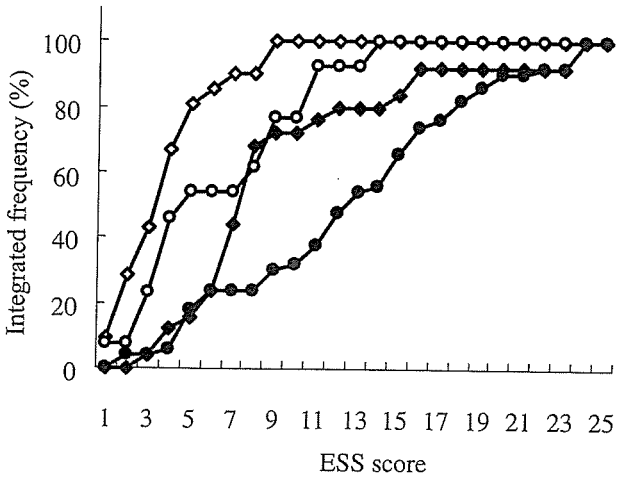

Fig. 1 Distribution of the baseline Epworth sleepiness scale (ESS) scores in men (*open square*) and women (*open circle*), and those of the cases who experienced H1-antihistamine-induced excessive daytime sleepiness in men (*closed square*) and women (*closed circle*). The baseline refers to the subjects without H1-antihistamine-induced adverse drug reactions. The Mann-Whitney U–test was used to compare the ESS scores between the baseline and the cases for each gender ($P<0.001$) and between men and women for baseline ($P=0.068$) and for cases ($P=0.018$)

noncarriers, Mann-Whitney U–test). The gender distribution did not differ among the *CYP2D6* genotypes ($P=0.285$, Fisher's exact test). In addition, the mean ESS scores in the cases that specified etiological H1-antihistamines were 12.2± 6.8 in men and 17.0±4.5 in women ($n=19$), which were significantly higher than in those unspecified cases, namely 7.7±3.8 in men ($n=15$) and 10.4±5.6 in women ($n=31$; specified vs. unspecified for men $P=0.046$ and for women $P<0.001$, Mann-Whitney U–test).

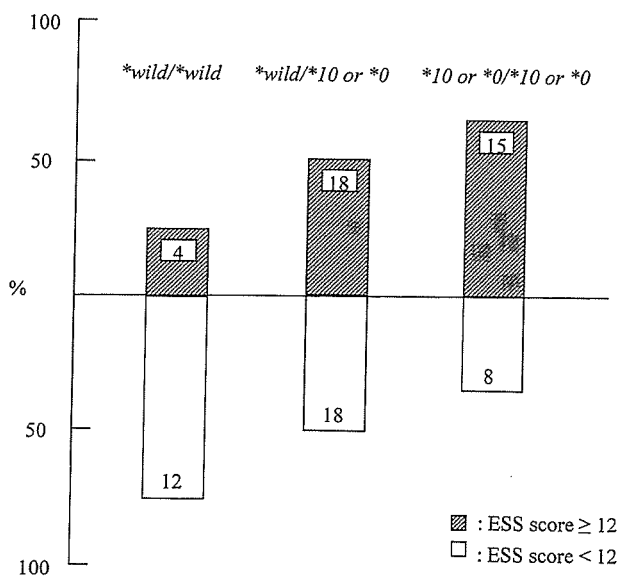

Fig. 2 Genotype distribution in the cases who experienced H1-antagonist-induced excessive daytime sleepiness corresponding to an Epworth sleepiness scale (ESS) score ≥12 (hypersomnia) or <12. A significant genotypic association between ≥12 and <12 of the total ESS score was identified ($P=0.045$, Fisher's exact test). A significant allelic association between ≥12 and <12 of the total ESS score was also identified ($P=0.015$, Fisher's exact test). The number of cases is shown in each bar

Discussion

This study investigated the relationship between the *CYP2D6* genotype and the occurrence of H1-antihistamine-induced ADRs while using the ESS to assess the most common symptom, daytime sleepiness. The main results showed that the *CYP2D6*10* allele was the risk factor for the ADRs caused by H1-antihistamines and that the occurrence of hypersomnia increased as the number of *CYP2D6* mutant allele increased. In addition to the extremes of the CYP2D6, the impact of the IM genotype or phenotype on the pharmacokinetics of β-blockers, morphine, antipsychotics, and such has been principally demonstrated [10–13, 22, 23]. As for antidepressants, an attempt toward dose adjustment based on *CYP2D6* genotype, including IM, was reported by providing distinct dose recommendations [24]. Moreover, evidence is emerging that *CYP2D6* genotyping can predict not only the pharmacokinetics but also the risk of adverse effects and treatment outcome [5–9]. However, all of these studies indicated that the IM genotype was not overrepresented in patients with the ADRs [5–9]. The IM genotype *CYP2D6*10* has been reported to be significantly associated with the tardive dyskinesia occurrence in Japanese psychotic populations [14, 15], but a recent meta-analysis reported that the risk of tardive dyskinesia was inconclusive for the *CYP2D6*10* allele [25]. Taken together, the present study showed a significant impact of the *CYP2D6*10* allele and the IM genotype on the H1-antihistamine-induced ADRs, but these results need to be interpreted carefully.

In the present study, the distribution of *CYP2D6*10* polymorphism in the cases was significantly different from that in healthy Japanese populations [4] (Table 1). The cases had about a 2.0-fold higher likelihood of being homozygotes for *CYP2D6*10* than healthy Japanese populations [4, 21]. These results indicate that *CYP2D6*10* was associated with H1-antihistamine-induced ADRs in Japanese. On the other hand, the frequencies of null alleles did not differ from the others. Although more than 95% of PMs in Caucasians can be diagnosed by four mutant alleles, *CYP2D6*3–*6*, only 30% in Asian PMs have these mutant alleles [4]. We therefore determined the most common mutant alleles, *CYP2D6*4, *5*, and **14*, which account for 95% of the known Japanese null alleles [4]. However, only 60% of the Japanese PMs could be explained by these null alleles, as uninvestigated mutant alleles might also exist [4].

Further study to identify these alleles is currently in progress.

The risks of H1-antihistamines demand particular attention because they are a top-selling class of drugs that are widely available over the counter [3, 26]. These drugs are indicated for allergic rhinitis, and approximately 20 million people use OTC antihistamines in the United States [27]. In Japan, the most frequently used antihistamine is chlorpheniramine, and 1,118 variations of chlorpheniramine are on the market as OTC drugs (Japan Pharmaceutical Information Center, http://www.japic.or.jp/). Clemastine follows chlorpheniramine. Promethazine is a component of a popular prescribed cold medicine in Japan, PL, which contains a four-times-daily dose of 13.5 mg of promethazine and was the etiological agent in all cases who complained of promethazine-induced adverse reaction in this study. The potentially dangerous sedating effect of first-generation H1-antihistamines (e.g., diphenhydramine, chlorpheniramine) among drivers has been documented [26, 28, 29]. Sedation, ranging from mild drowsiness to deep sleep, can occur even at the usual therapeutic doses in 25% of adults [26]. The first-generation H1-antihistamine-induced excess risk of injury is a potentially substantial public health problem because of the high frequency of injuries and their associated costs to patients, insurers, and society [26, 27]. A retrospective cohort study reported that the use of diphenhydramine contributes to 55% of the serious injuries in the patients taking the drug [27]. Our results suggest that the daytime sleepiness resulting from the first-generation H1-antihistamines may reach a level of hypersomnia at the usual therapeutic dose, and this therefore appears to be a substantial risk of serious injury, especially in females and/or in individuals with a reduced CYP2D6 activity.

The ESS, which we used to assess the level of H1-antihistamine-induced sedation, has been proposed as a simple method for measuring the general level of daytime sleepiness in adults [17, 18]. The mean ESS score of the cases was significantly higher than that of the baseline in both men and women. The occurrence of hypersomnia increased as the number of *CYP2D6* mutant alleles increased, in a manner of consistent with a gene-dose effect (Fig. 2). The mean ESS score also increased in the same trend of manner, and the score in the carriers of mutant allele(s) was significantly higher than that in the non-carriers. We wondered whether the inhibitory effect of H1-antihistamines on the CYP2D6 activity might amplify the influence of the IM genotype [3, 30–32], but we could not find any supportive information [33, 34].

Another possible risk factor for the H1-antihistamine-induced ADRs is the female gender. The mean female ESS score was significantly higher than that for males, and it was over the level of hypersomnia (Fig. 1). Previous studies have produced conflicting results regarding the influence of gender on the CYP2D6 activity: For some CYP2D6 substrates, such as propranolol and desipramine, the mean metabolism was lower in women than in men [35], whereas for dextromethorphan it was higher in women than in men [36], and for debrisoquine it was the same in both sexes [37]. In addition, gender-related differences also exist regarding other factors (e.g., transporters, receptors, sex hormones, body weight, history of drug use) [38]. Female gender has been shown to be a risk factor for ADRs, and women are also known to be more sensitive than men to the sedating effects of drugs [27, 39, 40]. Some studies have reported women to be more prone to an impairment of driving ability by H1-antihistamines than men are [27].

In general, the present study is limited by the self-reporting nature (potential participation bias) and the time delay (potential recall bias) between a history of adverse drug reaction and the time of survey. Specific limitations are as follows: First, we investigated only the cases. If we could choose a nested sample of H1-antihistamine users who did not report an ADR while taking the medication as the control group, it would be more informative. Second, the data were not controlled for H1-antihistamine exposure in this population. In this study, the incidence of ADRs induced by H1-antihistamines was significantly higher in women than in men, 63/835 (7.5%) and 37/1,239 (3.0%), respectively. The relative risk of women vs. men was 2.53 (95% CI, 1.70–3.76). However, we deleted the data from the results because we could not clarify the frequencies of the H1-antihistamine exposure in each sex. Third, we could not specify the etiological H1-antihistamines in 60% of the cases. The mean ESS score in the cases of the specified etiological drugs was 12.2 in men and 17.0 in women, which was 1.6 times higher than that in the unspecified cases in both genders. This suggests that some cases who complained of ADRs caused by drugs other than the classic H1-antihistamines might be included in the present study and that the impact of the classic H1-antihistamines might thus be underestimated. Consequently, a prospective study of a nested sample of H1-antihistamine users is further required.

In conclusion, we demonstrated the presence of the *CYP2D6*10* allele to be the risk factor of the ADRs caused by H1-antihistamines, and the occurrence of hypersomnia increased as the number of *CYP2D6* mutant alleles increased in Japanese. However, we obviously need further study to elucidate the relevance of the CYP2D6 phenotype and genotype to H1-antihistamine-induced ADRs.

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Sports and Culture, and by a grant-in-aid (no. 99-2) from the Organization for Pharmaceutical Safety and Research, Tokyo, Japan. The authors have identified no conflicts of interest in relation to this manuscript. This study complies with the current laws of Japan, where it was performed inclusive of ethics approval.

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Regular Article

In vivo Evaluation of Coumarin and Nicotine as Probe Drugs to Predict the Metabolic Capacity of CYP2A6 Due to Genetic Polymorphism in Thais

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

Summary: The association between the distribution characteristics of CYP2A6 catalytic activities toward nicotine and coumarin, and the frequency distribution of *CYP2A6* variant alleles reported was estimated in 120 healthy Thais. The distributions of the subjects as classified by the amounts of 7-hydroxycoumarin (7-OHC) excreted in the urine and by cotinine/nicotine ratio in the plasma were clearly bimodal. However, the numbers of apparently poor metabolizers for coumarin and nicotine were different. The inter-individual variability in the *in vivo* dispositions of coumarin and nicotine closely related to the *CYP2A6* genetic polymorphism. There was a close correlation between the rate of 7-OHC excretion in the urine and cotinine/nicotine ratio in the plasma among subjects ($R = 0.92$, $p < 0.001$). The frequency of *CYP2A6* allele found in the present study was: *CYP2A6*1A* = 32% (95% CI, 22.1–39.4%), *CYP2A6*1B* = 27% (95% CI, 19.4–33.5%), *CYP2A6*9* = 20% (95% CI, 17.6–23.3%), *CYP2A6*4* = 14% (95% CI, 9.6–17.8%), *CYP2A6*7* = 5% (95% CI, 3.7–9.4%), *CYP2A6*10* = 2% (95% CI, 0.8–5.1%). Subjects having *CYP2A6*1A/*1B* were found to have a higher rate of 7-OHC excretion, as well as a higher cotinine/nicotine ratio in the plasma compared with those of the other genotypes. In contrast, subjects with *CYP2A6*4/*7* and *CYP2A6*7/*7* almost lacked any cotinine formation, whereas urinary 7-OHC was still detectable. *CYP2A6*9* allele clearly resulted in reduced enzyme activities. Despite the absence of the homozygote for *CYP2A6*10* allele, the presence of *CYP2A6*10* allele significantly decreased the enzyme activities. The results of the present study demonstrate that *in vivo* phenotyping of CYP2A6 using nicotine and coumarin are not metabolically equivalent. Nicotine is a better probe according to its specificity, while coumarin is still valuable to be used for a routine CYP2A6 phenotyping since the test employs a non-invasive method.

Key words: All authors; hereby; declare no conflict of interest

Introduction

Cytochrome P450 (CYP) constitutes a large family of heam-containing monooxygenases that play an important role in the biotransformation of various xenobiotics and endogenous compounds.[1] The *CYP2A* gene subfamily, located on human chromosome 19, consists of *CYP2A6*, *CYP2A7*, *CYP2A13*, and two *CYP2A7* pseudogenes. Among them, only *CYP2A6* and *CYP2A13* genes encode active proteins.[2] Human

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CYP2A6 has been first identified as an enzyme which metabolizes coumarin to yield 7-hydroxycoumarin (7-OHC).[3] Following this observation, a number of other xenobiotics, including some drugs such as fadrozole, tegafur, and halothane, have been identified as substrates for CYP2A6.[4,5]

In addition to these clinically used drugs, nicotine and nicotine-derived nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are known to be metabolized to cotinine and mutagens, respectively.[5] A positive association between cigarette consumption and *CYP2A6* genetic polymorphism has been demonstrated.[6] However, a meta-analysis reviewing several case-control studies revealed no association between CYP2A6 variant alleles and smoking habit.[7]

Phenotyping study using coumarin and nicotine as probe drugs has shown a significant inter-individual variability which has been explained at least in part by *CYP2A6* genetic polymorphism.[8,9] Other factors contributing to the variation include physiological factors, diseases, and drug interactions.[10] Regarding the genetic polymorphism of *CYP2A6*, many variant alleles (*CYP2A6*1* to *CYP2A6*22*) have been reported so far (see http://www.imm.ki.se/CYPalleles).

The frequencies of alleles vary considerably among different ethnic populations. *CYP2A6*1* allele encoding a wild-type protein is relatively prevalent among Caucasians and black subjects, while they are found at lower frequency in Mongolians.[11] *CYP2A6*2* is a rare variant allele with an approximate frequency of 2% among Caucasians,[12] whereas the allele has not been found in Mongolians. The deletion allele (*CYP2A6*4*) is most common in Mongolians (up to 20%), resulting in an apparently low enzyme activity in these populations.[13] *CYP2A6*5* and *CYP2A6*6* alleles are relatively rare in Mongolians, with the frequencies of less than 1%.[14] *CYP2A6*7* and *CYP2A6*10* alleles are also reported to cause an impairment in nicotine metabolism and found at higher frequencies in Mongolians.[15]

*CYP2A6*9*, another variant allele possessing a single nucleotide polymorphism (SNP) in the TATA box found at a high frequency among Mongolians, is also reported to produce a small amount of the transcript CYP2A6 as proven both *in vitro* and *in vivo*.[15,16] *CYP2A6*11* that contains a 670T>C substitution has also been shown to have a reduced enzyme activity.[4] Similarly, *CYP2A6*12* allele which is probably originated through an unequal crossing over between *CYP2A6* and *CYP2A7* genes, is also described to cause a reduced CYP2A6 activity *in vivo*.[17] Four alleles, *CYP2A6*13*, **14*, **15*, and **16* containing a SNP that causes an amino acid substitution, have also been found in humans. However, the effects of these alleles on the enzyme activity have not yet been fully elucidated.[18] *CYP2A6*17*, **18*, **19*, and **20* have been found to have a decreased enzyme activity both *in vitro* and *in vivo*.[19-21] On the contrary, *CYP2A6*21* (K476R) has been found to have a minimal impact on nicotine disposition.[22]

The frequencies of all the major variant alleles of *CYP2A6* are rather low in Caucasians and black Africans; however, some of these alleles are highly prevalent in Mongolian populations. There are only a few reports on *in vivo* phenotyping of CYP2A6 using both coumarin and nicotine as probes in the same group of subjects, although several lines of evidence supporting the discordance between coumarin 7-hydroxylase activity and nicotine *C*-oxidase activity have been reported.[23,24] Moreover, no epidemiological study on *CYP2A6* polymorphism and its consequence in coumarin and nicotine dispositions has been done in Thais despite the clinical importance.

In the present study, we, therefore, performed a population study of coumarin metabolism in comparison to nicotine which was used as a reference probe, and *CYP2A6* genotyping in 120 Thais to clarify the association between the distribution characteristics of CYP2A6 activities toward coumarin and *CYP2A6* polymorphism in Thai population.

Methods

Chemicals: Venalot® tablets, each containing 15 mg coumarin and 90 mg troxerutin, were purchased from Schaper and Brummer GmbH & Co. KG (Salzgitter, Germany). Nicorette® chewing gums, each containing 2 mg of nicotine, were purchased from Pharmacia & Upjohn Co. (Tokyo, Japan). Other chemicals used were of analytical reagent grade unless indicated otherwise.

Subjects: One hundred twenty unrelated volunteers, 58 men and 62 women (age range 18–50 years), were recruited from the staff and students at the Faculty of Science, Mahidol University, to participate in the study. All subjects were classified as native Thais according to the familial history of their parents and grandparents. All subjects were non-smokers who never smoked at any time. They were healthy as defined by medical history, physical examination and routine blood chemistry analysis. All subjects were informed both verbally and in writing, about the experimental procedures and the purpose of the study. Written informed consent was obtained from all of them for their participation in the study. The subjects were asked to refrain from taking any medication for at least one week before and throughout the study period. The study protocol was approved by the Ethical Research Committee of Mahidol University, Thailand. All volunteers were asked to follow the experimental protocol as strictly as possible.

Genotyping for *CYP2A6* alleles: Genomic DNA was extracted from peripheral leukocytes using whole

blood samples by standard phenol-chloroform extraction method.[25] *CYP2A6*1A*, *CYP2A6*1B*, *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*8*, *CYP2A6*9*, and *CYP2A6*10* alleles were genotyped by the methods described previously.[16,23,26]

***In vivo* phenotyping for coumarin metabolism:** After an overnight fasting, the subjects emptied their bladders and took 15 mg of coumarin (Venalot®) with 200 mL of drinking water. Food was allowed at 2 hr postdose. All urine produced in the subsequent 8 hr was collected, and the total volumes were recorded. A 10 mL aliquot was taken and stored at −20°C until assayed. 7-OHC was determined by the method described previously.[27] All assays were performed in duplicate.

***In vivo* phenotyping for nicotine metabolism:** At least one week after the coumarin test, each subject was given a piece of 2 mg nicotine chewing gum (Nicorette®) after an overnight fasting. They chewed the gum for 20 sec per 30 sec for 30 min. Two hours after starting to chew, 10 mL blood samples were collected by venipuncture.[13] Approximately 5 mL of each sample was later used for DNA extraction and genotyping. The rest was used for the assay of cotinine/nicotine metabolic ratio according to the method previously described with some modifications.[28,29] Briefly, after centrifugation at 3,000 rpm for 10 min at 4°C, plasma sample (200 μL) was diluted with 500 μL of water and alkalinized with 10 μL of 10 M NaOH. The mixture was extracted with 1.5 mL of dichloromethane by vortex mixing for 2 min. After centrifugation at 3,000 rpm for 10 min at 4°C, a 750 μL portion of the lower phase was transferred to another tube. To this tube, 10 μL of conc. HCl was added. The organic fraction was evaporated with a vacuum evaporator at 40°C. The residue was dissolved in 25 μL of the mobile phase as shown below, and then an aliquot (25 μL) was injected to an Agilant 1100 series HPLC system (Agilent, Yokogawa, Tokyo, Japan) equipped with a 5 μm Mightysil RP-18 column (4.5 mm × 60 mm GP, Kanto Chemical, Tokyo, Japan) maintained at 40°C. The mobile phase consisted of acetate buffer (pH 4.5), methanol, and acetonitrile (740:245:16 v/v). The flow rate was 1.2 mL/min. The mass spectrometer was Finnigan LCQ^{DUO} (Thermoquest, Tokyo, Japan) equipped with Agillent HP-1100 series HPLC system (Yokogawa, Tokyo, Japan). The capillary potential was 4 kV, and the source temperature was 200°C. All assays were performed in duplicate.

Statistical analysis: Results were expressed as mean ±SD. The data were analyzed using the StatView 5.0 statistical software package (SAS Institute Inc., Cary, North Carolina, USA). The possibility of bimodality in the amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma was assessed using probit analysis.[30] The statistical significance of differences in the average amount of 7-OHC excreted in the urine from subjects with different genotypes was tested by Mann-Whitney *U* test. A correlation between the dispositions of coumarin and nicotine of the subjects was analyzed by Instat (GraphPad software, San Diego). Student *t*-test was used to examine significant levels for male-female differences in the amounts of 7-OHC excreted in urine, and the significant difference in the level of blood chemistry parameters of the ten subjects measured just before and 24 hr after a single oral administration of 15 mg coumarin. Chi-square test was used to evaluate significant levels for differences in the frequency distribution of males and females in genotypes, alleles, and phenotypes. A value of $p<0.05$ was considered statistically significant.

Table 1. Prevalence of *CYP2A6* genotypes in a Thai population

Genotypes	Males (n=58)	Females (n=62)	Total (n=120)
*CYP2A6*1A/*1A*	6	8	14 (11.7%)
*CYP2A6*1A/*1B*	11	11	22 (18.3%)
*CYP2A6*1B/*1B*	5	4	9 (7.5%)
*CYP2A6*1A/*4*	7	4	11 (9.2%)
*CYP2A6*1B/*4*	7	5	12 (10%)
*CYP2A6*1A/*7*	2	2	4 (3.3%)
*CYP2A6*1B/*7*	0	1	1 (0.8%)
*CYP2A6*1A/*9*	4	5	9 (7.5%)
*CYP2A6*1B/*9*	3	7	10 (8.3%)
*CYP2A6*1A/*10*	0	2	2 (1.7%)
*CYP2A6*1B/*10*	1	1	2 (1.7%)
*CYP2A6*4/*4*	2	2	4 (3.3%)
*CYP2A6*4/*7*	0	1	1 (0.8%)
*CYP2A6*4/*9*	1	1	2 (1.7%)
*CYP2A6*7/*7*	0	3	3 (2.5%)
*CYP2A6*9/*9*	9	5	14 (11.7%)

Results

Genotyping for *CYP2A6* in Thais: The genotype and allele frequencies of *CYP2A6* are shown in **Tables 1** and **2**, respectively. There was no subject homozygous for *CYP2A6*10*. No *CYP2A6*8* allele was found in this study. There was no gender difference in the frequencies of *CYP2A6* genotype and allele ($\chi^2=11.28$, $p=0.73$ for genotype frequency and $\chi^2=6.96$, $p=0.22$ for allele frequency).

Phenotyping for CYP2A6 in Thais: In the CYP2A6 phenotyping procedure, a number of subjects comprising of all *CYP2A6* genotypes developed nausea and/or mild headache after chewing nicotine gum. The symptoms gradually disappeared within 15 min after stopping nicotine gum chewing. There was no statistical significance of the *CYP2A6* genotype-related difference in the prevalence of such adverse effects of nicotine administration. The average amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma of male and female subjects as classified accord-

Table 2. Prevalence of *CYP2A6* alleles in Thais participating in the present study

Alleles	Males (n=58)	Females (n=62)	Total (n=120)	95% CI
*CYP2A6*1A*	36 (31%)	40 (32%)	76 (32%)	22.1–39.4%
*CYP2A6*1B*	32 (28%)	33 (27%)	65 (27%)	19.4–33.5%
*CYP2A6*4*	19 (16%)	15 (12%)	34 (14%)	9.6–17.8%
*CYP2A6*7*	2 (2%)	10 (8%)	12 (5%)	3.7–9.4%
*CYP2A6*8*	0 (0%)	0 (0%)	0 (0%)	0.0%
*CYP2A6*9*	26 (22%)	23 (19%)	49 (20%)	17.6–23.3%
*CYP2A6*10*	1 (1%)	3 (2%)	4 (2%)	0.8–5.1%
Total	116(100%)	124(100%)	240(100%)	ND

ND: not determined

ing to *CYP2A6* genotypes are shown in **Table 3.** 7-OHC in the 8-hour urine was detectable in the urine of all subjects, ranging from 0.03 to 15 mg (7.25±2.94 mg). There was no significant gender-related difference in the amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma when compared after classifying subjects according to the *CYP2A6* genotypes.

The probit plot of 7-OHC excreted in the urine is shown in **Fig. 1A.** The distribution of 7-OHC excreted in the urine showed bimodality. The probit plots of cotinine/nicotine ratio in the plasma was also clearly curved **(Fig. 1B).** By the antimode, four subjects who

Table 3. Coumarin 7-hydroxylase and nicotine *C*-oxidase activities of males and females in certain *CYP2A6* genotypes

Genotypes	Total 7-hydroxycoumarin excreted in 8 hr urine (mg)*			Cotinine/nicotine ratio in the plasma		
	Males (n=58)	Females (n=62)	Total (n=120)	Males (n=58)	Females (n=62)	Total (n=120)
*CYP2A6*1/*1*	9.57±1.38 (22)	9.90±2.74 (23)	9.52±2.59 (45)	8.11±1.70 (22)	8.66±2.04 (23)	8.39±1.88 (45)
*CYP2A6*1/*4*	5.02±0.90 (14)	5.26±1.71 (9)	5.11±1.25* (23)	4.40±0.90 (14)	4.95±1.51 (9)	4.62±1.18* (23)
*CYP2A6*1/*7*	6.63±2.21 (2)	6.57±1.38 (3)	6.60±1.48* (5)	5.28±0.61 (2)	5.11±0.18 (3)	5.18±0.34* (5)
*CYP2A6*1/*9*	7.84±1.60 (7)	7.74±1.61 (12)	7.78±1.56* (19)	6.58±1.20 (7)	6.43±1.03 (12)	6.48±1.06* (19)
*CYP2A6*1/*10*	5.51 (1)	6.86±1.58 (3)	6.52±1.45* (4)	5.19 (1)	5.07±0.20 (3)	5.10±0.17* (4)
*CYP2A6*4/*4*	0.29±0.37 (2)	0.80±0.10 (2)	0.55±0.36** (4)	0.00 (2)	0.00 (2)	0.00** (4)
*CYP2A6*4/*7*	— (0)	3.27 (1)	3.27 (1)	— (0)	0.94 (1)	0.94 (1)
*CYP2A6*4/*9*	3.97 (1)	3.37 (1)	3.67±0.42 (2)	3.22 (1)	2.79 (1)	3.01±0.30 (2)
*CYP2A6*7/*7*	— (0)	4.42±1.10 (3)	4.42±1.10* (3)	— (0)	1.64±0.54 (3)	1.64±0.54** (3)
*CYP2A6*9/*9*	6.30±1.90 (9)	6.82±1.94 (5)	6.48±1.73* (14)	5.04±0.93 (9)	5.11±0.32 (5)	5.06±0.79* (14)
Total	7.00±2.79 (58)	7.48±3.09 (62)	7.25±2.94 (120)	6.04±2.35 (58)	6.22±2.77 (62)	6.14±2.56 (120)

The average value of total amounts of 7-OHC excreted in the 8 hr urine (mg)±SD. The numbers in parentheses indicate the numbers of subjects. *=$p<0.05$, **=$p<0.01$ compared with *CYP2A6*1/*1*.

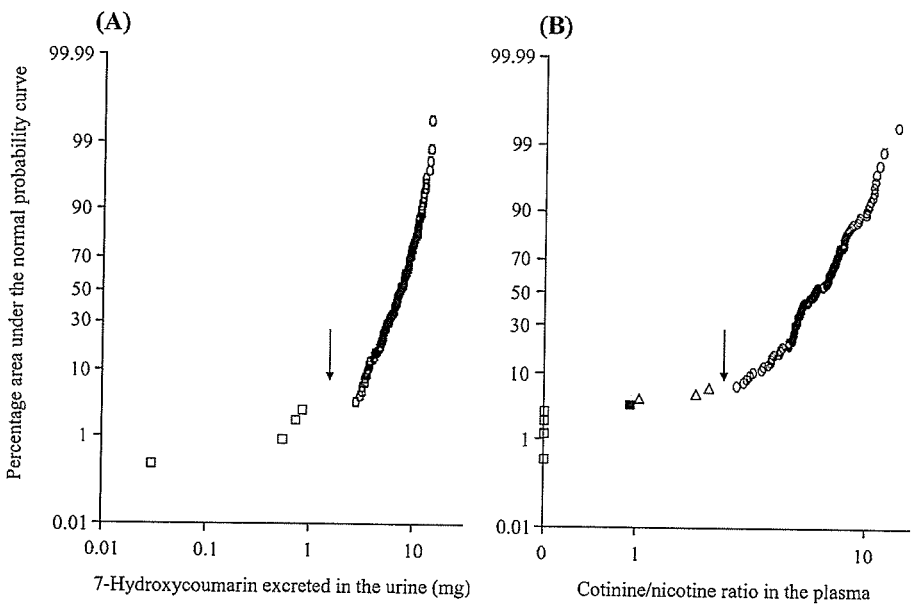

Fig. 1. Probit analysis for CYP2A6 activity toward coumarin and nicotine in the Thai subject population. The transparent square, black square, and transparent triangle represent the subjects genotyped as *CYP2A6*4/*4*, *CYP2A6*4/*7*, and *CYP2A6*7/*7*, respectively. The other genotypes are represented by ovals. (A) Analyzing for total 7-OHC excreted in the subsequent 8 hr urine after taking a 15-mg coumarin tablet. (B) Analyzing for cotinine/nicotine ratio in the plasma 2 hr after chewing a piece of nicotine chewing gum. The arrow represents the antimode of the probit plot.

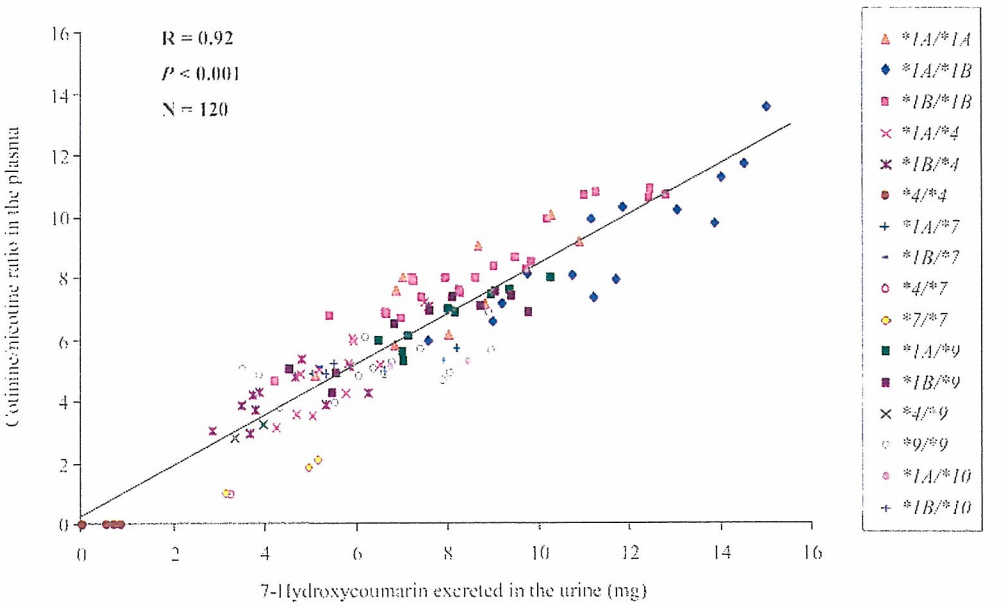

Fig. 2. Correlation between the rate of 7-OHC excretion in the urine and cotinine/nicotine ratio in the plasma in 120 Thai subjects classified according to *CYP2A6* genotypes.

were homozygotes of the *CYP2A6*4* allele, were phenotyped as PM for coumarin (**Fig. 1A**), whereas eight subjects (four *CYP2A6*4/*4*, one *CYP2A6*4/*7*, and three *CYP2A6*7/*7*) were judged as PM for nicotine (**Fig. 1B**). The numbers of apparent PM toward coumarin were lower in this population when compared to the data with nicotine as a reference probe (**Fig. 1B**). This indicates discordance between CYP2A6 catalytic activity toward coumarin and nicotine metabolisms. The reason(s) for this phenomenon is not known.

The *in vivo* oxidation activities toward coumarin and nicotine: The correlation between the rate of 7-OHC *excretion* in the urine and cotinine/nicotine ratio in the plasma among subjects as classified according to *CYP2A6* genotypes is shown in **Fig. 2**. There was no statistically significant difference of the *in vivo* disposition of coumarin and nicotine between male and female subjects (**Table 3**). The mean 7-OHC excreted in the urine and cotinine/nicotine ratio in plasma of the subjects genotyped as *CYP2A6*1A/*1B* were higher than those of the subjects with other genotypes (11.61 ± 2.26 mg for 7-OHC excreted in the urine and 9.10 ± 2.17 for cotinine/nicotine ratio in the plasma). The homozygotes of *CYP2A6*1A* allele showed CYP2A6 activity toward coumarin (7-OHC excreted in the urine; 8.06 ± 1.82 mg) and nicotine (cotinine/nicotine ratio in the plasma; 7.51 ± 1.72) at approximately 77% and 91% of those with *CYP2A6*1A/*1B*, respectively. Similarly, the homozygotes of *CYP2A6*1B* allele exhibited the CYP2A6 activity toward coumarin (7-OHC excreted in the urine; 8.79 ± 2.30 mg) and nicotine (cotinine/nicotine ratio in the plasma; 8.31 ± 1.65) at approximately 86% and 89% of those with *CYP2A6*1A/*1B*, respectively. There was no statistically significant difference in the CYP2A6 activity toward both probes among *CYP2A6*1A/*1A* subjects ($p = 0.29$ for coumarin metabolism and $p = 0.74$ for nicotine metabolism compared with *CYP2A6*1A/*1B* subjects), *CYP2A6*1A/*1B* subjects, and *CYP2A6*1B/*1B* subjects ($p = 0.42$ for coumarin metabolism and $p = 0.58$ for nicotine metabolism compared with *CYP2A6*1A/*1B* subjects). The subjects genotyped as *CYP2A6*1A/*1A*, *CYP2A6*1A/*1B*, and *CYP2A6*1B/*1B* were, therefore, combined as the wild-type (*CYP2A6*1/*1*). On the contrary, compared with the wild-type, all other variant genotypes excreted significantly lower 7-OHC as well as exhibited lower cotinine/nicotine ratio, especially four subjects with *CYP2A6*4/*4*. The amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma of subjects genotyped as *CYP2A6*1/*4* were reduced to 54% and 55% of the wild-type ($p < 0.05$), respectively (**Table 3**). Four subjects genotyped as *CYP2A6*4/*4* totally lacked cotinine formation, whereas they still excreted a small amount of 7-OHC (0.55 ± 0.36 mg) in the urine, (**Table 3**).

Three subjects who were homozygous for *CYP2A6*7* allele (*CYP2A6*7/*7*) showed the rate of 7-OHC excretion of approximately 46%, but only 20% of cotinine/nicotine ratio as compared to those homozygously having the wild-type allele. Subjects with *CYP2A6*4/*7* and *CYP2A6*4/*9* also showed a markedly reduced capacity of 7-OHC as well as cotinine formation. As can be seen in **Table 3**, the amount of 7-OHC excreted

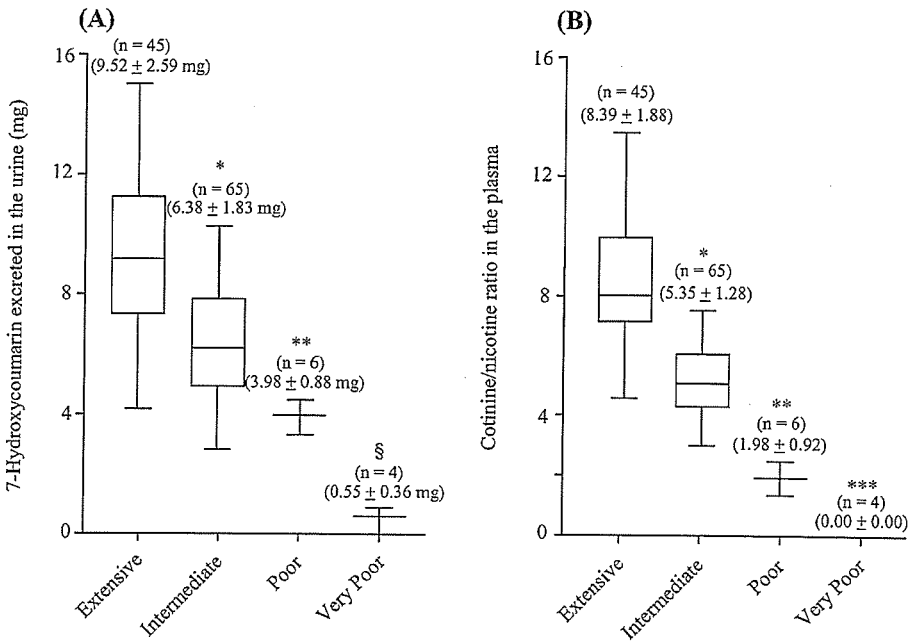

Fig. 3. Comparison of CYP2A6 activity toward coumarin (A) and nicotine (B) among CYP2A6 phenotypes. Data are shown as box plots representing medians (——), minimal and maximal values (-) with 25th and 75th percentiles. The extensive phenotypic group = *CYP2A6*1/*1*. The intermediate phenotypic group = *CYP2A6*1/*4*, *CYP2A6*1/*7*, *CYP2A6*1/*9*, *CYP2A6*1/*10* and *CYP2A6*9/*9*. The poor phenotypic group = *CYP2A6*4/*7*, *CYP2A6*4/*9*, *CYP2A6*7/*7*. The very poor phenotypic group = *CYP2A6*4/*4*. *; $p<0.05$ compared to the extensive phenotypic group, **; $p<0.05$ compared to the intermediate phenotypic group, ***; $p<0.05$ compared to the poor phenotypic group, §; $p<0.01$ compared to the poor phenotypic group. The numbers of the subjects in each phenotypic group and the average amounts of 7-OHC excreted in the urine (A) and cotinine/nicotine ratio in the plasma (B) are shown in the parentheses.

in the urine of a subject genotyped as *CYP2A6*4/*7* (3.27 mg) was relatively comparable to those of subjects genotyped as *CYP2A6*4/*9* (3.67 ± 0.42 mg). Cotinine/nicotine ratio in the plasma of subjects with *CYP2A6*4/*7* (0.94) was clearly lower than those of the subjects with *CYP2A6*4/*9* (3.01 ± 0.30). When compared with the wild-type subjects, the subjects with *CYP2A6*4/*7* and *CYP2A6*4/*9* showed markedly decreased capacity of 7-OHC formation to about 34% and 39%, respectively, while the same subjects showed cotinine formation only approximately 11% and 36% of the wild-type subjects, respectively.

The capacity of *in vivo* dispositions of coumarin and nicotine of the subjects with *CYP2A6*1/*9* were 82% and 77% of the wild-type subjects, respectively. Thirteen subjects genotyped as *CYP2A6*9/*9* also showed even less capacities of the 7-OHC formation and cotinine formation of about 68% and 60%, respectively (**Table 3**). The subjects homozygous for *CYP2A6*9* allele showed a level of coumarin disposition similar to those genotyped as *CYP2A6*1/*7* and *CYP2A6*1/*10* (**Table 3**). Compared with the wild-type subjects, the average amount of 7-OHC excreted in the urine and the average cotinine/nicotine ratio in the plasma of the subjects genotyped as *CYP2A6*1/*10* significantly decreased to about 68% and 61%, respectively.

Figure 3 shows the capacities of 7-OHC and cotinine formation of subjects classified into four phenotypic groups according to *CYP2A6* genotypes; the extensive phenotypic group (homozygotes for *CYP2A6*1*), the intermediate phenotypic group (heterozygotes for the wild-type and certain variant alleles, and homozygotes for *CYP2A6*9*), the poor phenotypic group (heterozygotes for certain variant alleles and homozygotes for *CYP2A6*7*), and the very poor phenotypic group (homozygotes for *CYP2A6*4*). The average amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma of the subjects in each phenotypic group are also indicated (**Fig. 3**). Significant differences in the average amounts of 7-OHC excreted in the urine as well as cotinine/nicotine ratio in the plasma in subjects across all phenotypic groups were observed. The data indicate that the presence of *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, and *CYP2A6*10* alleles clearly reduces the *in vivo* disposition of coumarin and nicotine.

Inter-ethnic variation in *CYP2A6* allele frequency: Frequency percentage of reported *CYP2A6* alleles in different populations is summarized in **Table 4**. In the present study, *CYP2A6* alleles were found to display distinct inter-ethnic differences among Mongolian, Caucasian, and black African populations. The most conspicuous differences was that not only the deleted

Table 4. Frequency percentage of reported *CYP2A6* alleles in different populations

Alleles	Thais[a] (%)	Japanese[b] (%)	Koreans[b] (%)	Caucasians[c] (%)	Black Africans[d] (%)
*CYP2A6*1*	58.8	48.3	59.2	88.8–97.1	92.4
*CYP2A6*2*	ND	0.0	0.0	1.0–3.0	0.0
*CYP2A6*4*	14.2	20.1	11.0	1.0–4.0	1.9
*CYP2A6*5*	ND	0.0	0.5	ND	0.0
*CYP2A6*7*	5.0	6.5	3.6	0.0	0.0
*CYP2A6*8*	0.0	2.2	1.4	0.0	0.0
*CYP2A6*9*	20.4	21.3	22.3	5.2–7.2	5.7
*CYP2A6*10*	1.6	1.1	0.5	0.0	0.0
*CYP2A6*11*	ND	ND	ND	0.0	0.0
*CYP2A6*17*	ND	0.0	0.0	ND	ND
*CYP2A6*18*	ND	0.0	0.5	ND	ND
*CYP2A6*19*	ND	0.5	1.0	ND	ND
*CYP2A6*20*	ND	0.0	0.0	ND	ND

a: Current study, b: Yoshida *et al.* 2002[14], Yoshida *et al.* 2003[15], Fukami *et al.* 2004[19], Fukami *et al.* 2005[20] and Fukami *et al.* 2005[21], c: unpublished data obtained in our laboratory, d: Gyamfi *et al.* 2004[11], ND: not determined

*CYP2A6*4* allele, but also *CYP2A6*7* and *CYP2A6*9* alleles were detected at a higher frequency in Mongolians than Caucasians and black Africans. On the other hand, *CYP2A6*2* was not found in Mongolians and black Africans, while *CYP2A6*7*, *CYP2A6*8*, and *CYP2A6*10* did not exist at all in the Caucasian and black African subjects.

Discussion

In this study, we investigated the effects of the *CYP2A6* group of polymorphism on *in vivo* metabolism of coumarin as well as nicotine in the same group of Thai subjects. Coumarin (15 mg) and nicotine (2 mg in chewing gum) were sequentially given to the same. The urine (for coumarin test) and plasma (for nicotine test) samples were collected at certain time points, and the amounts of produced metabolites were determined. Since the mean plasma elimination half-life of nicotine is approximately 2 hr and does not significantly vary with age and gender,[31] the *in vivo* disposition of nicotine has been, therefore, investigated by collecting blood samples at 2 hr after a nicotine challenge. Regarding adverse effects of nicotine and the fact that all subjects were absolute non-smokers, nausea and mild headache after a nicotine challenge were predictable. However, those who experienced such symptoms comprised of all *CYP2A6* genotypes and the number of affected subjects in each genotype was commensurate with the number of subjects of that particular genotypic group, the adverse effects of nicotine at the challenging dose seemed to less likely be contributed by *CYP2A6* polymorphism.

The mean value of the amount of 7-OHC excreted in the urine of our study was slightly higher than that of the previous study reported by Ujjin *et al.*[32] who recruited 202 Thais and collected the urine samples 3 hr after coumarin administration. This suggests that 7-OHC is not completely excreted from the body within 3 hr after coumarin administration. Even though it was proposed that up to 95% of 7-OHC would be excreted in the urine within 4 hr,[8] we collected the urine samples up to 8 hr to avoid the effect of urinary retention since, in our preliminary experiment, some subjects did not void their urine in the first 4 hr. In addition, total creatinine excretion in 8 hr urine was measured in order to verify the urine samples' eligibility.

In the present study, we determined several *CYP2A6* variant alleles, which had recently been reported to practically contribute to the *CYP2A6* polymorphism and CYP2A6-mediated enzymatic activity. However, the number of *CYP2A6*4/*4* subjects reported by Ujjin *et al.* was the same as that of the present study.

It should be noted that the subjects who participated in this study were confirmed to be native Thai but came from different parts of the country, whereas Thai subjects recruited by Ujjin *et al.* were not identified for their origin and domicile. However, considering the fact that the frequency of *CYP2A6* alleles in the present study was comparable to that previously reported in Chinese and Koreans, it may be reasonable to report no considerable difference in the *CYP2A6* allele frequency between the present and the previous study by Ujjin *et al.* It may be regarded as a general concept that the wild-type allele is found at a greater frequency among Caucasians and black Africans while the variant alleles are found at a lower frequency when compared to that of Mongolians.

Even though both coumarin and nicotine have been regarded as specific probes for CYP2A6, phenotyping study using both coumarin and nicotine in the same group of subjects revealed that they were not metabolically equivalent (**Fig. 1**). The data from the present study were consistent with those of the study conducted by Xu *et al.*,[24] who have shown in a small group of subjects that the *CYP2A6*7* allele causes different con-

sequences in the *in vivo* dispositions of coumarin and nicotine. However, such report did not identify the health status of subjects and smokers were also recruited in their study. Moreover, the probe drugs used were not standardized. Thus, to give a strong support to the existence of the discordance between coumarin and nicotine dispositions, we provided the data and information in this regard by performing the study in a larger scale population. Inter-individual differences in the postulated enzymatic activity of CYP2A6 proved the existence of the close association between the phenotype and genotype.

Although the *CYP2A6* genotype-phenotype correlation was found to be rather clear, the homozygotes of the *CYP2A6*4* allele were totally deficient in cotinine formation, whereas the amounts of 7-OHC excreted in the urine in these subjects were, though, extremely low but still detectable (**Fig. 2**). Taken together with *in vitro* studies employing genetically expressed human *CYP2A13* gene system,[33,34] our data confirmed the role of other enzymes in the *in vivo* 7-OHC formation. However, the contribution of CYP2A13 to the *in vivo* dispositions of coumarin and nicotine is obviously minimal since the *in vitro* intrinsic activity of the enzyme toward coumarin is about 10-fold less than that of CYP2A6.[35] Moreover, the subjects with *CYP2A6*4/*4* showed total absence of the *in vivo* metabolism toward nicotine.

The discordance between CYP2A6 catalytic activities toward coumarin and nicotine did exist at a large sample population. The change in substrate specificity of the *CYP2A6*7* allele has been hypothesized to account for the discordance in the rates of coumarin 7-hydroxylase and nicotine *C*-oxidase.[36] Since the I471T variant of CYP2A6.7 is not located in the substrate-recognition site,[37] the mechanism(s) of functional changes dependent of substrates such as coumarin and nicotine needs further investigation. Despite an exception for the *CYP2A6*7* allele, the correlation study ($R=0.92$, $p<0.001$) indicated that in human both 7-OHC and cotinine formations were mostly catalyzed by CYP2A6 (**Fig. 2**). This means that both coumarin and nicotine could be clinically and experimentally used as probe drugs in a routine CYP2A6 phenotyping test.

Even though no subject genotyped as a homozygote for *CYP2A6*10* allele was found in the present study, the effects of *CYP2A6*10* allele on the rates of 7-OHC and cotinine formation were observed (**Table 3**). The frequency of *CYP2A6*9* allele found in this study was corresponding to that of Japanese (**Table 4**). Individuals possessing *CYP2A6*9* allele showed a lower rate of 7-OHC excretion and cotinine formation (**Table 3**). The data were consistent with the *in vivo* studies performed in healthy volunteers that displayed reduced enzyme activities in the subjects genotyped as *CYP2A6*1/*9* and *CYP2A6*9/*9*.[27] Taken together, this confirmed the effect of *CYP2A6*9* allele on the decrease in the CYP2A6 activity *in vivo*. Our classification of the subjects into four phenotypic groups; extensive, intermediate, poor and very poor phenotypic group, was more advantageous, reliable, and useful for clinical application. As can be seen in **Fig. 3**, the average amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma of the subjects in each phenotypic group was significantly different from the other phenotypic groups.

Since CYP2A6 is capable of bioactivating tobacco-specific procarcinogens, CYP2A6 PM could be theoretically protected from developing cancer at the target sites mainly the lungs.[36] Results reported by Fujieda *et al.*[6] clearly indicated that Japanese smokers with *CYP2A6* variant alleles had significantly lower risk of lung cancer. However, a meta-analysis conducted by Carter *et al.*[7] showed that CYP2A6 mutation had no protective effect toward the risk of lung cancer. The discordance in the literature may, therefore, be resulted from various aspects of study design such as broad definitions of smokers, ethnic stratification, genotype groups, and genotyping assays.

We, hereby, confirmed that the frequency distribution of *CYP2A6* variant alleles was consistent over Mongolians, and these ethnic groups may, therefore, have similar potential tobacco-related lung cancer risk, pharmacological as well as toxicological responses to some anticancer agents such as coumarin, fadrozole, and tegafur. The data presented in the present study, therefore, may be applicable to drug development and therapy with drugs metabolized mainly by CYP2A6.

Since there are still large CYP2A6-activity variations among the subjects with the same genotype, particularly *CYP2A6*1/*1* (**Fig. 3**), it appears that other factors may also contribute to the CYP2A6 enzyme activity. Among those, gender and environmental contaminants have been found to influence CYP2A6. The average amounts of 7-OHC excreted in the urine in females was higher than that in males,[32,38] while the exposure to cadmium from environmental pollutant and diet resulted in the induction of the enzyme activity.[39] In contrast, plant substances seemed to have a minimal influence on CYP2A6 activity.[40] In addition to those host and/or environmental factors, the intra-genotypic variation of CYP2A6 activity among the subjects may possibly be marked due to the presence of unknown lack-functioned or gain-functioned mutations in the *CYP2A6* gene. The complex etiology of the intra-genotypic variation of CYP2A6 activity may also be resulted from the potential consideration of epigenetics.

In conclusion, we performed a population study of *CYP2A6* genotype-phenotype analysis in 120 Thais who took both coumarin and nicotine sequentially. This

procedure provided a precise evaluation of *CYP2A6* genetic polymorphism in the metabolism of coumarin and nicotine. The CYP2A6 activity toward coumarin was markedly low in the subjects homozygous for either *CYP2A6*4* or *CYP2A6*9*, or heterozygous for these alleles, whereas the enzyme activity toward nicotine was dramatically reduced in the homozygotes for *CYP2A6*4*, *CYP2A6*7*, and *CYP2A6*9* or the heterozygotes with these alleles. The presence of *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, or *CYP2A6*10* was a critical factor determining inter-individual variations in drug oxidation of CYP2A6 substrates. We, therefore, propose that nicotine is a better probe according to its specificity, while coumarin is still valuable to be used for a routine CYP2A6 phenotyping since the test employs a non-invasive method.

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Activation of p53 as a causal step for atherosclerosis induced by polycyclic aromatic hydrocarbons

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Abstract **This study was performed to prove our hypothesis that the metabolite(s) of polycyclic aromatic hydrocarbons (PAHs) caused the activation or phosphorylation of p53 via DNA damage to suppress the liver X receptor (LXR)-mediated signal transductions as a probably more direct mechanism. We found that LXR-mediated *trans*-activation was inhibited by 3-methylchoranthrene (MC) and doxorubicin (Dox) in HepG2 cells carrying wild-type p53, but not in Hep3B cells possessing mutant p53. The exogenous expression of wild-type p53 suppressed the LXR-mediated *trans*-activation in Hep3B cells. The expression of mRNA for ATP binding cassette A1 was suppressed by MC and Dox in HepG2 cells. The protein expression of retinoid X receptor (RXR), a partner of LXR to form a heterodimer, was suppressed by MC and Dox in HepG2 cells.**

Keywords: ABCA1; AHR; Atherosclerosis; DNA damage; Luciferase assay; MC; Quantitative RT-PCR

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]-pyrene (B[*a*]P) and 3-methylcholanthrene (MC), are ubiquitous contaminants in the environment. PAHs are detected in multiple sources, including cigarette smoke, exhaust emissions, industrial wastes and the pyrolysates of foods [1–3]. They cause a wide variety of toxicities, including carcinogenesis, atherogenesis and teratogenesis [4]. These toxic effects are known to be mediated by aryl hydrocarbon receptor (AHR), a ligand-dependent basic helix–loop–helix transcription factor [5–7]. A ligand-activated AHR translocates into the nucleus, forms a heterodimer complex with AHR nuclear translocator (ARNT), and finally interacts with xenobiotic responsive elements in the 5′-flanking regions of the AHR-target genes [8]. As one of the important mechanisms of PAH-induced toxicities, AHR up-regulates drug metabolizing enzymes such as cytochrome P450 (CYP), especially CYP1A1, which metabolizes PAHs to yield reactive intermediates causing DNA damage [9].

Tumor suppressor p53 is also known to be a transcription factor activated or phosphorylated by many types of stresses, including DNA damage [10,11]. The activated p53 subsequently *trans*-activates target-genes responsible for growth arrest or apoptosis [10,11]. Recently, it has been reported that p53 interacts with nuclear receptor, glucocorticoid receptor (GR), which is activated by glucocorticoids and maintains homeostasis in response to internal or external stresses [12]. The interaction results in the promotion of the proteosomal degradation of both proteins [12]. In addition, p53 is reported to inhibit the specific binding of nuclear receptor, androgen receptor (AR), which binds to androgens and is critical for the development, growth and maintenance of the male reproductive system, to DNA as a result of the inhibition of AR dimerization [12]. These studies provide evidence for a negative cross-talk between p53 and nuclear receptors.

PAHs, including B[*a*]P and MC, was reported to induce the atherosclerosis in several experimental animals [13–15]. Previously, we reported that MC inhibited liver X receptor (LXR)-mediated signal transductions, which are known to maintain cholesterol homeostasis, through AHR to cause atherosclerosis [16]. We also reported that the metabolism, probably the metabolic activation, of MC by CYP1A1 was a necessary step to repress the LXR-originated signal transductions by MC [17]. In the present study, we hypothesized that p53 activated by the metabolite(s) of PAHs suppressed LXR as well as GR or AR [12]. In this paper, we show evidence supporting our idea that p53 activated by PAHs acts as a negative regulator of LXR-mediated signal transductions to cause atherosclerosis via suppression of retinoid X receptor (RXR) expression, which is a partner of LXR to form a heterodimer.

2. Materials and methods

2.1. Cell culture

Human hepatoma-derived HepG2 and Hep3B cells were purchased from RIKEN (Tsukuba, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmacy, Tokyo, Japan) supplemented with 10% fetal bovine serum (Bio Whittaker, Warkersville, MD), non-essential amino acids (ICN, Aurora, OH) and 1 mM sodium pyruvate (Gibco-BRL, Rockville, MD) in 5% CO_2 at 37 °C.

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Abbreviations: ABCA1, ATP binding cassette A1; AHR, aryl hydrocarbon receptor; AR, androgen receptor; ARNT, AHR nuclear translocator; B[*a*]P, benzo[*a*]pyrene; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; Dox, doxorubicin; GR, glucocorticoid receptor; LXR, liver X receptor; LXRE, LXR response element; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; RT-PCR, reverse transcriptase-polymerase chain reaction; RXR, retinoid X receptor; T1317, TO-901317; TK, thymidine kinase

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2.2. Plasmids

The p(LXRE)$_2$–thymidine kinase (TK)–Luc was constructed as described previously [16]. Full-length human p53 cDNA was obtained by PCR with a sense primer, p53-*Xho*I-S (5′-CGGGCTCGAGCCATGGAGGAGCCGCAGTC-3′), and an antisense primer, p53-*Xho*I-AS (5′-GTGGCTCGAGTCAGTCTGAGTCAGGCCCT-3′). The resultant fragment was digested with *Xho*I, and inserted into the *Xho*I site of the pcDNA 3.1 mammalian expression vector (pcDNA-p53) (Invitrogen, Carlsbad, CA).

2.3. Transient transfection and luciferase assay

One day before transfection, cells were plated at a density of 1×10^5 cells/well in a 12-well plate. HepG2 cells were transfected with 350 ng of p(LXRE)$_2$–TK–Luc, 100 ng of pcDNA-hLXRα and 50 ng of pRL-TK vector (as an internal control for transfection) by using Fugene6 (Roche Diagnostics, Indianapolis, IN). After the transfection, the medium was changed to fresh DMEM containing 1 μM TO-901317 (T1317), a LXR ligand (Sigma–Aldrich, St. Louis, MO), 1 μM MC, an AHR ligand (Sigma–Aldrich) and 1, 10 or 100 nM doxorubicin (Dox), a known p53 activator (Sigma–Aldrich). Hep3B cells were transfected with 350 ng of p(LXRE)$_2$–TK–Luc, 100 ng of pcDNA-hLXRα, 50 ng of pRL-TK vector and 0.1, 1 or 10 ng of pcDNA-p53. After the transfection, the medium was changed to fresh DMEM containing 1 μM T1317 and 0.1 or 1 μM MC. Cells were harvested after incubation for 36 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

2.4. Real-time RT-PCR analysis

HepG2 cells were treated with 10 μM T1317, 10 μM MC and 100 nM Dox. After incubation for 24 h, total RNA from these cells was prepared using a GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich). Reverse transcription reaction was performed by using a First Strand cDNA Synthesis Kit for reverse transcriptase-polymerase chain reaction (RT-PCR) (AMV) (Roche Diagnostics). Quantitative real-time PCR was performed as described previously [16].

2.5. Western blot analysis

HepG2 cells were treated with 10 μM T1317 and 10 μM MC or 100 nM Dox. Nuclear extracts from these cells were prepared after incubation with MC for 0, 6, 12 or 24 h and with Dox for 24 h according to the method of Dignam et al. [18]. Protein concentration was determined using bovine serum albumin as a standard by BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed using antibodies to LXRα (C-19), RXRα (D-20), p-p53 (Ser 15), p21 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (AC-15) (Abcam, Cambridge, MA).

3. Results and discussion

To examine a possibility of whether the activation of p53 was a process critical for the suppression of LXR-mediated signal transductions, the effects of Dox, one of the representative p53 activators, on LXR-mediated transcriptional activity were investigated by a luciferase reporter assay using a reporter plasmid, p(LXRE)$_2$–TK–Luc (Fig. 1). When T1317, a LXR ligand, was added to a culture containing HepG2 cells transfected with the pcDNA-hLXRα,the luciferase activity seen with p(LXRE)$_2$–TK–Luc was elevated to a level of 12-fold higher than that of control (Fig. 1). The luciferase activity seen with p(LXRE)$_2$–TK–Luc in the presence of T1317 was decreased to a level of approximately 20–30% by co-treatment with MC (Fig. 1). Similarly, the luciferase activity was decreased by co-treatment with Dox in a dose-dependent manner (Fig. 1). These results support the idea that the activation of p53 is a process responsible for the suppression of LXR-mediated signal transductions by PAHs.

To further support the idea that p53 was involved in the suppression of LXR-mediated signal transductions by PAHs, the effects of MC and the exogenous expression of p53 on LXR-mediated transcriptional activity were investigated in Hep3B cells, a human hepatoma-derived cell line lacking wild-type p53 [19]. When T1317 was added to a culture containing Hep3B cells transfected with the pcDNA-hLXRα, the luciferase activity seen with the p(LXRE)$_2$–TK–Luc was increased to a level 15-fold higher than that of control (Fig. 2A). The co-treatment of Hep3B cells with MC did not affect the luciferase activity seen with the p(LXRE)$_2$–TK–Luc (Fig. 2A). When Hep3B cells were transfected with increasing amounts

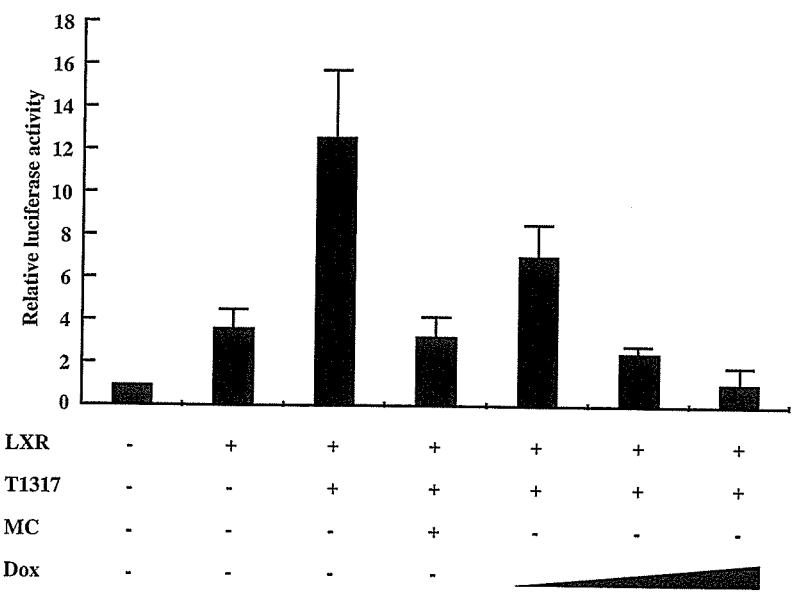

Fig. 1. Inhibition of LXR-mediated transcriptional activity by MC and Dox, which is the activator of p53. HepG2 cells were transfected with p(LXRE)$_2$–TK–Luc and pcDNA-hLXRα, and to the culture were added 1 μM T1317, 1 μM MC and 1, 10 or 100 nM Dox. Luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ± S.D. from three independent experiments.

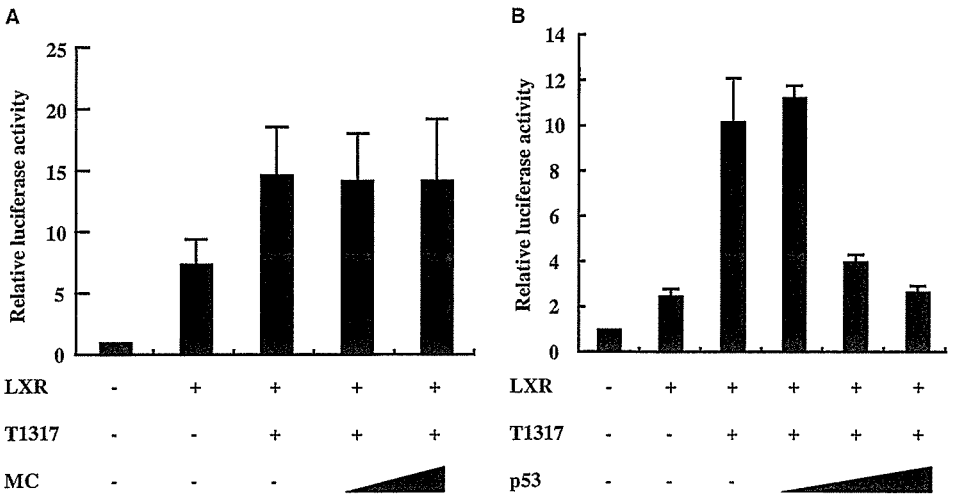

Fig. 2. Effects of MC and the exogenous expression of p53 on LXR-mediated transcriptional activity in Hep3B cells, hepatoma-derived cells carrying mutant p53. (A) A luciferase reporter plasmid, $p(LXRE)_2$–TK–Luc, was co-transfected into Hep3B cells with pcDNA-hLXRα. The Hep3B cells were treated with MC (0.1 or 1 μM). The luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ± S.D. from three independent experiments. (B) A luciferase reporter plasmid, $p(LXRE)_2$–TK–Luc, was co-transfected into Hep3B cells with pcDNA-hLXRα and pcDNA-p53 (0.1, 1 or 10 ng). The luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ± S.D. from three independent experiments.

of pcDNA-p53, the luciferase activity seen with $p(LXRE)_2$–TK–Luc was decreased depending on the amount of pcDNA-p53 (Fig. 2B). These results suggest that p53 plays a key role in the suppression of LXR-mediated signal transductions by PAHs.

To further support the results of reporter gene experiments, in which p53 was involved in the transcriptional down-regulation of the LXR-target genes, we examined the effects of Dox on the expression of mRNA for ATP binding cassette A1 (ABCA1), one of the LXR-target genes [20] (Fig. 3). The expression of ABCA1 mRNA was induced by treatment of HepG2 cells with T1317 and was suppressed by co-treatment with MC and Dox (Fig. 3).

It has been reported that p53 interacts with GR to promote the degradation of GR [12]. To examine the possibility of whether p53 promoted the degradation of LXR or its heterodimeric partner, RXR, the expression of LXR and RXR was investigated by western blot analysis (Fig. 4). Nuclear extracts were prepared from HepG2 cells after incubation for 0, 6, 12 or 24 h with MC, and 24 h with Dox. The increase in the amounts of phosphorylated p53 and p21 expression, which is known to be a typical p53-target gene, was seen after incubation for 6, 12 or 24 h with MC, indicating that p53 was activated by MC (Fig. 4). The expression of LXR was not decreased after incubation for 12 or 24 h with MC, while the notable decrease of RXR expression was found after incubation for 12 or 24 h with

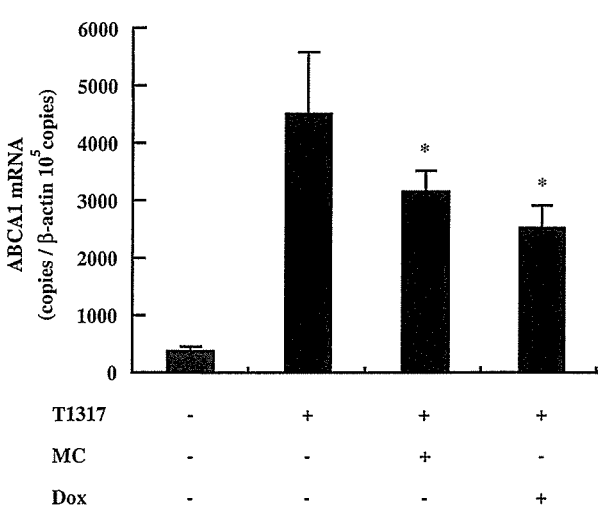

Fig. 3. Suppression of the expression of mRNA for ABCA1 by MC and Dox. The expression of mRNA for ABCA1 was quantified by a real-time RT-PCR. HepG2 cells were incubated with 10 μM T1317 and 10 μM MC or 100 nM Dox. After incubation for 24 h, total RNA was prepared from the cells and subjected to the real-time RT-PCR. Values in the figure represent the average ± S.D. from three independent experiments. *, Statistically different ($P < 0.05$) relative to the cells treated with T1317 alone.

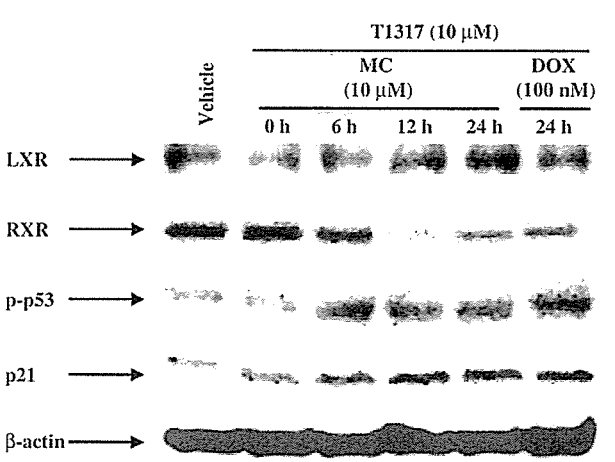

Fig. 4. Effects of MC and Dox on the protein expression of LXR and RXR. HepG2 cells were treated with 10 μM T1317 and 10 μM MC or 100 nM Dox. Nuclear extracts were prepared after incubation for 0, 6, 12 or 24 h with MC, or 24 h with Dox as indicated in the figure. Nuclear extracts (50 μg) prepared from the cells were subjected to SDS–PAGE and analyzed by western blot using antibodies to LXR, RXR, p-p53, p21 and β-actin.

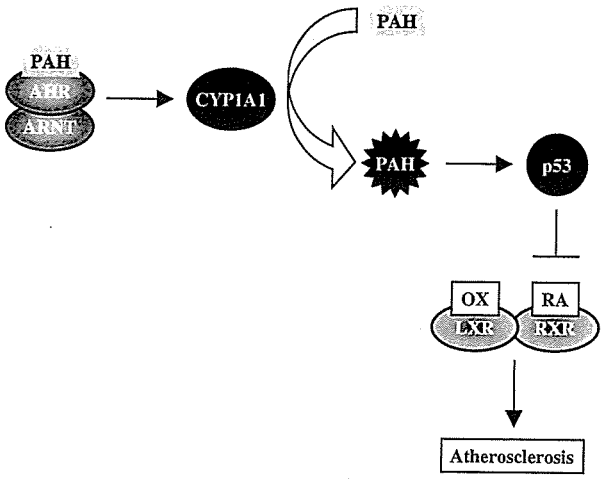

Fig. 5. Proposed mechanism(s) for the PAH-induced suppression of LXR-mediated signal transductions. PAH, polycyclic aromatic hydrocarbon; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; OX, oxysterols; RA, retinoic acid; LXR, liver X receptor; RXR, retinoid X receptor.

MC (Fig. 4). When HepG2 cells were treated with Dox for 24 h, the expression of RXR, but not LXR, was decreased (Fig. 4). These results suggest that the activated p53 suppresses the expression of RXR, which is a heterodimeric partner of LXR.

In the present study, we found that the activated p53 suppressed the expression of RXR to cause the suppression of LXR-mediated signal transductions. Yahagi et al. [21] reported that p53 and its target genes in adipocytes of *ob/ob* mice, which develop obesity, insulin resistance and glucose intolerance owing to an inherited deficiency of the appetite-suppressing hormone, were highly induced. They also found that the activation of p53 was responsible for the suppression of the lipogenic genes which were regulated by LXR. In addition, p53 is reported to negative-regulate nuclear receptors including GR and AR [12]. Together with these results, it may be possible to assume the mechanism of atherosclerosis induced by PAHs as follows (Fig. 5). First, PAHs bind to AHR and induce the expression of CYP1A1. Second, PAHs are metabolized by CYP1A1 to generate a reactive intermediate(s) and the resultant PAH-metabolites cause DNA damage to activate p53. Third, the activated p53 suppresses the protein expression of RXR, which is a heterodimeric partner of LXR. Finally, the expression of the LXR-target genes is suppressed to cause atherosclerosis.

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Dose dependent inhibitory effects of dietary 8-methoxypsoralen on NNK-induced lung tumorigenesis in female A/J mice

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Abstract

We have reported that pretreatment by stomach tube with 8-methoxypsoralen (methoxsalen; 8-MOP), a potent human CYP2A6 inhibitor, strongly suppresses lung tumorigenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice (Cancer Res. 2003). Here, we examined inhibitory effects with adminstration in the diet. When the mice were 7 weeks of age, they received dietary supplementation with 8-MOP at concentrations of 1, 10 or 100 ppm for 3 days prior to a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or an equal volume of saline (vehicle control). The experiment was terminated 16 weeks after the first 8-MOP treatment and lung proliferative lesions were analyzed. The incidences and multiplicities in the 8-MOP 100 ppm-treated group were significantly reduced as compared with values for the NNK alone group ($P<0.001$). Multiplicities of NNK-induced lung proliferative lesions were also reduced in a dose dependent manner (Spearman rank correlation coefficient; $\rho=-0.806$, correction $P<0.0001$). Mouse CYP2A4 and CYP2A5 differ from each other only 11 amino acids, and are closely related to the human CYP2A6. One hour after the last of three daily doses of 8-MOP (0.5, 5 or 50 mg/kg body weight in 0.2 ml corn oil, given by stomach tube) or an equal volume of corn oil (vehicle control), given to the mice at 7 weeks of age, isolation of lung and liver RNAs demonstrated no effects on CYP2A4 and CYP2A5 mRNA levels with 8-MOP. In conclusion, the results of this study showed that clear dose response inhibitory effects of 8-MOP on NNK-induced lung tumorigenesis in female A/J mice fed diets containing 8-MOP, due to inhibition of enzyme activity of CYP2A4 and CYP2A5, rather than their gene expression.

Keywords: A/J mice; Lung carcinogenesis; NNK; CYP2A6; 8-Methoxypsoralen

1. Introduction

Lung cancer is one of the most common causes of cancer mortality in the world, with cigarette smoking

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generally regarded as the most important risk factor. Adenocarcinoma of the lung is second in frequency, after squamous cell lung cancer, and recently there appears to have been a shift to this histologic type, with an absolute increase, especially in females [1–3]. The incidence of squamous cell carcinoma, in contrast has been decreasing. The most likely explanation is the shift from high-tar non-filter to low-tar filter cigarettes. A case control study has shown that the risk of squamous cell carcinoma for smokers is lower with filter than non-filter cigarettes, but this is not the case for adenocarcinomas [4]. The change in cigarette type has led to a reduction in exposure to polycyclic aromatic hydrocarbons (PAHs) and an increase in nitrogen oxides and tobacco-specific *N*-nitrosamines, especially NNK, in the inhaled smoke [5]. Thus, the increment in adenocarcinomas may be *N*-nitrosamine related. The conclusion that NNK conceivably plays an important role in tobacco-related human lung cancer is supported by its strong potential to induce lung tumorigenesis in rodents [6].

Japanese male smokers with a cytochrome P450 2A6 (CYP2A6) gene deletion-type polymorphism have been shown to be at reduced lung cancer risk in a hospital-based case control study [7,8]. CYP2A6 is in fact recognized as being involved in the mutagenic activation of promutagens such as tobacco-specific *N*-nitrosamines [9], and we earlier demonstrated that pretreatment with 8-MOP, a potent human CYP2A6 inhibitor, strongly inhibits lung tumorigenesis by NNK in female A/J mice. In the previous study, we administrated 8-MOP by the stomach tube, but this is impractical for chemoprevention in human lung cancer. Therefore, in the present investigation, we examined effects of dietary 8-MOP on NNK-induced lung tumorigenesis in female A/J mice.

Mouse CYP2A4 and CYP2A5 differ from each other in only 11 amino acids [10], and these enzymes are closely related to the human CYP2A6 [11]. Since mouse CYP2A5 is a specific and efficient catalyst of coumarin 7-hydroxylation, while CYP2A4 is not [12], the former is considered to be the mouse ortholog of human CYP2A6. However, both isoforms catalyzed methylene hydroxylation of NNK [13], considered to be responsible for carcinogen activation in the A/J mouse lung [14]. It has already been shown that 8-MOP inhibits the coumarin 7-hydroxylase activity of human CYP2A6 [15,16] and mouse CYP2A5 [17], presumably in a mechanism-based, non-competitive fashion [16]. To determine whether 8-MOP might also influence the mRNA expression of CYP2As in vivo, an assessment of CYP2A4 and CYP2A5 mRNA induction was included in the present study.

2. Materials and methods

2.1. Chemicals

8-MOP was purchased from Sigma (St Louis, MO) and NNK from Toronto Research Chemicals (Toronto, Canada).

2.2. Animals

Female A/J mice (5 weeks of age), purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in the Narita Laboratory (Kanagawa, Japan) Animal Facility, according to the institutional animal care guidelines. All animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, Labo MR (Narita Laboratory, Kanagawa, Japan), under controlled conditions of humidity (60±10%), lighting (12 h light/dark cycle) and temperature (24±2 °C).

2.3. Experimental design

The design for the lung tumorigenesis experiment is outlined in Fig. 1. When the mice were 7 weeks of age, they were fed diets supplemented with 8-MOP at concentrations of 100, 10 or 1 ppm (Groups 1, 2 and 3) for 3 days. 3 days after the first treatment, each group was given a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or an equal volume of saline (vehicle control). Group 4 was treated with NNK alone and Group 5 with 8-MOP 100 ppm+saline. They were then fed basal diets and maintained without further treatment until the termination after 16 weeks, when all surviving mice were killed under ether anesthesia. At autopsy, their lungs were excised and weighed and then infused with 10% neutral buffered formalin, and carefully inspected grossly. All macroscopically detected lung nodules were counted under