valuable tool in the search for metabolic bioactivators of carcinogens [5]. In order to understand the metabolism of xenobiotics and endogenous compounds, information on the expression of individual members of the CYP superfamily in larynx tissue is required as the individual CYPs may protect the body against orally ingested xenobiotics as well as influence the bioavailability of therapeutic compounds. The aim of this study was to detect the presence of mRNAs for *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* as an indication of the potential expression in human larynx tissue and to compare them with hepatic CYP expression using the reverse transcriptase-polymerase chain reaction (RT-PCR) method.

Materials and Methods

Subjects

A total of ten larynx tissue samples (two malignant, four premalignant and four normal) of about 10–100 mg from individuals between the ages of 33 and 84 were excised with direct laryngoscopy under general anesthesia. A total of six liver biopsy specimens about 10–100 mg were obtained and pooled from the hepatectomic material of patients undergoing liver transplantation at Ege University Hospital, Izmir, Turkey. Samples tissues were frozen immediately in liquid nitrogen. A written informed consent was obtained from each patient and the study was approved by the Ethics Committee of Ege University, Izmir, Turkey.

RT-PCR

Tissues were homogenized in a microtube using a homogenizer (Ika-Werke, Staufen, Germany). Total RNA was isolated using a Highpure RNA tissue kit (Roche, Penzberg, Germany) and cDNA was synthesized using a Takara RNA PCR kit (AMV.Ver.2) (Takara, Otsu-Shiga, JA) according to the manufacturer's specifications. The primers for detecting *CYPs -1A1, -1A2, -2B6, -2C, -2D6, -2E1, -3A3/4, -3A5, -3A7, -4B1* and *GAPDH* were purchased as a human cytochrome P450 competitive RT-PCR kit (Takara, Otsu-Shiga, JA). The human cytochrome P450 RNA competitor with a 3′-poly (A) tail (1.0×10^8 copies/μl) was purchased from Takara Bio Inc (Takara, Otsu-Shiga, JA). It covered the primer sequences necessary to amplify all the object templates in a single molecule with +25% to −25% size differences with target mRNAs in order to have similar amplification efficiency for each fragment. The *CYP2A6* primers (2A6-KK) were designed using the Genbank database (accession number; NM 00762 and NG 000008); 5′-CCCTCA TGAAGATCAGTGAGC-3′ (forward) and 5′-GC GCTCCCCGTTGCTGAATA-3′ (reverse), giving a band of 200 bop upon PCR amplification. Another set of *CYP2A6* primers (2A6-DS) were synthesized according to Vondracek and his co-workers [6]. The PCR conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and polymerization at 72 °C for 1 min.Both cDNA synthesis and PCR amplifications included negative control reactions, which were set up by excluding RNA and DNA templates, respectively. The products were analysed on 2% agarose gels in TBE buffer and gels were photographed under UV light after staining with ethidium bromide (EtdBr) (0.5 μg/ml). All reactions were carried out in nuclease-free microcentrifuge tubes.

Data evaluation

Band intensities were quantified from gel photographs using BioRad Multianalyst (Ver.1.1). The lane with the same band intensity between the products derived from the target mRNA and from the RNA competitor was determined. The expression level in copies per ng total RNA for a given CYP was determined using the following formula [7].

Expression level (copies/ng total RNA)=RNA competitor concentration (copies/μl) × volume of competitor (μl)/the amount of total RNA (ng).

Quantification for *CYP2A6* was carried out by comparing with the *GAPDH* amplification upon densitometric analyses. To control the reproducibility, all amplifications were repeated at least once.

Results

In this study a competitive RT-PCR method was employed to analyse the expression of *CYP* genes

Copyright © 2006 John Wiley & Sons, Ltd.

DOI: 10.1002/bdd

in larynx tissues by including a competitor RNA with a 3′-poly (A) tail for reverse transcription. Primers were used covering *CYPs -1A1, -1A2, -2A6, -2B6, -2C, -2D6, -2E1, -3A3/4, -3A5, -3A7* and *-4B1* (Table 1). A representative agarose gel photograph of the amplified *CYPs* using a RNA competitor in larynx tissue is given in Figure 1A. All the amplified products were detected at their expected molecular weights (Table 1). In Figure 1A, the PCR products of *CYPs -2C, -2E1, -3A5, -2D6, -4B1, -1A1* and *-2B6* are shown as two bands (Figure 1A, lanes 2–8). The lower bands in the lanes for *CYPs -2D6, -4B1, -1A1* and *-2B6* (Figure 1A, lanes 5, 6, 7 and 8, respectively) originated from their corresponding mRNAs while the competitor gave rise to the upper DNA bands in this group of *CYPs* (Table 1). The mRNAs for *CYPs -2C, -2E1* and *-3A5* (Figure 1A, lanes 2–4, respectively) gave products with slower mobility due to the higher molecular weight relative to the competitor (Table 1). There were no detectable reaction products for the *CYPs -1A2, -3A3/4* and *-3A7* either in tumor or healthy larynx tissues (data not shown). On the other hand, the amplification for *CYP2A6* was carried out using two sets of primers, 2A6-KK and 2A6-DS, with expected PCR amplification products of 200 bp and 423 bp, respectively (Figure 1A, lanes 9 and 10, respectively). The two other bands detected above 200 bp were due to non-specific amplification products in the course of polymerization (Figure 1A, lane 9). The competitor was not included in this particular reaction as its sequence did not cover *CYP2A6*-specific primers. The products of *GAPDH* primers, used as a positive control, are shown in Figure 1A, lane 11. No amplification was detected in the negative control reaction which was set up without including the template (Figure 1A, lane 12).

The procedure was repeated using a pooled liver tissue homogenate (Figure 1B). Being the major organ of CYP synthesis, the liver tissue gave rise to the additional *CYP* bands. The products for the *CYPs -2C, -2E1, -3A5, -2D6, -3A3/4, -1A1, -2B6, -1A2* and *-3A7* were amplified in liver in the presence of RNA competitor (Figure 1B, lanes 3–7, 9–12, respectively). Unlike larynx samples, *CYP1A2, CYP3A3/4* and *CYP3A7* mRNAs were detected (see Figure 1B, lanes 11, 7,

Table 1.

Primer	Amplified product (bp)	
	Competitor	mRNA
CYP1A1	507	433
CYP1A2	370	309
CYP2B6	445	376
CYP2C	287	333
CYP2D6	387	339
CYP2E1	300	366
CYP3A3/4	284	324
CYP3A5	384	471
CYP3A7	545	475
CYP4B1	442	398
CYP2A6-KK	NA	200
CYP2A6-DS	NA	423
GAPDH	486	546

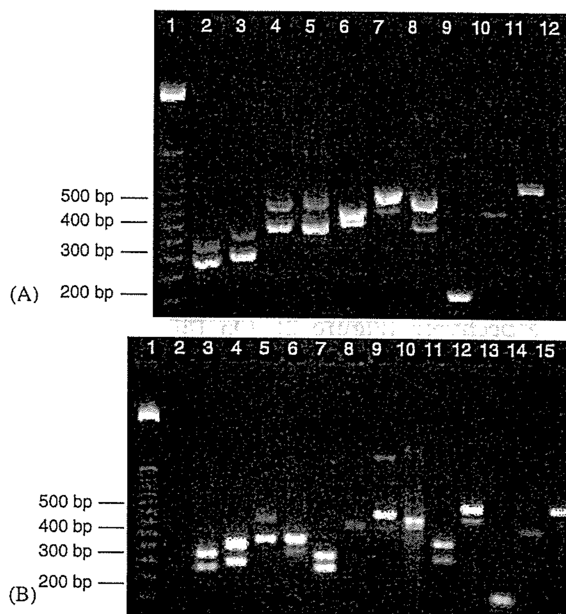

Figure 1. A representative agarose gel photograph of the amplified products in larynx (A) and liver tissues (B). (A) The PCR products (20 μl) were applied on to 2% agarose gel, run in TBE (5 V/cm) and stained with EtdBr (0.5 μg/ml). Lanes (1) 50 bp. DNA standards, PCR products with (2) CYP2C-; (3) CYP2E1-; (4) CYP3A5-; (5) CYP2D6; (6) CYP4B1-; (7) CYP1A1-; (8) CYP2B6-; (9) CYP2A6-KK; (10) CYP2A6-DS; (11) GAPDH-specific primers; (12) negative control reaction. (B) Lanes (1) 50 bp. DNA standards; (2) negative control reaction, PCR products with (3) CYP2C-; (4) CYP2E1-; (5) CYP3A5-; (6) CYP2D6-; (7) CYP3A3/4-; (8) CYP4B1-; (9) CYP1A1-; (10) CYP2B6-; (11) CYP1A2-; (12) CYP3A7-; (13) CYP2A6-KK; (14) CYP2A6-DS; (15) GAPDH-specific primers

Copyright © 2006 John Wiley & Sons, Ltd.

DOI: 10.1002/bdd

12, respectively) while no *CYP4B1* mRNA was found in the liver (Figure 1B, lane 8). The *CYP2A6* amplification using both 2A6-KK and 2A6-DS primer sets gave bands at the expected molecular weights (Figure 1B, lanes 13 and 14, respectively, and Table 1). No product was detected in the negative control reaction (Figure 1B, lane 2) and *GAPDH* amplification was used as a positive control for *CYP2A6* (Figure 1B, lane 15).

The next step was to compare target RNA-based amplifications with competitive RNA-based amplifications by using a competitor of known copy number. The results for the individual *CYPs* for larynx and liver tissues in averaged copy numbers per μl obtained from ten larynx tissue samples are given in Figure 2. As seen in Figure 2, the liver had an average of approximately 2.3 fold mRNA copy number for *CYP2C* (9.0×10^6 copy/μl vs 3.9×10^6 copy/μl, for liver and larynx, respectively) and 3.4 fold mRNA copy number for *CYP2E1* (2.5×10^6 copy/μl vs 8.4×10^6 copy/μl, in larynx and liver, respectively) (Figure 2). The *CYP2D6* and *CYP1A1* mRNA copy numbers were comparable in both tissues. Larynx and liver tissues gave *CYP2D6* mRNAs of 3.4×10^6 copy/μl and 3.6×10^6 copy/μl, respectively, and *CYP1A1* mRNAs of 2.7×10^6 copy/μl and 2.4×10^6 copy/μl, respectively (Figure 2). On the other hand, *CYP3A5* and *CYP2B6* mRNAs were found to be slightly higher in larynx (1.5 and 1.6 fold, respectively) compared with that of the liver (Figure 2). Comparison for *CYP2A6* was carried out from the average band intensities of the larynx samples relative to *GAPDH*. There was a lower average detection of *CYP2A6* mRNA in larynx based on the comparison of the amplified products to *GAPDH* control (5.9 vs 9.8 pmol/μl, in larynx and liver, respectively) (data not shown).

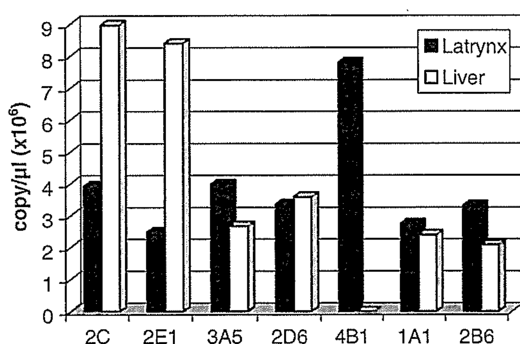

Figure 2. Comparative CYP expression levels in larynx and liver tissues. Band intensities were quantified from gel photographs using BioRad Multianalyst (Ver.1.1) (see Materials and Methods for the details of the experiments)

Discussion

The expression of CYPs can be determined by various methods including immunoblotting, immunohistochemistry and RT-PCR [8]. Given that interpretation of antibody-requiring methods is influenced by the non-specific binding of antibodies, RT-PCR is generally considered as a highly sensitive method for the detection of small amounts of RNA molecules. However, accurate quantification with normal cycling is difficult as the amount of amplified product does not reflect the amount of template because of the plateau phase of PCR, which indicates that a similar amount of amplified products would be obtained, regardless of the initial template levels by performing sufficient cycling of PCR. Competitive RT-PCR, on the other hand, is a sensitive and specific method developed to overcome this difficulty in quantification [7].

The human liver samples used were not obtained from the same individuals that the larynx samples came from. It should be taken into account that the liver samples were used as positive control for the amplifications, as it is the major organ of CYP expression, i.e. reporting a minus result for a particular CYP in larynx would be valuable if the same primer set was capable of amplifying the same form of CYP already known to be expressed in liver. Therefore, it was not intended to compare individual's CYP profile between laryngeal and liver tissues. Liver tissue also served as a reference in the quantification of CYP mRNA levels in larynx. A particular CYP isoform, unless shown to be expressed in the larynx, was not subjected to quantification.

Because of its histological resemblance to nasal mucosa, trachea and lung, the CYP content of larynx is potentially comparable to this group of

Copyright © 2006 John Wiley & Sons, Ltd.

extrahepatic tissues. Many microsomal *CYPs* were reported in human lung, including *CYPs -1A1, -1A2, -2B6, -2A6, -2E1, -3A4, -4B1, -2C* and *-2D6,* while the expression of *CYPs -2A6, -2A13* and *-2B6* were detected in trachea at mRNA level [3,9,10]. The expression of *CYPs -2A6, -2A13, -2B6, -2C, -2J2* and *-3A* were reported in human nasal mucosa [11,12]. Reports on CYP expressions in these reports were only qualitative.

The results demonstrated a consistent estimation of mRNAs for the *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* in larynx. Although the range of individual CYP forms, covered in our study, do not exactly match those searched by the others, detecting a comparable subset of CYP mRNAs in larynx that is reported to be preferentially expressed in the respiratory tract strengthens its physiological resemblances to lung, trachea and nasal mucosa [12]. This study shows that human larynx is most comparable to the lung tissue in terms of CYP transcriptional levels. The *CYPs -1A2, -3A3/4* and *-3A7* were not detected in larynx. The absence of mRNA for *CYP1A2* in human lung was reported by a number of other studies [13–15]. Moreover, the absence of *CYP3A7* in larynx is supported by the CYP subset of respiratory tract organs, reported so far [16,17].

The mRNA copy numbers for *CYP2C* and *CYP2E1* in larynx were below that of their hepatic counterparts because these individual *CYP* forms, like several other individual forms, are predominantly expressed in liver [18]. However, a slightly higher copy number of mRNAs was detected for *CYP3A5* and *CYP2B6* in larynx compared with liver (1.5 fold and 1.6 fold, respectively). On the other hand, CYP3A5 is the main extrahepatic form of the CYP3A subfamily enzymes and not induced in human liver while inducible in other tissues such as human lung [17,19]. A number of other studies reported low to undetectable levels of hepaticCYP3A5 in 80%–90% of Caucasian populations [20,21], confirming our finding of high *CYP3A5* transcription in the larynx. This particular CYP was also reported to be polymorphically expressed in human liver [17]. Therefore the polymorphic nature of CYP3A5 should also be considered when interpreting its expression in the larynx.

Among the individual CYP mRNAs reported here, the human CYP2B6 is known as a minor drug-metabolizing enzyme accounting for only 0.2% to 2% of total hepatic CYP [22,23] while CYP1A1 is known for its role in the oxidative activation of polycyclic aromatic hydrocarbons and nitrosamines [1,24]. Together with CYP3A4, CYP1A1 forms ketoaldehydes. Both CYP1A1 and CYP4B1 forms have been detected mainly in extrahepatic tissues [2,25]. There is no published result on the expression of CYP4B1 protein, but its mRNA was reported in the human lung [19,26].

The CYP2Cs are an important subfamily of P450 enzymes that metabolize approximately 20% of clinically used drugs and it comprises four members in humans (CYP2C8, CYP2C9, CYP2C18, CYP2C19), however, virtually no role in bioactivation of toxic and carcinogenic chemicals is attributed to CYP2C [27]. Both CYP2D6 and CYP2E1 expressions were reported in several other tissues [21,28,29].

The CYP2A6 represents a relatively minor component (~4%) of the human CYPs and but it is a highly important CYP member because CYP2A6 catalyses the metabolic activation of a number of clinically used drugs and several procarcinogens, such as nitrosamines [30]. Besides the liver, CYP2A6 expression was also reported in human nasal and bronchial mucosa, trachea and lung [9–12]. In agreement with its profound localization in the liver, the larynx *CYP2A6* mRNA level was found to be half that of liver *CYP2A6* mRNA. However, the CYP2A6 is also a polymorphic enzyme [30], therefore its expression needs further analyses in extended research.

Studies correlating the quantitative levels of CYP expression with the amount of cigarette smoking were reported. Among the CYPs, the -1A1, -2E1, -3A5 and -2D6 were inducible by cigarette smoking in human lung [12]. Some other studies reported comparative CYP expression in tumor and non-tumor tissues [12]. The mRNA copies for individual CYP forms investigated in our study did not vary significantly due to either smoking habits or health conditions among individuals (data not shown). However, it should be noted that a total of ten tissue samples was not adequate to make a

Copyright © 2006 John Wiley & Sons, Ltd.

comparison for the subjects with different health conditions and habitual characteristics.

In conclusion, this is the first report on the expression profile of *CYPs* in larynx tissue. Our detection method was limited to monitoring *CYP* mRNAs. The current data show that *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* are transcribed in human larynx tissue. Although it is difficult to assess *in vitro* detection of mRNAs to *in vivo* protein expression, the results establish a base for further studies on the role of CYPs in larynx tissue.

Acknowledgements

This work was supported by the Turkish State Planning Organization (DPT) and Ege University Science and Technology Research Center (EBILTEM). The authors acknowledge Professor Dr Ulus Akarca of the Gastroenterology Department of Ege University Hospital, Izmir, TR for providing liver tissues and Dr Metiner Tosun for his useful comments.

References

1. Nelson DR, Koymans L, Kamataki T, *et al.* P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 1996; **6**: 1–42.
2. Gonzales FJ. Human cytochrome P450: problems and prospects. *Trends Pharm Sci* 1992; **13**: 346–352.
3. Koskela S, Hakkola J, Hukkanen J, *et al.* Expression of CYP2A genes in human liver and extrahepatic tissues. *Biochem Pharmacol* 1999; **57**: 1407–1413.
4. Kahane JC. Otolaryngology head and neck surgery. In *Functional Histology of the Larynx and Vocal Cords*, Cummings CW, Frederickson JM, Krause CJ, Harker LA, Schuller DE, Richardson MA (eds). Mosby Inc: St Louis, 1999; 1853–1868.
5. Gajeka M, Rydzanicz M, Rydzanicz Jaskula-Sztul R, Kujawski M, Szyfter W, Szyfter K. CYP1A1, CYP2D6, CYP2E1, NAT2, GSTM1 and GSTT1 polymorphisms or their combinations are associated with the increased risk of the laryngeal squamous cell carcinoma. *Mutat Res* 2005; **574**: 112–123.
6. Vondraček M, Xi Z, Larsson P, *et al.* Cytochrome P450 expression and related metabolism in human buccal mucosa. *Carcinogenesis* 2001; **22**: 481–488.
7. O'Connell J. *RT-PCR Protocols*. Humana Press: Totowa, NJ, USA, 2002; 380.
8. Senft AP, LeVine AM. Basic molecular biology. *Paediatr Respir Rev* 2005; **6**: 199–208.
9. Gervot L, Rochat B, Gautier JC, *et al.* Human CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics* 1999; **9**: 295–306.
10. Su T, Bao Z, Zhang QY, Smith TJ, Hong JY, Ding X. Human cytochrome P450 CYP2A13: predominant expression in the respiratory tract and its high efficiency metabolic activation of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res* 2000; **60**: 5074–5079.
11. Su T, Sheng JJ, Lipinskas TW, Ding X. Expression of CYP2A genes in rodent and human nasal mucosa. *Drug Metab Dispos* 1996; **24**: 884–890.
12. Ding X, Kaminsky S. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol* 2003; **43**: 149–173.
13. Landi MT, Sinha R, Lang NP, Kadlubar FF. Human cytochrome P4501A2. *IARC Sci Publ* 1999; **148**: 179–195.
14. Raunio H, Hakkola J, Hukkanen J, Lassila A, Paivarinta K. Expression of xenobiotic-metabolizing CYPs in human pulmonary tissue. *Exp Toxicol Pathol* 1999; **51**: 412–417.
15. Hukkanen J, Lassila A, Paivarinta K, *et al.* Induction and regulation of xenobiotic-metabolizing cytochrome P450s in the human A549 lung adenocarcinoma cell line. *Am J Respir Cell Mol Biol* 2000; **22**: 360–366.
16. Kitada M, Kamataki T. Cytochrome P450 in human fetal liver: significance and fetal-specific expression. *Drug Metab Rev* 1994; **26**: 305–323.
17. Anttila S, Hukkanen J, Hakkola J, *et al.* Expression and localization of CYP3A4 and CYP3A5 in human lung. *Am J Respir Cell Mol Biol* 1997; **16**: 242–249.
18. Guengerich FP. Enzyme systems that metabolize drugs and other xenobiotics. In *Cytochrome P450*, Ioannides C (ed.). John Wiley and Sons: Chichester, 2002; 33-67.
19. Hukkanen J, Pelkonen O, Raunio H. Expression of xenobiotic-metabolizing enzymes in human pulmonary tissue: possible role in susceptibility for ILD. *Eur Respir J* 2001; **18**: 122–126.
20. Mace K, Bowman ED, Vautravers P, Shields PG, Harris CC, Pfeifer AM. Characterization of xenobiotic-metabolizing enzyme expression in human bronchial mucosa and peripheral lung tissues. *Eur J Cancer* 1998; **34**: 914–920.
21. Lambda JK, Lin YS, Schultz EG, Hummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev* 2002; **54**: 1271–1294.
22. Mimura M, Baba T, Yamazaki H, *et al.* Characterization of cytochrome P-450 2B6 in human liver microsomes. *Drug Metab Dispos* 1993; **21**: 1048–1056.
23. Ekins S, Vandenbranden M, Ring BJ, *et al.* Further characterization of the expression in liver and catalytic activity of CYP2B6. *J Pharmacol Exp Ther* 1998; **286**: 1253–1259.
24. Guo Z, Smith TJ, Thomas PE, Yang CS. Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by inducible and constitutive cytochrome enzymes in rats. *Arch Biochem Biophys* 1992; **298**: 279–286.
25. Willey JC, Coy E, Brolly C, *et al.* Xenobiotic metabolism enzyme gene expression in human bronchial epithelial

Copyright © 2006 John Wiley & Sons, Ltd.

and alveolar macrophage cells. *Am J Respir Cell Mol Biol* 1996; **14**: 262–271.
26. Nhamburo PT, Gonzalez FJ, McBride OW, Gelboin HV, Kimura S. Identification of a new P450 expressed in human lung: complete cDNA sequence, cDNA-directed expression, and chromosome mapping. *Biochemistry* 1989; **28**: 8060–8066.
27. Goldstein JA. Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* 2001; **52**: 349–355.
28. Lo Guidice JM, Marez D, Sabbagh N, *et al.* Evidence for CYP2D6 expression in human lung. *Biochem Biophys Res Commun* 1997; **241**: 79–85.
29. Crawford EL, Weaver DA, DeMuth JP, *et al.* Measurement of cytochrome P450 2A6 and 2E1 gene expression in primary human bronchial epithelial cells. *Carcinogenesis* 1998; **19**: 1867–1871.
30. Topcu Z, Chiba I, Fujieda M, *et al.* CYP2A6 gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka. *Carcinogenesis* 2002; **23**: 595–598.

Copyright © 2006 John Wiley & Sons, Ltd.

DOI: 10.1002/bdd

Eur J Clin Pharmacol (2006) 62:995–1001
DOI 10.1007/s00228-006-0210-3

PHARMACOGENETICS

Impact of *CYP2D6*10* on H1-antihistamine-induced hypersomnia

Junji Saruwatari · Masayuki Matsunaga · Kazuyuki Ikeda · Masashi Nakao · Kentaro Oniki · Takayuki Seo · Shuichi Mihara · Toru Marubayashi · Tetsuya Kamataki · Kazuko Nakagawa

Received: 21 May 2006 / Accepted: 17 August 2006 / Published online: 7 November 2006
© Springer-Verlag 2006

Abstract
Objective This study investigated the relevance of the cytochrome P450 (CYP) 2D6 genotype to the adverse drug reactions (ADRs) of H1-antihistamines and the level of sedation.
Methods Japanese participants in a health screening program were asked to describe any past history of ADRs. Any subjects reporting ADRs induced by H1-antihistamines were then individually interviewed and defined as cases. Excessive daytime sleepiness, which had occurred in the cases as an H1-antihistamine-induced ADR, was assessed by the Epworth sleepiness scale (ESS), and an ESS score ≥12 was considered hypersomnia. *CYP2D6*4*, **5*, **14*, and **10* were genotyped by a panel of polymerase chain reaction techniques.
Results Out of 2,074 participants, 100 cases (M:F=37:63, mean age 51.9±9.2 years) were eligible for analysis. The most common etiological drug was chlorpheniramine, which is the most frequently used H1-antihistamine in Japan. *CYP2D6*10* allele and genotypes were more frequently found in the cases than in the healthy Japanese population in a large study ($P<0.005$ and $P=0.039$, respectively), but no difference was observed in the null alleles and genotypes. The ESS scores in 75 cases (M: F=25:50) who had experienced excessive daytime sleepiness were 9.5±5.5 in men and 12.9±6.1 in women ($P<0.001$, cases vs. 34 subjects without symptoms; $P=0.001$ men vs. women). The occurrence of hypersomnia increased as the number of *CYP2D6* mutant alleles increased ($P=0.045$).
Conclusion The results suggest that the presence of the *CYP2D6*10* allele is a risk factor for development of H1-antihistamine-induced ADRs in Japanese.

Keywords Cytochrome P450 2D6 · Gene polymorphism · H1-antihistamine · Adverse drug reaction · Epworth sleepiness scale

J. Saruwatari · K. Ikeda · M. Nakao · K. Oniki · T. Seo · K. Nakagawa (✉)
Division of Pharmacology and Therapeutics, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Oe-honmachi 5-1, Kumamoto 862-0973, Japan
e-mail: kazukon@gpo.kumamoto-u.ac.jp

M. Matsunaga · T. Kamataki
Laboratory of Drug Metabolism, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

S. Mihara · T. Marubayashi
Japanese Red Cross Kumamoto Health Care Center, Kumamoto, Japan

Present address:
T. Kamataki
Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan

Introduction

Cytochrome P450 (CYP) 2D6 is involved in the metabolism of more than 50 clinically important drugs, including antidepressants, antipsychotics, β-blockers, and H1-antihistamines [1–3]. It is polymorphically expressed, thus causing marked interindividual and interethnic variations [1, 4]. Many case reports as well as retrospective and prospective studies have demonstrated that individuals exhibiting extremes in CYP2D6 activity tend to have significantly more problems when they receive medications metabolized by CYP2D6 [5–9].

The frequency of a poor metabolizer (PM) phenotype has been estimated to be 5–10% in Caucasians and less than 1% in Japanese [1, 2, 4]. In Caucasians, *CYP2D6*3*, **4*, **5*, and **6* alleles account for over 95% of PMs [4]. In Japanese PMs, the allele frequencies of *CYP2D6*3*, **4*, **5*, **6*, and **14* are 0.000, 0.002, 0.051, 0.000, and 0.014, respectively; these variants are too rare to be epidemiologically relevant in the Japanese population [4]. Approximately 10–15% of Caucasians and up to 50% of East Asians appeared to exhibit impaired CYP2D6 activity [i.e., intermediate metabolizer (IM)] [1, 2, 4]. Several common IM alleles (e.g., *CYP2D6*9*, **10*, **17*, and **41*) have been identified [1, 2, 4]. The most common allele in Asians (allele frequency in Chinese of >50%) and thus perhaps the most common *CYP2D6* allele in the world is *CYP2D6*10*, with a frequency of about 40% in Japanese [2, 4]. *CYP2D6*10* has been shown to influence the pharmacokinetics of propranolol, codeine, haloperidol, and tramadol [10–13]. Although previous studies have reported that Japanese IMs were prone to antipsychotic-induced tardive dyskinesia [14, 15], the clinical relevance of the IM phenotype and/or the genotype to the CYP2D6 substrate-induced adverse drug reactions (ADRs) remains to be elucidated.

H1-antihistamines are frequently used as over-the-counter (OTC) or prescribed drugs, and many of them are metabolized either mainly (e.g., promethazine, mequitazine) or partially (e.g., chlorpheniramine) by CYP2D6 [3, 16]. The most common ADR of the first-generation H1-antihistamine is sedation, which is clearly governed by dose-response relationships [3, 16]. Therefore, individuals with a PM and/or IM phenotype of CYP2D6 might be identified from a large population by their past history of H1-antihistamine-induced excessive daytime sleepiness, without the loading of a probe drug.

In the present study, we investigated the impact of *CYP2D6* polymorphisms on the risk of ADRs induced by H1-antihistamines in a Japanese population, while paying special attention to the level of the sedating effect of H1-antihistamines.

Methods

Subjects

Approximately 3,300 participants in a health screening program at the Japanese Red Cross Kumamoto Health Care Center (Kumamoto, Japan) were asked by mail in 2003 about their past history of ADRs. Completed replies were available from 2,074 subjects (1,239 men and 835 women; mean age 52.0±10.2 years) for a response rate of 63.1%. The subjects who reported a past history of ADRs related to H1-antihistamines were then individually interviewed by pharmacists. A semistructured questionnaire was used to record pertinent data, the name of the etiological drugs, the doses, the indications, concomitant diseases, comedications, adverse symptoms, and detailed relationships between the symptoms and the H1-antihistamine use. The records were reevaluated by investigators, consisting of two pharmacists and a physician. The decision regarding inclusion and exclusion was made before genotyping *CYP2D6*. The following criteria were used to define cases that were included: an association of adverse reactions with H1-antihistamine treatment at standard doses, the absence of potent inhibitors of CYP2D6, and the absence of renal and hepatic failure. The subjects were excluded if sedation was a possible reason for receiving promethazine or other antihistamines. The study protocol was approved by the ethical review boards of the Graduate School of Medical and Pharmaceutical Sciences of Kumamoto University, the Japanese Red Cross Kumamoto Health Care Center, and Hokkaido University. In addition, all subjects gave their written informed consent to participate in the study.

Assessment of daytime sleepiness caused by H1-antihistamines

Excessive daytime sleepiness that had occurred in the cases as a symptom of the H1-antihistamine-induced ADR was assessed using the Epworth sleepiness scale (ESS) [17]. The ESS is a self-reported scale to measure sleep propensities in eight different real-life situations (range of an item score, 0–3). The ESS score is the sum of the eight item scores (range, 0–24); higher scores indicate being more sleepy. The ESS has a high sensitivity and high specificity, with a cut-off score ≥11 or 12 for daytime sleepiness [18]. In the present study, those who showed an ESS score ≥12 were diagnosed as having impairment in their daily activities due to their extraordinary sleepiness (hypersomnia). To determine the baseline ESS score, 34 age-matched controls without ADRs completed the ESS.

Determination of *CYP2D6* genotype

Genomic DNA was isolated from peripheral blood samples obtained from the cases with the use of an extraction kit (Wako Pure Chemical Industries, Osaka, Japan). The null allele **5*, in which the *CYP2D6* gene is deleted, was identified by long-range allele-specific polymerase chain reaction (PCR) using the method of Steen et al. [19]. The genotyping of null alleles **4* and **14*, as well as the reduced function allele **10*, was performed by a combined approach of PCR-restriction fragment length polymorphisms (RFLP) and allele-specific PCR. Briefly, the point mutation 188C>T that is associated with these alleles (i.e.,

*4, *10, and *14) was detected by the PCR-RFLP method as described by Wang et al. [20]. Next, these three alleles were discriminated by identifying mutations 1934G>A and 1846G>A using the PCR-RFLP method of Wang et al. [20] and the allele-specific PCR method of Kubota et al. [21], respectively. When these variant alleles were absent, the allele was identified as wild type allele with a normal function (e.g., *1 or *2).

A large study on the genotype and allele frequencies of general Japanese populations was used to calculate the expected genotype frequencies [4].

Data analysis

All data are given as the mean ± standard deviation. The chi-square test was used to compare allele and genotype frequencies, along with other categorical tests. Two-way analyses of variance, the Mann-Whitney U–test, and Fisher's exact test were used to analyze any differences in the ESS scores. These statistical analyses were done using the SPSS software package (version 12.0, SPSS, Chicago, USA). A score of $P<0.05$ was defined as statistically significant.

Table 1 Genotype and allele frequencies in the cases and references

	Cases[a] (n=100) [n (%)]	References[b] [%]	Cases vs. references[c]
CYP2D6 genotype			
**Wild/*wild*	22 (22.0)	29.7	P=0.039
**Wild/*10*	41 (41.0)	42.3	χ^2=10.101
**Wild/*0*	6 (6.0)	7.3	
**10/*10*	26 (26.0)	15.1	
**10/*0*	5 (5.0)	5.2	
**0/*0*	0	0.4	
CYP2D6 allele[d]			
**Wild*	91 (45.5)	54.5	P=0.013
**10*	98 (49.0)	38.8	χ^2=8.765
**0*	11 (5.5)	6.7	
*CYP2D6*10* allele			
Noncarriers	28 (28.0)	37.5	P=0.005
Heterozygous carriers	46 (46.0)	47.5	χ^2=10.399
Homozygous carriers	26 (26.0)	15.1	

[a] Cases: subjects with a past history of H1-antihistamine-induced adverse drug reactions
[b] References: healthy Japanese populations from a large study [4]
[c] The chi-square goodness of fit test and test of homogeneity were used to assess the genotype and allele frequencies of healthy Japanese. [4]
[d] Total allele number =200

Results

One hundred cases (37 men and 63 women; mean age 51.9± 9.2 years) were thus eligible for analysis. All cases were judged to be healthy based on physical examination and blood chemistry and urinalysis data. The etiological drugs were chlorpheniramine (n=20), promethazine (n=16), clemastine (n=5), and mequitazine (n=2). Five cases had had repeated ADRs caused by the different H1-antihistamines, and 62 cases could not specify the drug name(s) of the etiological H1-antihistamine(s). Indications for the H1-antihistamines were upper respiratory tract infection (common cold) in 56 cases, allergic rhinitis in 36 cases, and urticaria in eight cases. These cases had had no concomitant diseases or comedications that might have interacted with the H1-antihistamines. They had had one or more adverse symptoms. The most prevalent was excessive daytime sleepiness (75/100), followed by fatigue (18/100), mouth dryness (15/100), gastrointestinal symptoms (12/100), and others (18/100).

The frequencies of alleles and genotypes in the present study and in a referential study of healthy Japanese populations [4] are shown in Table 1. The observed genotype frequency distribution was consistent with Hardy-Weinberg equilibrium. The genotype and allele frequencies in the cases significantly differed from those in the healthy Japanese populations. The *CYP2D6*10* allele and its genotypes were observed significantly more frequently in these cases than in the healthy Japanese, but those of the null alleles (**0*: i.e., **4*, **5*, and **14*) or genotypes containing null alleles did not differ between the groups.

The distribution of the ESS scores in the 75 cases (25 men and 50 women; mean age 51.3±9.2 years) who had experienced excessive daytime sleepiness and the baseline scores in 34 subjects (21 men and 13 women; mean age 49.4±6.9 years) without ADRs are shown in Fig. 1. The mean ESS scores of the female and male cases were 12.9± 6.1 and 9.5±5.5, respectively, and the female and male baseline scores were 6.5±4.1 and 4.0±2.3, respectively. The mean ESS score of the cases was significantly higher than the baseline and was higher in women than in men. We assessed the effect of the *CYP2D6* genotypes among three groups of the homozygotes for wild type alleles (**wild/*wild*), heterozygotes for a wild type allele and a variant allele (**wild/*0* or **10*), and heterozygotes or homozygotes for variant alleles (**0* or **10/*0* or **10*), because the frequencies of the null alleles were low. The ratio of cases with hypersomnia, as diagnosed by an ESS score ≥12, increased as the number of variant alleles increased (P=0.045; Fig. 2). The mutant allele frequency in cases with hypersomnia (0.65) was also higher than that of controls (0.44; P=0.015). The mean ESS scores of the **wild/*wild*, the **wild/*0* or **10*, and the **0* or **10/*0* or **10* were 8.9±4.6, 11.9±6.2, and 13.5±6.3, respectively (P=0.085, Kruskal-Wallis test), and that of mutant allele(s) carriers was 12.5±6.2 (P=0.045 vs.

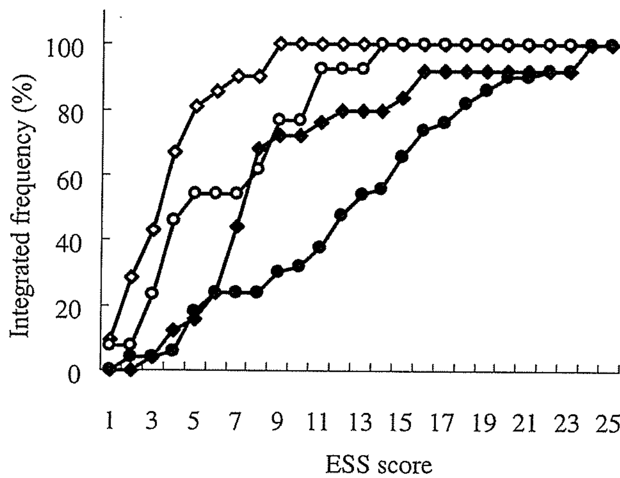

Fig. 1 Distribution of the baseline Epworth sleepiness scale (ESS) scores in men (*open square*) and women (*open circle*), and those of the cases who experienced H1-antihistamine-induced excessive daytime sleepiness in men (*closed square*) and women (*closed circle*). The baseline refers to the subjects 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).

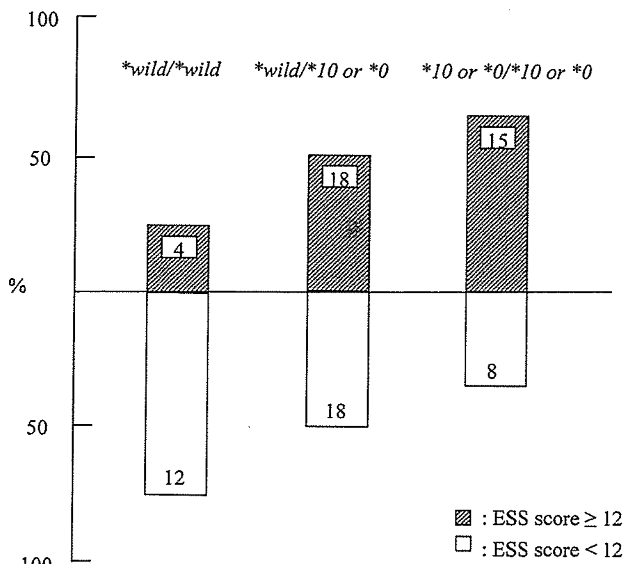

Fig. 2 Genotype distribution in the cases who experienced H1-antagonist-induced excessive daytime sleepiness corresponding to an Epworth sleepiness scale (ESS) score ≥12 (hypersomnia) or <12. A significant genotypic association between ≥12 and <12 of the total ESS score was identified ($P=0.045$, Fisher's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

Acknowledgment We gratefully acknowledge the staff of the Japanese Red Cross Health Care Center for their assistance. The valuable cooperation of the students Mirai Hashimoto, Yoko Higa, Yuko Tomiyasu, Keigo Yurube, and Koji Yoko is also acknowledged. This work was supported by a grant-in-aid (no. 16590438-0) for scientific research from the Japanese Ministry of Education, Science,

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.

References

1. Zanger UM, Raimundo S, Eichelbaum M (2004) Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. Naunyn-Schmiedebergs Arch Pharmacol 369:23–37
2. Ingelman-Sundberg M (2005) Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. Pharmacogenomics J 5:6–13
3. Sharma A, Hamelin BA (2003) Classic histamine H1 receptor antagonists: a critical review of their metabolic and pharmacokinetic fate from a bird's eye view. Curr Drug Metab 4:105–129
4. Shimizu T, Ochiai H, Åsell F, Shimizu H, Saitoh R, Hama Y et al (2003) Bioinformatics research on inter-racial difference in drug metabolism I. Analysis on frequencies of mutant alleles and poor metabolizers on CYP2D6 and CYP2C19. Drug Metab Pharmacokinet 18:48–70
5. Chou WH, Yan FX, de Leon J, Barnhill J, Rogers T, Cronin M et al (2000) Extension of a pilot study: impact from the cytochrome P450 2D6 polymorphism on outcome and costs associated with severe mental illness. J Clin Psychopharmacol 20:246–251
6. Rau T, Wohlleben G, Wuttke H, Thuerauf N, Lunkenheimer J, Lanczik M et al (2004) *CYP2D6* genotype: impact on adverse drug reactions and nonresponse during treatment with antidepressants—a pilot study. Clin Pharmacol Ther 75:386–393
7. Wuttke H, Rau T, Heide R, Bergmann K, Böhm M, Weil J et al (2002) Increased frequency of cytochrome P450 2D6 poor metabolizers among patients with metoprolol-associated adverse drug reactions. Clin Pharmacol Ther 72:429–437
8. Schillevoort I, de Boer A, van der Weide J, Steijns LSW, Roos RAC, Jansen PAF et al (2002) Antipsychotic-induced extrapyramidal syndromes and cytochrome P450 2D6 genotype: a case-control study. Pharmacogenetics 12:235–240
9. Brockmöller J, Kirchheiner J, Schmider J, Walter S, Sachse C Müller-Oerlinghausen B et al (2002) The impact of the *CYP2D6* polymorphism on haloperidol pharmacokinetics and on the outcome of haloperidol treatment. Clin Pharmacol Ther 72:438–452
10. Lai ML, Wang SL, Lai MD, Lin ET, Tse M, Huang JD (1995) Propranolol disposition in Chinese subjects of different *CYP2D6* genotypes. Clin Pharmacol Ther 58:264–268
11. Tseng CY, Wang SL, Lai MD, Lai ML, Huang JD (1996) Formation of morphine from codeine in Chinese subjects of different CYP2D6 genotypes. Clin Pharmacol Ther 60:177–182
12. Suzuki A, Otani K, Mihara K, Yasui N, Kaneko S, Inoue Y et al (1997) Effects of the *CYP2D6* genotype on the steady-state plasma concentrations of haloperidol and reduced haloperidol in Japanese schizophrenic patients. Pharmacogenetics 7:415–418
13. Fliegert F, Kurth B, Göhler K (2005) The effects of tramadol on static and dynamic pupillometry in healthy subjects-the relationship between pharmacodynamics, pharmacokinetics and CYP2D6 metaboliser status. Eur J Clin Pharmacol 61:257–266
14. Ohmori O, Suzuki T, Kojima H, Shinkai T, Terao T, Mita T et al (1998) Tardive dyskinesia and debrisoquine 4-hydroxylase (CYP2D6) genotype in Japanese schizophrenics. Schizophr Res 32:107–113
15. Mihara K, Kondo T, Higuchi H, Takahashi H, Yoshida K, Shimizu T et al (2002) Tardive dystonia and genetic polymorphisms of cytochrome P4502D6 and dopamine D2 and D3 receptors. Am J Med Genet 114:693–695
16. Yasuda SU, Zannikos P, Young AE, Fried KM, Wainer IW, Woosley RL (2002) The roles of CYP2D6 and stereoselectivity in the clinical pharmacokinetics of chlorpheniramine. Br J Clin Pharmacol 53:519–525
17. Johns MW (1991) A new method for measuring daytime sleepiness: the Epworth sleepiness scale. Sleep 14:540–545
18. Johns MW (2000) Sensitivity and specificity of the multiple sleep latency test (MSLT), the maintenance of wakefulness test and the epworth sleepiness scale: failure of the MSLT as a gold standard. J Sleep Res 9:5–11
19. Steen VM, Andreassen OA, Daly AK, Tefre T, Borresen AL, Idle JR et al (1995) Detection of the poor metabolizer-associated CYP2D6 (D) gene deletion allele by long-PCR technology. Pharmacogenetics 5:215–223
20. Wang SL, Huang JD, Lai MD, Liu BH, Lai ML (1993) Molecular basis of genetic variation in debrisoquin hydroxylation in Chinese subjects: polymorphism in RFLP and DNA sequence of *CYP2D6*. Clin Pharmacol Ther 53:410–418
21. Kubota T, Yamadura Y, Ohkawa N, Hara H, Chiba K (2000) Frequencies of *CYP2D6* mutant alleles in a normal Japanese population and metabolic activity of dextromethorphan O-demethylation in different *CYP2D6* genotypes. Br J Clin Pharmacol 50:31–34
22. Zineh I, Beitelshees AL, Gaedigk A, Walker JR, Pauly DF, Eberst K et al (2004) Pharmacokinetics and *CYP2D6* genotypes do not predict metoprolol adverse events or efficacy in hypertension. Clin Pharmacol Ther 76:536–544
23. Fux R, Mörike K, Pröhmer AM, Delabar U, Schwab M, Schaeffeler E et al (2005) Impact of *CYP2D6* genotype on adverse effects during treatment with metoprolol: a prospective clinical study. Clin Pharmacol Ther 78:378–387
24. Kirchheiner J, Brøsen K, Dahl ML, Gram LF, Kasper S, Roots I et al (2001) CYP2D6 and CYP2C19 genotype-based dose recommendations for antidepressants: a first step towards subpopulation-specific dosages. Acta Psychiatr Scand 104:173–192
25. Patsopoulos NA, Ntzani EE, Zintzaras E, Ioannidis JP (2005) *CYP2D6* polymorphisms and the risk of tardive dyskinesia in schizophrenia: a meta-analysis. Pharmacogenet Genomics 15:151–158
26. Milgrom H, Bender B, Wamboldt F (2002) Of injuries and antihistamines and dosing. Ann Allergy Asthma Immunol 89:221–223
27. Lee LS, Nafziger AN, Bertino JS Jr (2005) Evaluation of inhibitory drug interactions during drug development: genetic polymorphisms must be considered. Clin Pharmacol Ther 78:1–6
28. Vuurman EF, Rikken GH, Muntjewerff ND, de Halleux, Ramaekers JG (2004) Effects of desloratadine, diphenhydramine, and placebo on driving performance and psychomotor performance measurements. Eur J Clin Pharmacol 60:307–313
29. Finkle WD, Adams JL, Greenland S, Melmon KL (2002) Increased risk of serious injury following an initial prescription for diphenhydramine. Ann Allergy Asthma Immunol 89:244–250
30. He N, Zhang WQ, Shockley D, Edeki T (2002) Inhibitory effects of H1-antihistamines on CYP2D6-and CYP2C9-mediated drug metabolic reactions in human liver microsomes. Eur J Clin Pharmacol 57:847–851
31. Nakamura K, Yokoi T, Kodama T, Inoue K, Nagashima K, Shimada N et al (1998) Oxidation of histamine H1 antagonist mequitazine is catalyzed by cytochrome P450 2D6 in human liver microsomes. J Pharmacol Exp Ther 284:437–442
32. Hamelin BA, Bouayad A, Drolet B, Gravel A, Turgeon J (1998) In vitro characterization of cytochrome P450 2D6 inhibition by classic histamine H1 receptor antagonists. Drug Metab Dispos 26:536–539
33. Hamelin BA, Bouayad A, Méthot J, Jobin J, Desgagnés P, Poirier P et al (2000) Significant interaction between the nonprescription antihistamine diphenhydramine and the CYP2D6 substrate meto-

prolol in healthy men with high or low CYP2D6 activity. Clin Pharmacol Ther 67:466–477

34. Walle T, Walle UK, Cowart TD, Conradi EC (1989) Pathway-selective sex differences in the metabolic clearance of propranolol in human subjects. Clin Pharmacol Ther 46:257–263
35. Labbe L, Sirois C, Pilote S, Arseneault M, Robitaille NM, Turgeon J et al (2000) Effect of gender, sex hormones, time variables and physiological urinary pH on apparent CYP2D6 activity as assessed by metabolic ratios of marker substrates. Pharmacogenetics 10:425–438
36. Bebia Z, Buch SC, Wilson JW, Frye RF, Romkes M, Cecchetti A et al (2004) Bioequivalence revisited: influence of age and sex on CYP enzymes. Clin Pharmacol Ther 76:618–627
37. Schwartz JB (2003) The influence of sex on pharmacokinetics. Clin Pharmacokinet 42:107–121
38. Montastruc JL, Lapeyre-Mestre M, Bagheri H, Fooladi A (2002) Gender differences in adverse drug reactions: analysis of spontaneous reports to a Regional Pharmacovigilance Centre in France. Fund Clin Pharmacol 16:343–346
39. Anderson GD (2005) Sex and racial differences in pharmacological response: where is the evidence? Pharmacogenetics, pharmacokinetics, and pharmacodynamics. J Womens Health 14:19–29
40. Vermeeren A, Ramaekers JG, O'Hanlon JF (2002) Effects of emedastine and cetirizine, alone and with alcohol, on actual driving of males and females. J Psychopharmacol 16:57–64

Drug Metab. Pharmacokinet. 21 (6): 475–484 (2006).

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

Sompop PEAMKRASATAM[1,2], Kampon SRIWATANAKUL[1], Kazuma KIYOTANI[2], Masaki FUJIEDA[2], Hiroshi YAMAZAKI[2], Tetsuya KAMATAKI[2] and Krongtong YOOVATHAWORN[1,*]

[1]*Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand,*
[2]*Laboratory of Drug Metabolism, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan*

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

This work was taken from a dissertation submitted by Sompop Peamkrasatam in a partial fulfillment of the requirements for the Ph.D. degree from the Mahidol University in 2005 and partially supported by the Medical Scholars Program, Mahidol University. This work was supported in part by a Grant-in-Aid (No. 99-2) from the Organization for Pharmaceutical Safety and Research (OPSR) and Ministry of Education, Science, Sports and Culture of Japan. This work was also supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan and an SRF Grant for Biomedical Research in Japan.

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

Received; March 18, 2006, Accepted; July 7, 2006
**To whom correspondence should be addressed:* Dr. Krongtong YOOVATHAWORN, *Department of Pharmacology, Faculty of Science, Mahidol University, Rama VI Road, Rajthevi, Bangkok 10400, Thailand. Tel. +66-02-2015650, Fax. +66-02-3547157, E-mail: sckyo@mahidol.ac.th*

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*.

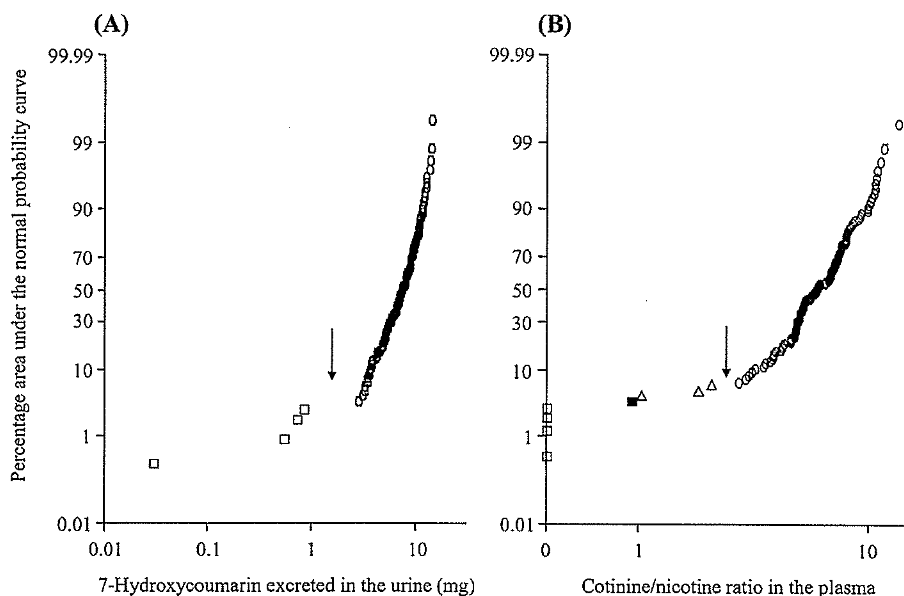

Fig. 1. Probit analysis for CYP2A6 activity toward coumarin and nicotine in the Thai subject population. The transparent square, black square, and transparent triangle represent the subjects genotyped as *CYP2A6*4/*4*, *CYP2A6*4/*7*, and *CYP2A6*7/*7*, respectively. The other genotypes are represented 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.

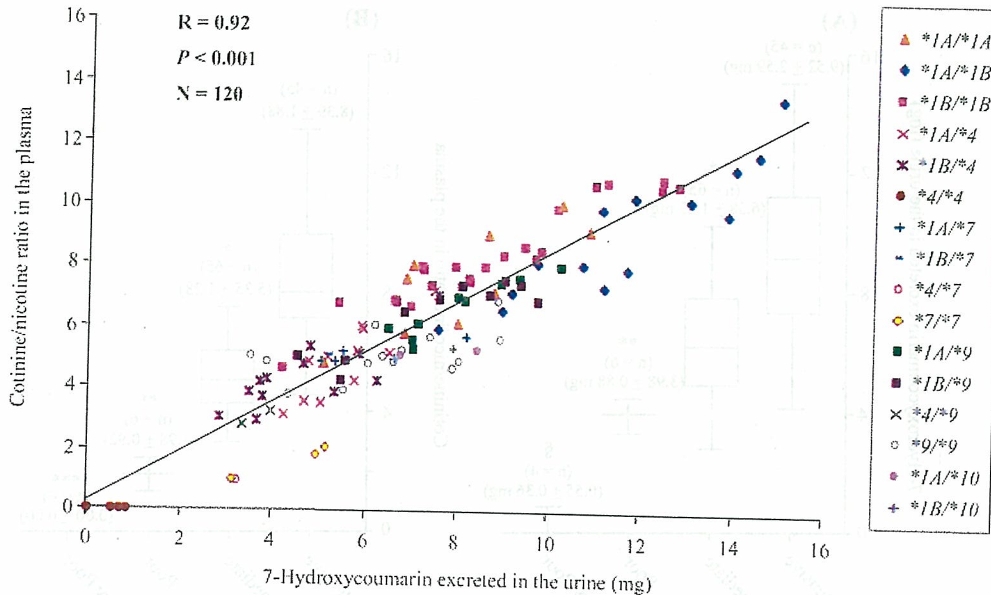

Fig. 2. Correlation between the rate of 7-OHC excretion in the urine and cotinine/nicotine ratio in the plasma in 120 Thai subjects classified according to *CYP2A6* genotypes.

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

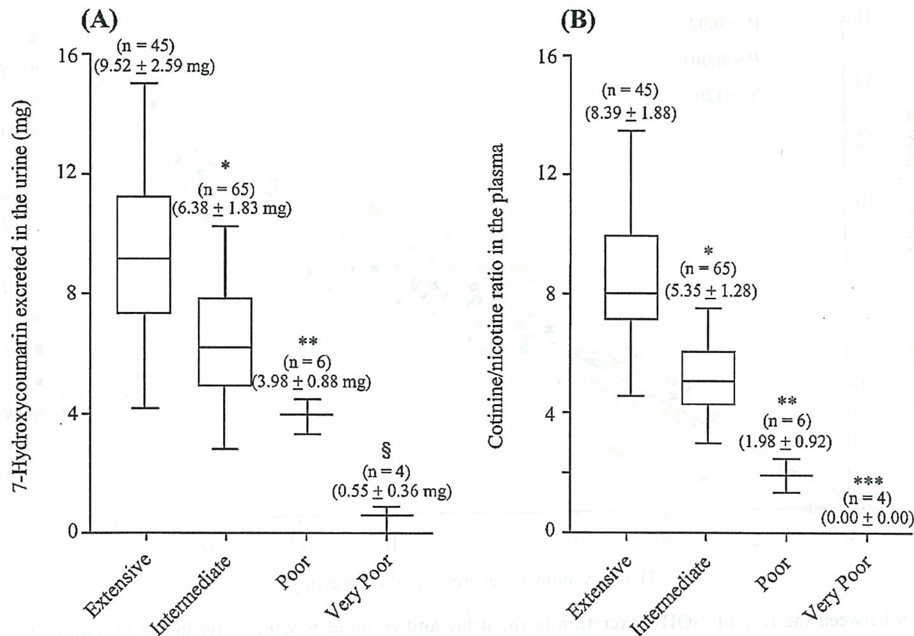

Fig. 3. Comparison of CYP2A6 activity toward coumarin (A) and nicotine (B) among CYP2A6 phenotypes. Data are shown as box plots representing medians (——), minimal and maximal values (-) with 25th and 75th percentiles. The extensive phenotypic group = *CYP2A6*1/*1*. The intermediate phenotypic group = *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-