) [†]	0.36 (0.15–0.88) [†]
Alleles				
<i>non*4</i>	307 (79.9)	302 (74.4)	1.00	
<i>*4</i>	77 (20.1)	104 (25.6)	0.73 (0.52–1.02)	
<i>CYP2A13</i>				
<i>*1/*1</i>	163 (84.9)	166 (81.8)	1.00	1.00
<i>*1/*2</i>	18 (9.4)	25 (12.3)	0.73 (0.39–1.40)	0.75 (0.39–1.44)
<i>*1/*3</i>	6 (3.1)	8 (3.9)	0.76 (0.26–2.25)	0.77 (0.26–2.27)
Rare genotypes ^c	5 (2.6)	4 (2.0)	—	—
Alleles				
<i>*1</i>	355 (92.4)	369 (90.9)	1.00	
<i>*2</i>	18 (4.7)	25 (6.2)	0.75 (0.40–1.40)	
<i>*3</i>	6 (1.6)	8 (2.0)	0.78 (0.27–2.27)	
<i>CYP4B1</i>				
<i>*1/*1</i>	52 (27.1)	48 (23.6)	1.00	1.00
<i>*1/*2</i>	67 (34.9)	61 (30.0)	1.01 (0.60–1.71)	1.00 (0.59–1.70)
<i>*1/*3</i>	23 (12.0)	32 (15.8)	0.66 (0.34–1.29)	0.65 (0.33–1.27)
<i>*2/*2</i>	19 (9.9)	20 (9.9)	0.88 (0.42–1.84)	0.88 (0.42–1.84)
<i>*2/*3</i>	14 (7.3)	16 (7.9)	0.81 (0.36–1.83)	0.80 (0.35–1.81)
<i>*3/*3</i>	7 (3.6)	7 (3.6)	0.92 (0.30–2.83)	1.00 (0.32–3.14)
Rare genotypes ^d	10 (5.2)	19 (9.4)	—	—
Alleles				
<i>*1</i>	200 (52.1)	202 (49.8)	1.00	
<i>*2</i>	122 (31.8)	121 (29.8)	1.02 (0.74–1.40)	
<i>*3</i>	52 (13.5)	64 (15.8)	0.82 (0.54–1.24)	
<i>SULT1A1</i>				
<i>*1/*1</i>	120 (62.5)	132 (65.0)	1.00	1.00
<i>*1/*2</i>	70 (36.5)	68 (33.5)	1.13 (0.75–1.72)	1.12 (0.74–1.70)
<i>*2/*2</i>	2 (1.0)	3 (1.5)	0.73 (0.12–4.46)	0.76 (0.12–4.71)
Alleles				
<i>*1</i>	310 (80.7)	332 (81.8)	1.00	
<i>*2</i>	74 (19.3)	74 (18.2)	1.07 (0.75–1.53)	
<i>GSTM1</i>				
Present	106 (55.2)	101 (49.8)	1.00	1.00
Null	86 (44.8)	102 (50.2)	0.80 (0.54–1.19)	0.80 (0.54–1.20)
<i>GSTT1</i>				
Present	95 (49.5)	99 (48.8)	1.00	1.00
Null	97 (50.5)	104 (51.2)	0.97 (0.66–1.44)	0.97 (0.65–1.44)

OR, odds ratio; CI, confidence interval.

[†]*P* < 0.05 (vs. *CYP2A6 non*4/non*4*).^aChi-squared test. ^bBinominal logistic regression analysis adjusted by sex, age, and smoking status.^cRare genotypes included: *CYP2A13*1/*4*, *CYP2A13*1/*5*, *CYP2A13*1/*7*, and *CYP2A13*1/*10*.²⁵⁾^dRare genotypes included: *CYP4B1*1/*5*, *CYP4B1*1/*6*, *CYP4B1*1/*7*, *CYP4B1*2/*5*, *CYP4B1*2/*6*, *CYP4B1*2/*7*, and *CYP4B1*3/*7*.

Table 3. Effect of CYP2A6 and CYP2A13 genotypes by smoking status on lung cancer risk

Genotypes	Smokers			Non-smokers		
	Cases/controls	Adjusted OR ^a (95% CI)	P-value	Cases/controls	Adjusted OR ^a (95% CI)	P-value
<i>CYP2A6</i>						
non *4/non *4	87/52	1.00		22/53	1.00	
non *4/*4	43/43	0.61 (0.35–1.05)	0.074	15/18	2.35 (0.96–5.72)	0.060
*4/*4	5/10	0.32 (0.10–0.99)	0.049	2/7	0.74 (0.14–4.01)	0.724
<i>CYP2A13</i>						
*1/*1	109/80	1.00		38/69	1.00	
*1/*2	17/19	0.68 (0.33–1.40)	0.293	0/5	—	—
*1/*3	5/3	1.51 (0.35–6.58)	0.585	0/3	—	—

OR, odds ratio; CI, confidence interval.

^aBinominal logistic regression analysis adjusted by sex and age.

In the present study, there was no statistically significant association between either the *GSTM1* or *GSTT1* genotype and lung cancer risk in the Japanese subjects studied. To-Figuerras *et al.* reported that 14.4% of their cancer patients possessed homozygous deletion of both *GSTT1* and *GSTM1* (12.5% among healthy smokers),²⁸⁾ suggesting no potentiation between null genotypes for lung cancer risk, which is in agreement with our results. In contrast, Pinarbasi *et al.*²³⁾ and Kihara *et al.*²²⁾ reported that *GSTM1* null genotypes were associated with lung cancer risk in a Turkish population and in male Japanese smokers, respectively. These conflicting results, including ours, may be caused by some confounding factors such as ethnicity, selection of control group, characterization of cases, sample size, gene-gene and gene-environment interactions, and second-hand smoke conditions.

In conclusion, these results indicate that the *CYP2A6* *4/*4 genotypes, but not the *CYP2A13*, *CYP4B1*, *SULT1A1*, *GSTM1*, and *GSTT1* gene polymorphisms, were associated with decreased risk of lung cancer in the Japanese population studied. However, there is an element of chance in the results in the present study because the sample size was relatively small. Therefore, further studies with larger sample sizes will be required to confirm the present findings.

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Functional Characterization of CYP2B6 Allelic Variants in Demethylation of Antimalarial Artemether

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ABSTRACT:

Artemether (AM) is one of the most effective antimalarial drugs. The elimination half-life of AM is very short, and it shows large interindividual variability in pharmacokinetic parameters. The aim of this study was to identify cytochrome P450 (P450) isozymes responsible for the demethylation of AM and to evaluate functional differences between 26 CYP2B6 allelic variants in vitro. Of 14 recombinant P450s examined in this study, CYP2B6 and CYP3A4 were primarily responsible for production of the desmethyl metabolite dihydroartemisinin. The intrinsic clearance (V_{max}/K_m) of CYP2B6 was 6-fold higher than that of CYP3A4. AM demethylation activity was correlated with CYP2B6 protein levels ($P = 0.004$); however, it was not correlated with CYP3A4 protein levels ($P = 0.27$) in human liver microsomes. Wild-type CYP2B6.1 and 25 CYP2B6 allelic variants (CYP2B6.2-CYP2B6.21 and CYP2B6.23-

CYP2B6.27) were heterologously expressed in COS-7 cells. In vitro analysis revealed no enzymatic activity in 5 variants (CYP2B6.8, CYP2B6.12, CYP2B6.18, CYP2B6.21, and CYP2B6.24), lower activity in 7 variants (CYP2B6.10, CYP2B6.11, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.20, and CYP2B6.27), and higher activity in 4 variants (CYP2B6.2, CYP2B6.4, CYP2B6.6, and CYP2B6.19), compared with that of wild-type CYP2B6.1. In kinetic analysis, 3 variants (CYP2B6.2, CYP2B6.4, and CYP2B6.6) exhibited significantly higher V_{max} , and 3 variants (CYP2B6.14, CYP2B6.20 and CYP2B6.27) exhibited significantly lower V_{max} compared with that of CYP2B6.1. This functional analysis of CYP2B6 variants could provide useful information for individualization of antimalarial drug therapy.

Introduction

Malaria is a very serious problem in many countries, and there are more than 200 million cases that result in approximately 1 million deaths worldwide each year (World Health Organization, World Malaria Report 2009, http://www.who.int/malaria/world_malaria_report_2009/en/index.html). The management of malaria has traditionally relied on monotherapy with quinolines such as quinine, mefloquine, and chloroquine. However, the widespread and excessive use of these agents has resulted in drug resistance (Wernsdorfer, 1991; Price and Nosten, 2001; Le Bras and Durand, 2003). In several studies, artemisinin, unique sesquiterpene lactone endoperoxides, have been used in areas with multidrug-resistant *Plasmodium falciparum* malaria (Woodrow et al., 2005; Gautam et al., 2009; World Health Organization, 2010).

Artemisinin is a natural antimalarial agent derived from the Chinese medicinal plant *Artemisia annua* (Klayman, 1985). The artemisinin derivative artemether (AM) is the most effective antimalarial drug. AM has a fast onset of action, therapeutic efficacy against multidrug-resistant malaria, and few side effects, although neurotoxicity has been observed in experimental mammals (Hien and White, 1993; Brewer et al., 1994). AM is mainly converted to dihydroartemisinin (DHA) (Fig. 1), a desmethyl metabolite that contributes to the majority of the antimalarial activity. The conversion of AM to DHA is catalyzed by cytochrome P450 (P450) (van Agtmael et al., 1999b,c; Navaratnam et al., 2000). However, the elimination half-life of AM is very short, and it shows large interindividual variability in pharmacokinetic parameters (Na Bangchang et al., 1994; Mordi et al., 1997; van Agtmael et al., 1999a; Lefèvre et al., 2002; Ali et al., 2010; Mwesigwa et al., 2010).

The P450 isozymes CYP2B6 and CYP3A4 are thought to catalyze AM demethylation (Navaratnam et al., 2000). In contrast, it has been reported that CYP2D6 and CYP2C19 make no major contribution to this reaction (van Agtmael et al., 1998), and the role of other P450s remains unclear. CYP2B6 plays a major role in the biotransformation of several therapeutically important drugs, including cyclophosph-

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ABBREVIATIONS: AM, artemether; DHA, dihydroartemisinin; P450, cytochrome P450; ART, artemisinin; LC, liquid chromatography; MS/MS, tandem mass spectrometry.

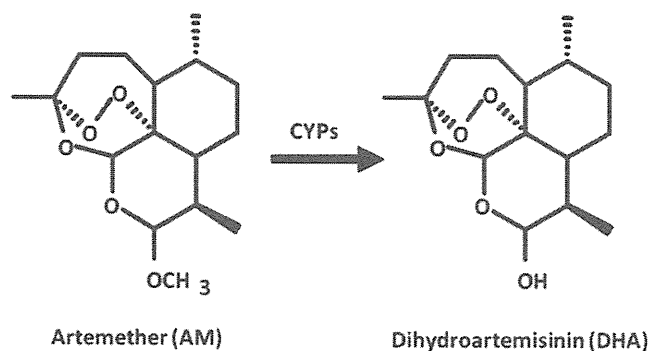


Fig. 1. Metabolic pathway of artemether to dihydroartemisinin by P450 enzymes.

amide, bupropion, selegiline, efavirenz, nevirapine, and methadone (Roy et al., 1999; Hesse et al., 2000; Hidestrand et al., 2001; Salonen et al., 2003). Many genetic polymorphisms in the *CYP2B6* gene have been reported, and these are thought to be responsible for interindividual and interethnic differences in responses to *CYP2B6* substrate drugs [Zanger et al., 2007; Human Cytochrome P450 (CYP) Allele Nomenclature Committee, 2008; Mo et al., 2009]. In the case of chemotherapy using cyclophosphamide, the increasing enzymatic activity of *CYP2B6* variants can be associated with the increased blood concentration of the active metabolite of the drug, resulting in a heightened risk of side effects (Xie et al., 2003, 2006; Nakajima et al., 2007).

Several functional analyses of *CYP2B6* variant proteins, using an in vitro expression system, have been reported. Watanabe et al. (2010) characterized the functional relevance of many *CYP2B6* variants, including *CYP2B6.1* to *CYP2B6.28*, using 7-ethoxy-4-trifluoromethylcoumarin and selegiline as substrates, and reported that *CYP2B6.8*, *CYP2B6.11*, *CYP2B6.12*, *CYP2B6.13*, *CYP2B6.15*, *CYP2B6.18*, *CYP2B6.21*, *CYP2B6.24*, and *CYP2B6.28* were inactive with regard to these compounds. These results were consistent with those of a number of in vitro studies using bupropion as a substrate. In contrast, *CYP2B6.16*, *CYP2B6.19*, and *CYP2B6.27* exhibited activity toward 7-ethoxy-4-trifluoromethylcoumarin and inability to detect selegiline metabolism. Several researchers have reported that these *CYP2B6* variants exhibited decreased protein expression/activity when bupropion was used as the *CYP2B6* substrate (Lang et al., 2004; Klein et al., 2005; Wang et al., 2006; Rotger et al., 2007). These results suggest that some allelic variants of *CYP2B6* are associated with a substrate-dependent decrease in the catalytic properties of the enzyme. To date, there have been no reports of functional characterization of *CYP2B6* variants in relation to AM demethylation activity.

In this study, we performed an in vitro analysis of 14 P450s (*CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP2J2*, *CYP3A4*, *CYP3A5*, and *CYP4A11*) to identify isoforms responsible for AM demethylation and evaluated functional differences among 26 *CYP2B6* allelic variants (Fig. 2).

Materials and Methods

Chemicals. AM, DHA, and artemisinin (ART) were purchased from Tokyo Chemical Industry Corporation (Tokyo, Japan). Recombinant *CYP1A1*, *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2D6*, *CYP2J2*, and *CYP4A11* Supersomes were purchased from BD Biosciences (Woburn, MA). *CYP1A2*, *CYP2C9*, *CYP2E1*, *CYP3A4*, and *CYP3A5* Baculosomes were purchased from Invitrogen (Carlsbad, CA). NADPH was obtained from Oriental Yeast (Tokyo, Japan). Protease Inhibitor Cocktail Set III was purchased from Merck Chemicals (Darmstadt, Germany). Methanol (CH₃OH) and acetonitrile (CH₃CN) of LC-mass spectrometry grade were obtained from Kanto Chemical (Tokyo, Japan). Ammonium formate (HCOONH₄) and formic acid (HCOOH) of LC-

mass spectrometry grade were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

DHA stock solution (5 mM) was prepared in CH₃CN/H₂O [50:50 (v/v)], and working solutions (1.0, 2.0, 5.0, 10, 25, 50, 100, and 200 μM) were prepared from the stock solution. These solutions were further diluted in 50 mM potassium phosphate buffer, pH 7.4, and the final calibration curves were obtained with 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 10, and 20 μM solutions. Working solutions (100 μl) were prepared in 1.5-ml plastic tubes, and 100 μl of CH₃OH, 5 μl of internal standard (ART at 100 μM), and 100 μl of H₂O were

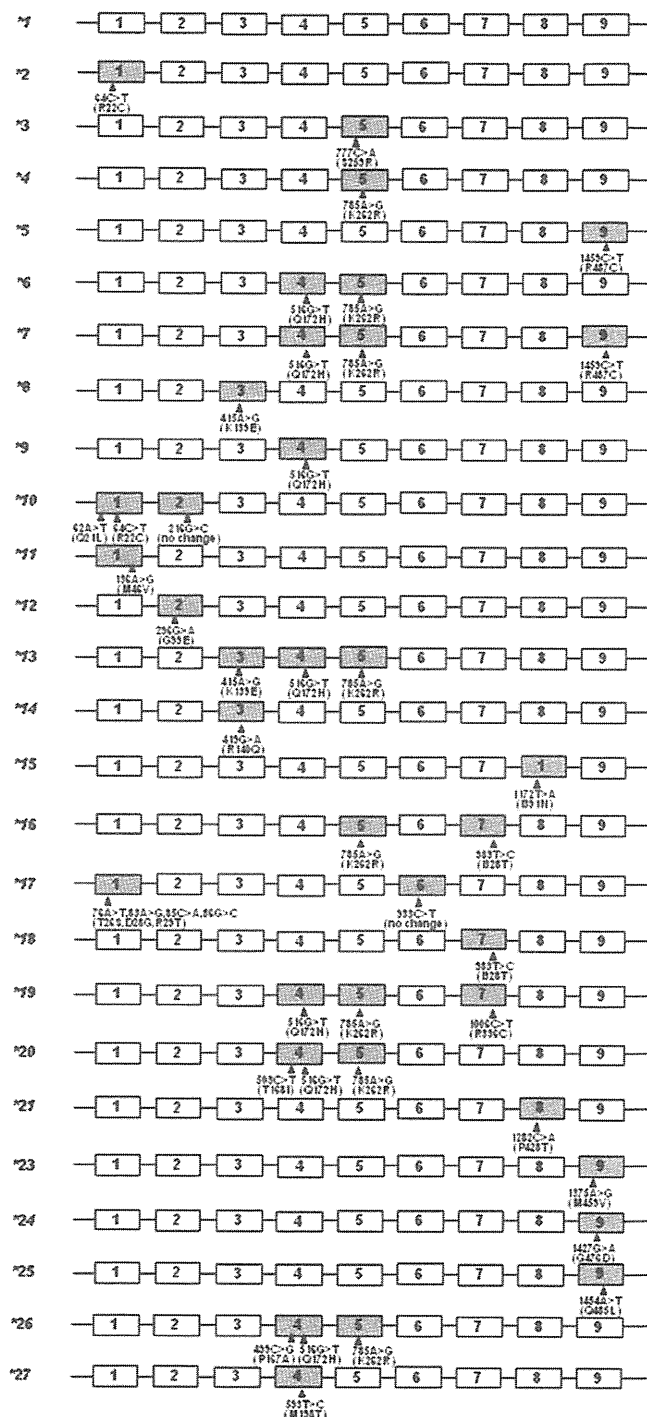


Fig. 2. Structures of *CYP2B6* alleles characterized in this study. The nine exons are indicated by numbered boxes. Some promoter and intronic polymorphisms are not shown.

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added. The resulting mixture was vortexed. After centrifugation at 12,000g for 10 min, 80 μ l of the supernatant was transferred to a new plastic tube and passed through a filter (pore size: 0.2 μ m; YMC Co., Ltd., Kyoto, Japan). Subsequently, 10 μ l of the filtered solution was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system for analysis. All peaks were integrated automatically by Xcalibur software (Thermo Fisher Scientific, Waltham, MA). Levels of DHA were calculated from the calibration curves by the ratios of their peak areas to that of ART. An eight-point calibration curve was plotted for DHA concentration (0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 10, and 20 μ M).

Sample Preparation for Analysis of Specific Activity. AM demethylation activity was determined by measurement of the formation of DHA, according to the method of Asimus and Ashton (2009), with minor modifications. AM stock solution (50 mM) was prepared in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ [50:50 (v/v)], and a working solution (500 μ M) was prepared by dilution of the stock solution in potassium phosphate buffer, pH 7.4. The incubation mixture contained AM as a substrate (1 and 50 μ M), recombinant P450 enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11; 0.5 pmol) or human liver microsomes (50 μ g), 0.5 mM NADPH, 5 mM MgCl_2 , and 50 mM potassium phosphate buffer to a final volume of 90 μ l. After preincubation (3 min at 37°C), the reaction was started by addition of NADPH. Reactions were performed for 30 min and terminated by the addition of 100 μ l of methanol. Then, 5 μ l of internal standard (ART at 100 μ M) and 100 μ l of H_2O were added. The resulting mixture was vortexed. After centrifugation at 12,000g for 10 min, 80 μ l of the supernatant was transferred to a new plastic tube and passed through a filter (pore size: 0.2 μ m; YMC). Then, 10 μ l of the filtered solution was injected into the LC-MS/MS system for analysis. All peaks were integrated automatically by Xcalibur software. Levels of DHA were calculated from the calibration curves by using the ratios of their peak areas to that of ART. Formation of DHA was in the linear range between 10 and 60 min and 30 and 50 μ g of microsomal protein.

Sample Preparation for Analysis of Kinetic Parameters of CYP2B6 and CYP3A4. AM stock solution (50 mM) was prepared in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ [50:50 (v/v)], and working solutions (0.25, 0.50, 1.25, 2.5, 5.0, 12.5, and 25 mM) were prepared by dilution of the stock solution. These solutions were diluted with 50 mM potassium phosphate buffer, pH 7.4, and the final calibration curves were obtained with 0.5, 1.0, 2.5, 5.0, 10, 25, 50, and 100 μ M. CYP2B6 and CYP3A4 activity was evaluated using the concentration ranges 0.5 to 50 and 2.5 to 100 μ M, respectively. Samples were prepared as described above.

Determination of DHA and ART Using Online Column-Switching LC-MS/MS. Levels of DHA were determined by the LC-MS/MS method described by Huang et al. (2009), with minor modifications. A Nanospace SI-2 LC system, comprising an LC pump, autosampler, column oven maintained at 40°C, and on-line degasser (Shiseido, Tokyo, Japan), was used. The on-line column-switching valve system consisted of an automated switching valve (six-port valve) connected to pump A and pump B. Pump A was connected via the switching valve to the trap column, a CAPCELL PAK C18 SG II (10 \times 2 mm i.d., 3- μ m particle size) (Shiseido, Tokyo, Japan), and pump B was connected via the switching valve to the analytical column, a Sunfire C18 (150 mm \times 2.1 mm i.d., 3.5- μ m particle size) (Waters, Milford, MA). The outlet of

the analytical column was connected to the mass spectrometer via a divert valve.

Sample loading. A 10- μ l aliquot of sample was injected onto the trap column using pump B with the switching valve in position 1. Impurities on the trap column were eluted to waste using 0.1% $\text{HCOOH}-\text{H}_2\text{O}/\text{CH}_3\text{CN}$ [5:95 (v/v)] at a flow rate of 200 μ l/min for 4 min. Concurrently, initial flow was maintained by pump A at 200 μ l/min with 10 mM $\text{HCOONH}_4-\text{H}_2\text{O}$ (adjusted to pH 4.1 using HCOOH)-0.1% $\text{HCOOH}-\text{CH}_3\text{CN}$ [20:80 (v/v)] via the analytical column.

Sample elution. At 4 min, the switching valve was switched to position 2 to allow the purified DHA and ART to be eluted from the trap column onto the analytical column and subsequently into the mass spectrometer. Isocratic flow was maintained by pump B at a rate of 200 μ l/min for 11 min. Concurrently, the flow from pump A was passed through the trap column and diverted directly to waste. At 11 min, the switching valve was switched back to position 1, and the configuration of the online column switching system reverted back to that in the initial conditions (described for the sample loading above). A divert valve was used to divert the LC effluent to waste during the first 4.5 min and last 0.5 min of the chromatographic run. The total run time was 11 min.

Quantification analyses by MS were performed in the selected reaction monitoring mode because of the high selectivity and sensitivity of selected reaction monitoring data acquisition, in which the transitions of the precursor ion into the product ion were monitored: m/z 302 \rightarrow 145 and 302 \rightarrow 267 for DHA and m/z 300 \rightarrow 151 and 300 \rightarrow 209 for ART. The optimized parameters for MS are as follows: positive heated electrospray ionization voltage, 3 kV; heated capillary temperature, 300°C; sheath gas pressure, 50 psi; auxiliary gas setting, 20 psi; and heated vaporizer temperature, 300°C. Both the sheath and auxiliary gases were nitrogen. The collision gas was argon at a pressure of 1.5 mTorr. The LC-MS/MS system was controlled by Xcalibur software, and data were also collected with this software. The retention times of DHA and ART were 7.0 and 7.5 min, respectively.

Liver Specimens. Human liver specimens were obtained from the Human and Animal Bridging Research Organization (HAB) in Chiba, Japan, using frozen human livers (most of the donors were white). Microsomes were prepared from these specimens using differential centrifugation. Research protocols were approved by the ethics committees of the Graduate School of Pharmaceutical Sciences, Tohoku University (Sendai, Japan).

Expression of CYP2B6 Variant Proteins in COS-7 Cells. CYP2B6 variant proteins were expressed in COS-7 cells as described by Watanabe et al. (2010).

Determination of Protein Expression Levels by Immunoblotting. Western blotting was performed according to standard procedures, with 10% SDS-polyacrylamide gel electrophoresis, and 30- μ g microsomal fractions were loaded into each lane. Recombinant CYP2B6 Supersomes reagent (BD Gentest) was coanalyzed as the standard on each gel and used to quantify the CYP2B6 protein. The CYP2B6 protein was detected using the antihuman CYP2B6 antibody (BD Gentest) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako Denmark A/S, Glostrup, Denmark). CYP3A4 Baculovirus reagent (Invitrogen) was coanalyzed as the standard on each gel and used to quantify the CYP3A4 protein. The CYP3A4 protein was detected using the antihuman CYP3A4 antibody (Nosan, Yokohama, Japan) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako Denmark A/S). Immuno-

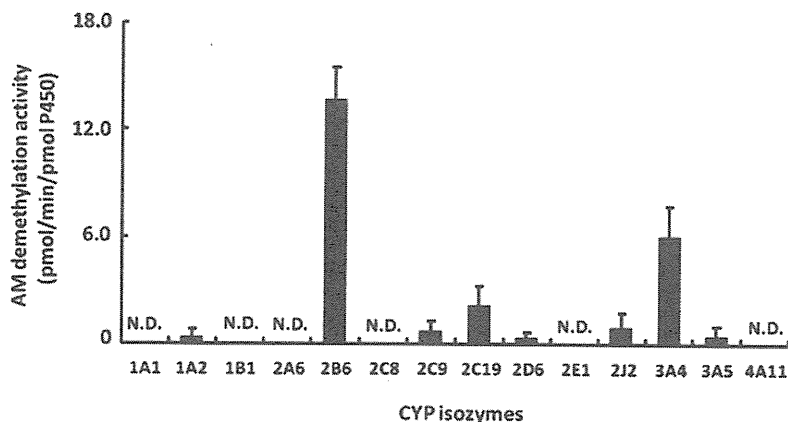


Fig. 3. AM demethylation activity of 14 P450 isozymes. The concentration of AM was 50 μ M. Each number corresponds to a P450 subtype. Results are presented as the mean \pm S.D. in triplicate. N.D., not detectable (activities were lower than 0.22 pmol \cdot min $^{-1}$ \cdot pmol P450 $^{-1}$).

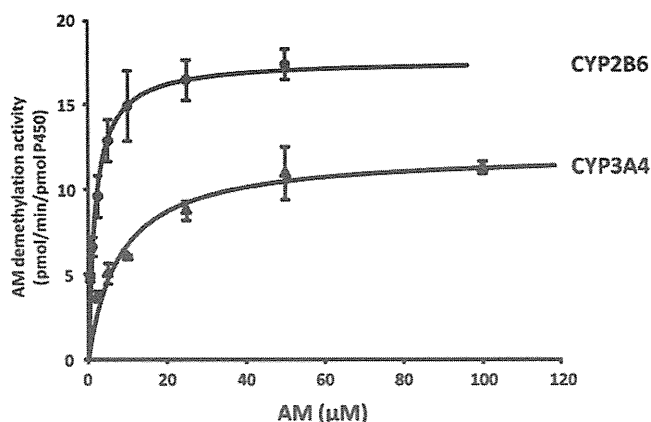


Fig. 4. The Michaelis-Menten curves for the demethylation of AM with recombinant CYP2B6 and CYP3A4.

blots were developed using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Chemiluminescence was quantified using a lumino-imaging analyzer (LAS-1000; Fujifilm, Tokyo, Japan) and ImageJ software (National Institutes of Health, Bethesda, MD).

Sample Preparation for Analysis of Kinetic Parameters of CYP2B6 Variants. Microsomal fractions (50 μg) obtained from COS-7 cells were used for evaluation of the activity of CYP2B6 variants. Samples were prepared as described above.

Statistical Analysis. Apparent K_m and V_{max} parameters were determined using nonlinear regression analysis. All data are the mean \pm S.D. in triplicate. Statistical analyses of enzymatic activities and kinetic parameters were performed by analysis of variance using the Dunnett method. $P \leq 0.05$ was considered significant.

Results

AM Demethylation by Recombinant Human P450s. The activities of AM demethylation were measured in 14 recombinant human P450 enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11) at 1 and 50 μM substrate concentrations. The lower concentration used was intended to approximate the plasma AM concentrations reported to be clinically relevant (0.3–1 μM) (Ali et al., 2010). At 1 μM AM, AM demethylation activities of recombinant CYP2B6 and CYP3A4 were 6.61 and 2.50 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$, respectively. Under the lower substrate conditions used in this study, DHA was not formed by the other P450 isoforms with the exception of CYP2B6 and CYP3A4. At a higher concentration (50 μM), AM was principally metabolized by CYP2B6, followed by CYP3A4. A low rate of demethylation was observed for

CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2J2, and CYP3A5; CYP1A1, CYP1B1, CYP2A6, CYP2C8, CYP2E1, and CYP4A11 were inactive (Fig. 3).

Kinetics of AM Demethylation by CYP2B6 and CYP3A4. The kinetics of AM demethylation were investigated for each of recombinant enzymes CYP2B6 and CYP3A4 by Michaelis-Menten plots (Fig. 4). Apparent K_m , V_{max} , and V_{max}/K_m values for CYP2B6 were estimated to be 1.95 μM , 17.9 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$, and 9.19 $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$, respectively; those for CYP3A4 were 8.24 μM , 12.3 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$, and 1.49 $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$, respectively, demonstrating a higher K_m and lower V_{max} , which resulted in an approximately one-sixth V_{max}/K_m value for CYP3A4 relative to that for CYP2B6.

Comparison of AM Demethylation Activities (at 50 μM AM) to Immunoquantified CYP2B6 and CYP3A4 Protein Levels in 13 Human Liver Microsomes. As shown in Fig. 5, AM demethylation activity in 13 human liver microsomes was correlated with immunoquantified CYP2B6 content ($r^2 = 0.548$, $P = 0.004$) but not with immunoquantified CYP3A4 content ($r^2 = 0.109$, $P = 0.272$).

Enzymatic Properties for AM Demethylation by Wild-Type and 25 Variant CYP2B6s. The demethylation activities of wild-type and 25 variant microsomal CYP2B6 proteins were determined using AM (50 μM) as a substrate (Fig. 6). For CYP2B6.8, CYP2B6.12, CYP2B6.18, CYP2B6.21, and CYP2B6.24, no AM demethylation activity was detected. The enzymatic activity of CYP2B6.3 could not be calculated because its expression level could not be determined by immunoblotting. CYP2B6.10, CYP2B6.11, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.20, and CYP2B6.27 exhibited significantly decreased activities compared with that of wild-type CYP2B6.

The Michaelis-Menten kinetics for AM demethylation were determined for CYP2B6.1, CYP2B6.2, CYP2B6.4, CYP2B6.5, CYP2B6.6, CYP2B6.7, CYP2B6.9, CYP2B6.10, CYP2B6.13, CYP2B6.14, CYP2B6.17, CYP2B6.19, CYP2B6.20, CYP2B6.23, CYP2B6.25, CYP2B6.26, and CYP2B6.27. The kinetic parameters are summarized in Table 1. The estimated kinetic parameters, apparent K_m , V_{max} , and $V_{max}/\text{apparent } K_m$ for AM demethylation by CYP2B6.1 were 3.10 μM , 36.0 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol CYP2B6}^{-1}$, and 12.4 $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{pmol CYP2B6}^{-1}$, respectively. The V_{max} values for CYP2B6.14, CYP2B6.20, and CYP2B6.27 were significantly decreased, whereas those for CYP2B6.2, CYP2B6.4, and CYP2B6.6 were significantly increased, relative to that for the wild-type enzyme.

Discussion

In this study, we have determined the human P450 enzymes responsible for AM demethylation. Among 14 human P450s, CYP2B6

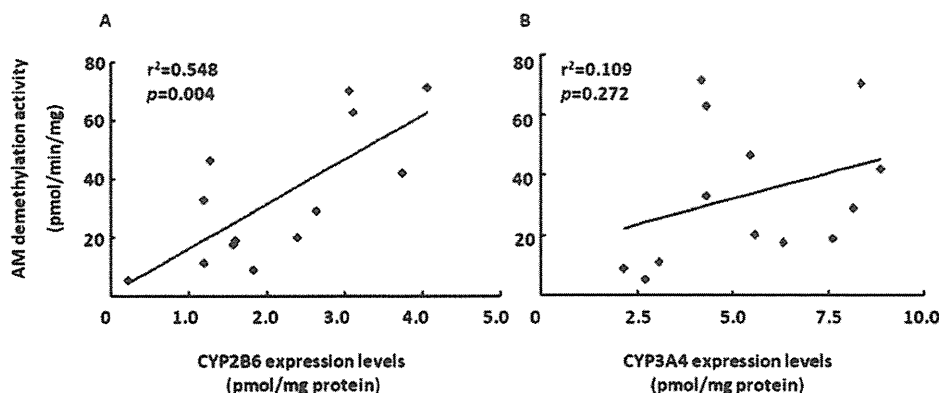


Fig. 5. Comparison of AM demethylation activities (at 50 μM AM) to immunoquantified CYP2B6 (A) and CYP3A4 (B) protein levels in 13 human liver microsomes. Correlation coefficients (r^2) obtained in these cases are shown.

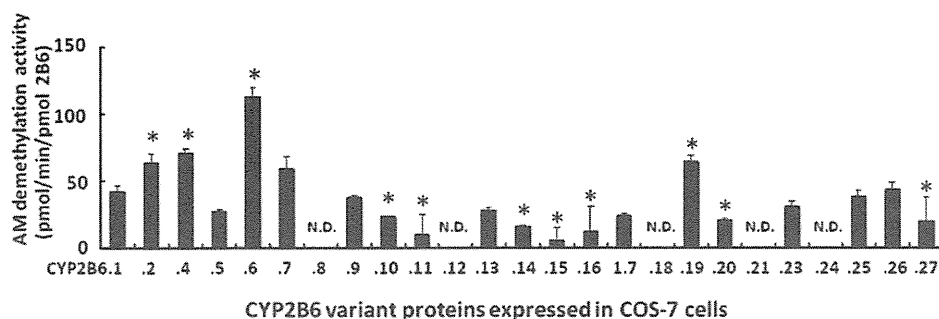


Fig. 6. AM demethylation activity of CYP2B6 proteins expressed in COS-7 cells. The concentration of AM was 50 μ M. Each number corresponds to CYP2B6 variant proteins. Results are presented as the mean \pm S.D. in triplicate. *, $P < 0.05$ compared with CYP2B6.1. N.D., not detectable.

has the highest intrinsic activity for AM demethylation, followed by CYP3A4. In the kinetic parameter analysis, the affinity of CYP2B6 was 4-fold higher than that of CYP3A4, and the V_{max} /apparent K_m of CYP2B6 was 6-fold higher than that of CYP3A4. These results suggest that AM demethylation is likely to be mainly catalyzed by CYP2B6 in the liver. The contribution of CYP2B6 to AM demethylation by human liver microsomes was further substantiated by showing a correlation to CYP2B6 protein content ($r^2 = 0.548$). However, van Agtmael et al. (1999b,c) have reported that administration of AM with grapefruit juice, a CYP3A4 inhibitor, increased the blood concentration of AM and DHA but not their elimination half-life. Thus, CYP3A4 in the small intestine might also play an important role in the metabolism of AM.

CYP2B6 is a genetically polymorphic enzyme (Zanger et al., 2007; Arenaz et al., 2010). In vitro functional characterization of polymorphically expressed CYP2B6 variants revealed that CYP2B6.4 and CYP2B6.6 increased AM demethylation activity, whereas CYP2B6.8, CYP2B6.11, CYP2B6.12, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.18, CYP2B6.20, CYP2B6.21, CYP2B6.24, and CYP2B6.27 exhibited no activity or decreased activity. These alterations were consistent with those of previous in vitro studies performed using bupropion, 7-ethoxy-4-trifluoromethylcoumarin, and selegiline as CYP2B6 substrates (Lang et al., 2004; Klein et al., 2005; Wang et al., 2006; Rotger et al., 2007; Watanabe et al., 2010). However, CYP2B6.2 exhibited increased AM demethylation activity, and the activity of CYP2B6.13 was similar to that of wild-type CYP2B6.1. There have been several reports that CYP2B6.2 exhibited no functional differences compared with CYP2B6.1 (Jinno et al., 2003;

Watanabe et al., 2010) and that CYP2B6.13 had no metabolic activity toward 7-ethoxy-4-trifluoromethylcoumarin and selegiline (Watanabe et al., 2010). These results suggest that these CYP2B6 variants show substrate-dependent changes in the catalytic properties of the enzyme.

Gay et al. (2010) recently determined the crystal structure of CYP2B6, allowing the prediction of precise locations within the three-dimensional structure at which amino acid substitutions occur. They suggested that the K262R substitution on the G/H loop is assembled into a hydrogen-bonding network with His252, Thr255, Asp263, and Asp266 and is involved in protein stability. In this study, the AM demethylation activities of CYP2B6.12 (G99E) and CYP2B6.24 (Q476D) were not detectable. In the CYP2B6 protein structure, the Gly99 and Gln476 residues are located in substrate recognition site 1 and 6, respectively. These amino acid changes may reduce the affinity of CYP2B6 for AM. However, a number of amino acids with altered AM demethylation activity are located far from substrate recognition sites. Indeed, the apparent K_m values of AM demethylation were not significantly different among the CYP2B6 variants (Table 1). We hypothesize that the functional effects of these variants are transduced via long-range hydrogen-bonding networks or through subtle differences in the placement of secondary structural elements. In addition, most of the amino acid substitutions that abolished enzymatic activity are conserved among human P450s and are therefore critical for CYP2B6 activity.

This is the first study to functionally analyze CYP2B6 genetic variants with respect to AM demethylation activity. If CYP2B6 has a significant role in the metabolism of AM in vivo as well as in vitro, individuals with poor CYP2B6 metabolism might have higher plasma

TABLE 1

Kinetic parameters of AM demethylation by CYP2B6 proteins expressed in COS-7 cells

Results represent the mean \pm S.D. of triplicate determinations.

Variants	Apparent K_m μ M	V_{max} $\text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol CYP2B6}^{-1}$	$V_{max}/\text{Apparent } K_m$ $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{pmol CYP2B6}^{-1}$	$V_{max}/\text{Apparent } K_m$ Ratio % CYP2B6.1
CYP2B6.1	3.10 \pm 1.1	36.0 \pm 5.67	12.4 \pm 4.11	
CYP2B6.2	4.29 \pm 2.7	64.4 \pm 3.92*	18.8 \pm 9.19	129
CYP2B6.4	2.73 \pm 0.45	70.6 \pm 9.29*	26.0 \pm 2.42	223
CYP2B6.5	6.87 \pm 6.8	19.8 \pm 3.06	8.91 \pm 11.3	24.8
CYP2B6.6	6.72 \pm 3.0	150 \pm 15.9*	24.2 \pm 6.84	192
CYP2B6.7	2.80 \pm 1.4	50.1 \pm 12.3	19.2 \pm 4.65	154
CYP2B6.9	4.44 \pm 1.7	33.1 \pm 5.20	8.38 \pm 3.94	64.2
CYP2B6.10	1.93 \pm 0.68	17.0 \pm 5.03	9.98 \pm 5.05	75.7
CYP2B6.13	7.33 \pm 4.1	18.2 \pm 5.54	2.93 \pm 1.73	21.3
CYP2B6.14	5.06 \pm 5.8	7.06 \pm 1.63*	6.70 \pm 9.40	12.0
CYP2B6.17	2.17 \pm 0.40	21.2 \pm 4.73	9.44 \pm 2.45	84.0
CYP2B6.19	8.06 \pm 8.9	36.9 \pm 21.9	7.38 \pm 6.15	39.4
CYP2B6.20	6.47 \pm 9.8	9.85 \pm 2.22*	8.79 \pm 7.22	13.1
CYP2B6.23	1.91 \pm 0.72	31.4 \pm 7.71	17.6 \pm 5.93	142
CYP2B6.25	2.04 \pm 1.9	25.7 \pm 7.31	21.6 \pm 15.7	108
CYP2B6.26	5.50 \pm 3.4	37.3 \pm 6.04	10.1 \pm 8.13	58.4
CYP2B6.27	4.50 \pm 0.98	10.6 \pm 6.16*	2.59 \pm 1.82	20.2

* $P < 0.05$ compared with CYP2B6.1.

AM concentrations than those with more active variants of this enzyme. However, because DHA also has an antimalarial effect, it would be difficult to assess the clinical outcome in subjects who polymorphically express CYP2B6 without *in vivo* data. To more fully understand the mechanistic basis of our findings, it would be of great value to clinically examine the relationship between CYP2B6 genotypes and the plasma concentration of AM and its metabolites.

In conclusion, demethylation of AM was mainly catalyzed by recombinant CYP2B6, although recombinant CYP3A4 also exhibited this metabolic activity. In addition, we performed a comprehensive analysis, using COS-7 cells as a heterologous expression system, to characterize nonsynonymous CYP2B6 variants. Many of the 26 variants expressed in COS-7 cells exhibited significantly altered AM demethylation activity. This study provides insights into the genotype-phenotype associations of CYP2B6 and lays a foundation for future clinical studies on interindividual variation in drug efficacy and toxicity.

Authorship Contributions

Participated in research design: Honda, Hirasawa, and Hiratsuka.

Conducted experiments: Honda, Muroi, and Tamaki.

Contributed new reagents or analytic tools: Saigusa, Suzuki, Tomioka, and Matsubara.

Performed data analysis: Honda, Oda, and Hiratsuka.

Wrote or contributed to the writing of the manuscript: Honda, Saigusa, and Hiratsuka.

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SNP Communication

Novel Single Nucleotide Polymorphism of
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Summary: Cytochrome P450 2A13 (*CYP2A13*) is a human CYP enzyme that is selectively expressed in the respiratory tract. It plays an active role in the metabolic activation of a tobacco-specific procarcinogen. In this study, the entire coding sequence and the exon-intron junctions of the *CYP2A13* gene obtained from 395 Japanese individuals were screened for genetic polymorphisms. Eight genetic polymorphisms were found, of which seven gave rise to known variant alleles: *CYP2A13**2, *CYP2A13**3, *CYP2A13**4, *CYP2A13**6, and *CYP2A13**7. We identified a novel single nucleotide polymorphism (SNP), 5792T>C, in exon 7 that caused an amino acid substitution (Ile331Thr). One of the 395 individuals included in the study was heterozygous for the variant allele, and therefore, the frequency of the allele in the study population was 0.13%.

Keywords: *CYP2A13*; genetic polymorphism; SNP; Japanese

Introduction

Cytochrome P450s (CYPs) play an important role in the metabolism of a variety of compounds, including therapeutic agents, environmental toxicants, and chemical carcinogens. *CYP2A13* is selectively expressed in the human respiratory tract; the highest level of expression is observed in the nasal mucosa, followed by the tracheal mucosa, and finally the lungs.^{1–5} *CYP2A13* plays an active role in the metabolism of many xenobiotic compounds such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is a major tobacco-specific procarcinogen, and *N*-nitrosomethylphenylamine and is also involved in the detoxification of carcinogens including *N,N*-dimethylaniline.³ Therefore, *CYP2A13* may play an

important role in xenobiotic toxicity and tobacco-related carcinogenesis in the respiratory tract and lungs.

The *CYP2A13* gene is located in a *CYP* gene cluster on chromosome 19.⁶ To date, nine alleles of *CYP2A13* have been identified (*CYP2A13**1–9).^{7–9} Genetic variations affecting *CYP2A13* enzyme function may lead to interindividual variability in susceptibility to diseases, including lung cancer. In the present study, we screened nine exons and exon-intron junctions of the *CYP2A13* gene from 395 Japanese individuals for genetic polymorphisms by using denaturing high-performance liquid chromatography (DHPLC). We identified one novel single nucleotide polymorphism (SNP) of the *CYP2A13* gene among the Japanese individuals included in the study; this novel polymorphism was nonsynonymous.

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On March 7, 2011, the variation was not found in the Japanese Single Nucleotide Polymorphism (JSNP) (<http://snp.ims.u-tokyo.ac.jp/>) database, the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>), the Human CYP Allele Nomenclature Committee (<http://www.cypalleles.ki.se/>) database, or the PharmGKB (<http://www.pharmgkb.org/>) database. The *CYP2A13* haplotype with 74G>A, 3375C>T, and 5792T>C was assigned as *CYP2A13**10 by the Human CYP Allele Nomenclature Committee.

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Table 1. Amplification and DHPLC conditions for *CYP2A13* SNP analysis of genomic DNA

Exon	Size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp. (°C)	PCR cycles	DHPLC temp. (°C)
1-5 ¹	4,817	GCTACACACTCCACCTCCCAGAAACTCCAC	AGGTGTGTCTGCTAACCAGGACATGAACGG	68	25	—
1 ²	257	AACCACCCAGCCATCACCA	CCCACCCCGTCCACCC	60	25	60.8, 62.8
2 ²	244	GGGGGCTGCTCCCTCTAACCA	ATCCACCTGGCCACCTTCCC	60	25	61.2, 64.2
3 ²	236	CGCCCCCTGACCTCTCTCCA	AGAAAGCGGGGTTCCCG	60	25	64.0
4 ²	241	TGACTCTCTCCCCAACCCCTTC	GTTGTGGTAGGGGCTCACTGG	60	25	61.6, 62.6
5 ²	260	TGACAGCTGTCTTCCCTTCCCA	CCTGGCTTTGCACCTGCCTG	60	25	59.7, 61.7
6-9 ¹	3,528	CCCTAGCTCAAACCTGGTCTCTCTGAGCC	TTCTCTCATCACAGCTCCTGAAGGACATC	65	25	—
6 ²	230	AAGAGCATGGAGAGTGAGCTTGGTCT	GAGGGTCTGGGGCCCTTCACTT	60	25	60.3, 62.3
7 ²	286	CATCCCTGCTCTAAGACCCTAGACAC	GAAGTCCCCTAGTCTGAGTGGTGG	60	25	58.5, 60.5
8 ²	224	CCCCAACCTGCCTCATTACACA	TGTGAGCCGTGGCCTGGC	60	25	59.4, 60.4
9 ²	271	GAGAGTGGGCTTACCTTACCC	GTTCCCTGGCCCGCC	60	25	59.8, 61.8, 63.8

¹First-round PCR. ²Second-round PCR.

Materials and Methods

Human DNA samples: In the present study, DNA samples were obtained at the autopsies of 395 diseased patients in the Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan, and were analyzed. The research protocols were approved by the Ethics Committees of the Tokyo Metropolitan Geriatric Hospital and the Graduate School of Pharmaceutical Sciences, Tohoku University.

Polymerase chain reaction (PCR) and DHPLC conditions: The primer pairs used to amplify the nine exons and the exon-intron junctions of the *CYP2A13* gene are listed in Table 1. These primers were designed on the basis of a genomic sequence reported in GenBank (NG_000008.7). The first-round long PCR was performed to specifically amplify exons 1-5 and 6-9 of the *CYP2A13* gene. Genomic DNA (10-50 ng) was amplified using LA-Taq DNA polymerase (TaKaRa, Otsu, Japan). The PCR thermal profile consisted of an initial denaturation at 95°C for 1 min; followed by 25 repetitive cycles of denaturation at 95°C for 15 s, annealing at 68°C or 65°C for 20 s, and extension at 72°C for 5 min; and then a final extension at 72°C for 7 min.

The first-round PCR products were diluted 1:500 in water and used as DNA templates for the second round of PCR for amplification of all the *CYP2A13* exons. The amplicons for each exon were generated using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR for the second round comprised an initial denaturation at 95°C for 10 min; followed by 25 repetitive cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and then a final extension at 72°C for 7 min. Heteroduplexes were generated by thermal cycling under the following conditions: 95°C for 1 min, followed by 45 temperature decrements of 1.5°C/min.

The PCR products were analyzed using the DHPLC system WAVE (Transgenomic Inc., Omaha, NE, USA). Amplified PCR samples (5 µL) were separated on a heated C18 reverse-phase column by using 0.1 M triethylammonium acetate (TEAA) in water and 0.1 M TEAA in 25% acetonitrile at a flow rate of 0.9 mL/min. We determined the temperature for heteroduplex separation of a heterozygous *CYP2A13* fragment using the software that was provided with the DHPLC system. The DHPLC running conditions for each amplicon are summarized in Table 1. The linear acetonitrile gradient was adjusted so that the retention time of the DNA peak was 3-5 min. The resultant chromatograms were compared with those of the wild-type DNA. We sequenced both strands of samples in which variants were detected using DHPLC.

To determine the linkage among the polymorphisms identified in this study, we amplified long fragments of DNA obtained from the individuals who were heterozygous for both the SNPs by using PCR. The fragments were run on gels, purified using columns, and then ligated to a pCR-XL-TOPO vector (Invitrogen Co., CA, USA). The ligation products were transfected into *Escherichia coli*, and single colonies (each containing a plasmid with only one of the two alleles) were collected and the plasmids were isolated, after which the plasmid DNA was sequenced.

Results and Discussion

We found a novel SNP during this study and provide its details below. SNP: 110224Hiratsuka013; GENE NAME: *CYP2A13*; ACCESSION NUMBER: NG_000008; LENGTH: 25 bases; SEQUENCE: 5'-TCCATGAGGAGAT/CTGACAGATGAT-3'.

DHPLC analysis showed that exons 1, 2, 3, 5, 7, and 9 in the *CYP2A13* gene obtained from the 395 DNA samples had distinct chromatographic profiles from those of the wild-type DNA. We tested the specificity of DHPLC for detecting the variant allele in these exons by comparing the