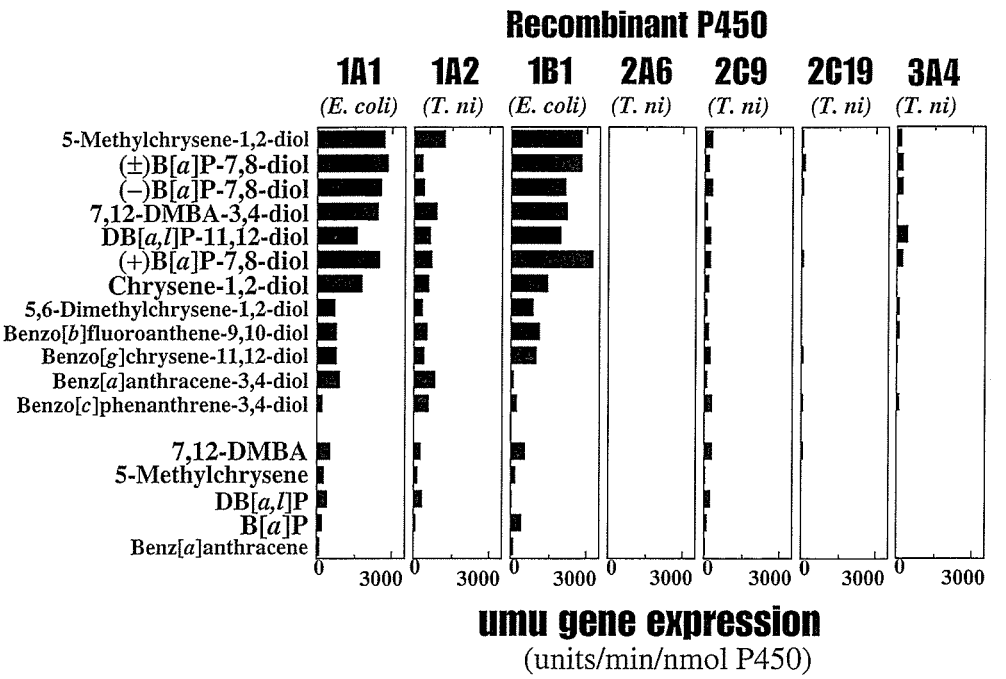

Fig. 3. Activation of PAHs and their dihydrodiol derivatives to DNA-damaging products by recombinant human P450 enzymes in *Salmonella typhimurium* NM2009. Recombinant P450s from *Escherichi coli* (CYP1A1 and 1B1) and from *Trichoplusia ni* cells (CYP1A2, 2A6, 2C9, 2C19, and 3A4) were used. Data were taken from Shimada *et al.*[39] with slight modifications.

thylchrysene.[15] Indeed, the reactive intermediates of these PAH dihydrodiols were activated by human CYPs to DNA-modifying products in *Salmonella typhimurium* NM2009 tester strain with the highest reaction rate for 5-methylchrysene 1,2-diol as a substrate (Fig. 3).[3, 16]

In this review, we wish to focus attention on the diol-epoxide pathway of PAH metabolic activation by CYPs. Readers who are interested in further pathways of PAH metabolism other than those described above may refer to additional review articles.[1, 10]

Roles of CYP1A1 and 1B1 in the metabolic activation of PAHs

Until early 1990, when CYP1B1 was first identified in mouse embryonic fibroblast cells and rat adrenal glands,[17] CYP1A1 had been thought to be uniquely responsible for the metabolic activation of most of the carcinogenic PAHs.[1, 4] In 1994, mouse CYP1B1 protein was purified from C3H/10T1/2 CL8 cells derived from an embryonic fibroblast.[18] The purified enzyme, when reconstituted with NADPH-P450 reductase on phospholipid vesicles, had a high turnover rate for the metabolism of 7,12-DMBA. Subsequently, the cDNA clone for mouse CYP1B1 was obtained and an expression plasmid of the cDNA was engineered for protein production in *Escherichia coli*.[19] The bacterially derived recombinant mouse CYP1B1 enzyme was found to transform 7,12-DMBA to several oxidized products at high rates. Additional spectroscopical studies suggested that purified mouse CYP1B1 was able to interact with several PAHs such as B[*a*]P, benz[*a*]anthracene, 7,12-DMBA, MC, and 1-ethynylpyrene.[19] In rat, CYP1B1 was first identified in adrenal microsomes as a novel ACTH-inducible P450RAP and was found to oxidize several PAHs including 7,12-DMBA.[17] RNA blot analysis using the isolated cDNA as a probe demonstrated that the mRNA for CYP1B1 was expressed ubiquitously in various tissues of rat, including adrenal, ovary, liver, lung, uterus, and kidney.[20]

Human CYP1B1 cDNA was isolated in 1994 and was introduced into a yeast expression vector.[3, 21] Yeast microsomes containing the expressed CYP1B1 protein activated diverse procarcinogens including PAHs, aryl- and heterocyclic amines and nitroarenes to mutagenic products as determined by the *umu* gene expression system using the *S. typhimurium* NM2009 tester strain.[3, 16, 22] Interestingly, a close resemblance was found in the substrate specificities of CYP1A1 and 1B1 towards various procarcinogens and promutagens, particularly when dihydrodiol derivatives of PAHs were used as substrates (Fig. 3).[3, 23] Human CYP1A2 displayed significant activity towards several PAH-diols, though to a lesser degree than CYP1A1 and 1B1. Other human CYPs such as CYP2A6, 2C9, 2C19, and 3A4 were all relatively weak at activating these PAH compounds (Fig. 3).

Is there any difference in the catalytic specificities of CYP1A1 and 1B1 toward the activation of PAHs? Buters *et al.*[24] reported that cultured embryonic fibroblast cells isolated from CYP1B1-null mice, which were incapable of metabolizing 7,12-DMBA, were resistant to the cytotoxicity caused by 7,12-DMBA. The inability of 7,12-DMBA to produce malignant lymphomas and other tumors was also observed in CYP1B1-null mice.[24] In a similar line of investigation, Doehmer and his associates reported that recombinant human CYP1A1 and 1B1 differ in their regio- and stereochemical selectivity for the activation of DB[*a,l*]P and further, that CYP1B1 played more important roles than CYP1A1 in the formation of bay (fjord) region DB[*a,l*]P-11,12-diol-13,14-epoxides.[14] In support of these findings, Buters *et al.* reported that DB[*a,l*]P did not produce any type of malignant or benign tumor in ovaries or lymphoid tissues of CYP1B1-null mice.[25] These results suggest that CYP1B1 may play important roles in the metabolic activation of two prototype carcinogens, DB[*a,l*]P and 7,12-DMBA, to toxic and carcinogenic metabolites.

Carcinogenesis induced by B[a]P in wild-type and AhR knockout mice

AhR is responsible for the inducible expression of several xenobiotic-metabolizing enzymes, including CYP1A1 and CYP1B1, by PAHs and TCDD.[1, 26] Mechanisms of inducible expression of CYP1A1 by these compounds have been extensively studied using TCDD as a prototype inducer.[27] It has been reported that there is an enhancer region with five conserved, putative XREs (xenobiotic response elements) in the upstream

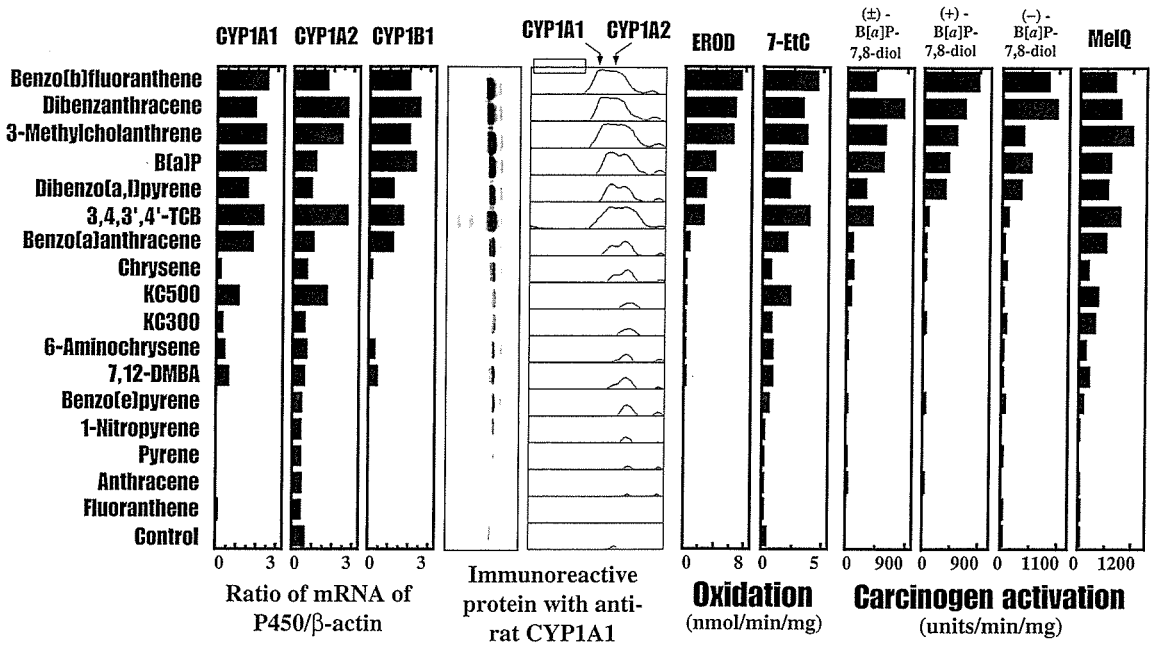

Fig. 4. Induction of mRNA, protein, and catalytic activity of liver CYP1A1, 1A2, and 1B1 by PAHs and 3,4,3′,4′-tetrachlorobiphenyl (3,4,3′,4′-TCB) and PCB mixtures Kanechloro 300 and 500 (KC300 and KC500) in AhR(+/+) mice. Levels of liver P450 mRNAs were determined relative to β-actin mRNA. Immunoblotting analysis to detect P450 proteins in liver microsomes of PAH-treated mice was done with anti-rat CYP1A1 IgG. Liver microsomal xenobiotic oxidation activities were determined with the substrates 7-ethoxyresorufin, 7-ethoxycoumarin, (±)-B[*a*]P-7,8-diol, (+)-B[*a*]P-7,8-diol, (−)-B[*a*]P-7,8-diol, and MeIQ. Data were taken from Shimada *et al.*[40] with slight modifications.

5′-flanking sequence of the *CYP1B1* gene closely resembling those of the *CYP1A1* gene.[28,29] Furthermore, CYP1B1 is induced by TCDD in human carcinoma cell lines such as mammary carcinoma,[30] renal adenocarcinoma (ACHN)[31] and squamous cell carcinoma (SCC12).[32] TCDD and other AhR agonists induce CYP1B1 through an AhR-dependent mechanism similar to that involved in the case of CYP1A1.[33] However, some differences were reported between the modes of induction of CYP1A1 and 1B1 by TCDD.[30] For example, the dose response relationship and the time course of CYP1B1 induction by TCDD were found to differ from those of CYP1A1 and CYP1A2 in rats.[34] Walker *et al.* have also reported that there are sex-related differences in the induction of CYP1B1 and CYP1A1 by TCDD in several organs of rats.[20] It remains unclear whether, in experimental animals and humans, different regulatory mechanisms govern the induction of CYP1A1 and CYP1B1 by TCDD and other chemicals.[35] Using a mouse Hepa-1 variant LA2 cell line defective in expression of Arnt, Eltom *et al.*[33] have shown that expression of CYP1A1 is extremely low and even unresponsive to TCDD, whereas basal CYP1B1 mRNA and protein levels were similar to those seen in wild-type Hepa-1 cells treated with TCDD. It has been reported that in mouse fibroblast C3H/10T1/2CL8 cells, the expression of CYP1B1 relies upon both AhR-dependent transcription activation and protein stabilization.[35] Different responses in the expressions of *CYP1A1* and *CYP1B1* genes to TCDD treatment have been reported in human breast cancer MCF-7 and MDA-MB 231 cell lines[36] and in human hepatoblastoma line HepG2.[31] AhR-dependent induction of *CYP1A1*, *CYP1B1*, and other genes has been reported to be influenced by interaction of AhR with negative and positive regulators in a tissue- and species-dependent manner.[26,37]

Shimizu *et al.*[38] have reported that topical application and subcutaneous injection of B[*a*]P induced skin tumors in AhR(+/+) mice, but not in AhR(−/−) mice. They suggest a possible role for CYP1A1 in B[*a*]P-induced carcinogenesis in AhR(+/+) mice because they observed no expression of CYP1A1 in the skin of the AhR-null mice. It would be interesting to see whether or not CYP1B1 is expressed in the skin of the AhR-null mice. Our recent studies showed that B[*a*]P and other carcinogenic PAHs, such as 7,12-DMBA, DB[*a,l*]P, MC, benzo[*b*]fluoranthene, 5-methylchrysene, and dibenz[*a,h*]anthracene, induce CYP1B1 as well as CYP1A1 in liver and lung of AhR(+/+) mice, but not in those of AhR(−/−) mice (Fig. 4).[39] Further studies on tissue-specific expression and induction of mRNAs of CYP1A1 and 1B1 by these PAHs suggested that CYP1A1 and CYP1B1 are differentially regulated in their expression in extrahepatic organs of mice.[40] For example, CYP1B1 is expressed at significant levels in heart, kidney, intestine, testis, and brain of AhR(−/−) mice as well as AhR(+/+) mice, in contrast with CYP1A1, whose expression is very low or below the detection limit in these organs of AhR(−/−) mice.[40] Further work will be required to determine whether CYP1A1 and 1B1 make different contributions to the occurrence of tumor formation in experimental animals and humans.

As described above, B[*a*]P is activated by CYPs to form bay region epoxides via two activation steps.[2,10] The first step is the formation of B[*a*]P-7,8-oxide by the action of CYP, followed by the formation of B[*a*]P-7,8-diol by microsomal epoxide hydrolase.[10] It has been shown that recombinant human CYP1B1 is more active than CYP1A1 in catalyzing oxidation of B[*a*]P to B[*a*]P-7,8-diol when human or rat liver epoxide hydrolase is present in the reaction mixture.[41] The dihydrodiol metabolites thus produced are activated by CYP1A1 and CYP1B1 to DNA-damaging products at similar rates in the *S. typhimurium* NM2009 tester strain.[3,16] Taken together, these results suggested that CYP1B1 may be more significant for the first step oxidation of B[*a*]P at the 7,8-position and thus play a more important role than CYP1A1 in the tumorigenesis caused by B[*a*]P. In this regard, it is interesting to note the recent work by Uno *et al.*,[42] who showed that CYP1A1-knockout mice produced increased levels of hepatic B[*a*]P-DNA adducts *in vivo*, as compared with those of wild-type mice.

Genetic polymorphisms of CYP1A1 and 1B1 and cancer susceptibilities

Many types of genetic polymorphisms in *CYP1A1* and *1B1* have been identified in humans and some of them are reported to be related to susceptibilities to lung and breast cancer.[43,44] However, it has also been reported that there is no relationship between genetic polymorphisms of *CYP1A1* and *1B1* and cancer susceptibility in humans.[45,46] Race-related differences in the occurrence of genetic polymorphisms in CYP species including CYP1A1 and 1B1 have been reported, and these differences make it difficult to interpret the possible roles of CYP genetic polymorphisms in cancer susceptibility in humans.[46,47] Biochemical studies have been carried out to investigate the catalytic activity that variant forms of CYP1A1 and 1B1 exhibit towards a number of environmental carcinogens.[48,49] Although much has been learned about the roles of variant forms of CYP1A1 and 1B1 in the metabolic activation of PAHs and other carcinogens, further work is required before any final conclusion can be drawn about the link between *CYP1A1* and *1B1* genetic polymorphisms and cancer susceptibility of individuals in the human population.

Chemoprevention

As described above, most procarcinogenic PAHs require metabolic activation by the oxidative action of CYPs to exhibit carcinogenicity, and chemicals which inhibit the CYP activity are expected to work as preventive agents against chemical carcinogenesis. Many chemicals, including[50,51] α-naphthoflavone, synthetic chemopreventive organoselenium compounds such as 1,2-, 1,3-, and 1,4-phenylenebis(methylene)selenocyanate (*o*-, *m*- and *p*-XSC, respectively), vinylic and acetylenic PAHs such as 1-(1-propynyl)pyrene, 2-(1-propynyl)phenanthrene, 1-ethynylpyrene, 2-ethynylpyrene, and 4-(1-propynyl)biphenyl, have been identified as inhibitors of CYP1A1 and 1B1. Recently, Chun *et al.* have reported that the natural products resveratrol and rhapontigenin are potent inhibitors of human CYP1A1.[52] Resverotrol was shown to inhibit human CYP1B1 in a noncompetitive manner, while it inhibits CYP1A1 competitively.[52] Chun *et al.* have also revealed that 2,4,3′,5′-tetramethoxystilbene is a potent and selective inhibitor of CYP1B1.[53]

It has been suggested that some of these inhibitors of CYP1A1 and 1B1 suppress tumorigenesis induced by a variety of chemical carcinogens.[54,55] In a rat mammary tumor model system using 7,12-DMBA as a prototype carcinogen, 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC) has been shown to have chemopreventive activity,[55] while *o*- and *m*-XSC appear to be better inhibitors of 7,12-DMBA-DNA adduct formation in rat mammary gland.[56] These synthetic organoselenium compounds were found to be potent inhibitors of human CYP1A1 and CYP1B1 with selectivity in inhibiting CYP1B1.[50] Likewise, 1-ethynylpyrene inhibits covalent binding of 7,12-DMBA and B[*a*]P to the epidermal DNA[57] and consequently prevents tumor formation caused by 7,12-DMBA and B[*a*]P in mouse skin.[58] 1- and 2-Ethynylpyrenes are relatively selective inhibitors for CYP1B1. As described above, resveratrol, which is found in red grapes, and is known to have cancer-preventive activity, is a potent inhibitor of human CYP1A1 and 1B1.[52,59]

Conclusion

CYP1A1 and 1B1 are the two enzymes involved in the activation of a number of carcinogenic PAHs to reactive electrophiles that initiate cell transformation. These enzymes have similar, but not identical, substrate specificity towards various types of PAHs. CYP1A1 and 1B1 are expressed mainly in extrahepatic organs of animals and are highly induced by carcinogenic PAHs and TCDD through AhR. Genetic polymorphisms in CYP1A1 and 1B1 may determine the different susceptibilities of individual humans to carcinogenesis caused by the action of PAHs. Further study is needed to define the impact of variant forms of CYP1A1 on the incidence of cancers in humans.

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

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We reported previously that subjects homozygous for the *cytochrome P450 2A6* (*CYP2A6*) **4* have a lower risk of lung cancer. The purpose of this study was to clarify whether or not the alterations of smoking behavior and risk for lung cancer could be found in subjects possessing novel *CYP2A6* variants discovered recently. An epidemiological study was performed with 1094 cases and 611 controls in male Japanese smokers. It was found that the amounts of daily cigarette consumption in subjects who harbored *CYP2A6*4/*7*, **4/*10*, **7/*7*, **7/*9* and **4/*4* genotypes were significantly less than those in subjects carrying the **1/*1* genotype ($P < 0.01$). Even after adjustment with cigarette consumption, the adjusted odds ratios (ORs) for lung cancer were significantly lower in subjects who harbored *CYP2A6*1/*4*, **1/*7*, **1/*9*, **1/*10*, **4/*4*, **4/*7*, **4/*9*, **7/*7* and **7/*9* genotypes than those who possessed the **1/*1* genotype ($P < 0.05$). When participants were classified into four groups according to the *CYP2A6* genotypes, group 1 (1/*1*), group 2 (heterozygotes for the **1* and a variant allele), group 3 (heterozygotes and homozygotes for variant alleles except for **4/*4*) and group 4 (**4/*4*), lung cancer risk was found to be less in subjects with the variant of *CYP2A6* alleles {group 2, OR of 0.59 [95% confidence interval (CI), 0.44–0.79]; group 3, OR of 0.52 (95% CI, 0.37–0.72); group 4, OR of 0.30 (95% CI, 0.16–0.57)}. The reduced risk for lung cancer was seen more clearly in heavy smokers than in light smokers. Additional stratification analysis showed that the ORs for squamous cell carcinoma (OR of 0.07) and small cell carcinoma (OR of 0.10) were lower than that of adenocarcinoma (OR of 0.39) in group 4. These results suggest that the CYP2A6 is one of the principal determinants affecting not only smoking behavior but also susceptibility to tobacco-related lung cancer.**

Introduction

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

The existence of a genetic polymorphism of *CYP2A6* was first suggested by evidence that there was a large inter-individual difference in the capacity of coumarin 7-hydroxylation (10,11). In fact, various variants of the *CYP2A6* gene have been found in recent years (1,12–18). Analyzing the genes of subjects who showed a poor metabolizer phenotype toward SM-12502, we found two novel deletion-type variants of the *CYP2A6* gene, *CYP2A6*4B* and *CYP2A6*4C* (19,20); the *CYP2A6*4C* was one of the major variants in Japanese. Following the discovery of *CYP2A6*4C*, we discovered two additional alleles, *CYP2A6*7* and *CYP2A6*11*, showing a decrease in enzymatic activity (21,22). A novel variant, the *CYP2A6*9*, has a −48T to G nucleotide substitution in the TATA box of the 5′-flanking region of the *CYP2A6*, which reduced the expression levels of CYP2A6 mRNA and protein in human livers (23). The *CYP2A6*10* allele possessing two simultaneous amino acid substitutions seen in the *CYP2A6*7* and *CYP2A6*8* also shows decreased enzymatic activity (24).

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

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

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

Materials and methods

Subjects

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

Genotyping

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

Genotyping of the *CYP2A6*1A*, *CYP2A6*1B*, *CYP2A6*4C*, *CYP2A6*7* and *CYP2A6*9* alleles was carried out by the method developed in our laboratory (21,23,36). Genotyping of the *CYP2A6*10* allele was carried out by the method reported by Xu *et al.* (24). The genotyping method, based on the PCR–restriction enzyme fragment length polymorphism (RFLP) for the *CYP2A6*4B* allele, was newly developed in this study. The reaction mixture (25 μl) for PCR contained LA PCR buffer II, 2.5 mM $MgCl_2$, 2.0 mM dNTPs, 2A6JDG sense (5′-GCA CAA TAG GGT GAA TGT AGT TAA CA-3′) and 2A6JDG AS6 (5′-GGA ATA ACT GAA TTT CCT TAA GG-3′) primers (0.2 μM), 1.0 U of LA *Taq* DNA polymerase (Takara, Kyoto, Japan) and ~50 ng of the genomic DNA. PCR was carried out under the following conditions: initial denaturation at 94°C for 5 min followed by 25 cycles of reactions composed of cycle denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. After amplification of a PCR fragment (1080 bp), restriction digestion using two restriction enzymes, *Bsp*HI and *Cla*I, was carried out. The resulting fragments were analyzed on a 1.5% agarose gel. *CYP2A6*1/*4B* yielded 1080, 885, 698, 382 and 195 bp fragments, while *CYP2A6*1/*1* or *CYP2A6*1/*4C* yielded 1080, 698 and 382 bp fragments. *CYP2A6*4B/*4B*, *CYP2A6*4B/*4C* and *CYP2A6*4C/*4C* yielded 885 and 195 bp fragments, 885, 698, 382 and 195 bp fragments, and 698 and 382 bp fragments, respectively.

A new genotyping method based on the PCR–RFLP for the *CYP2A6*11* allele was developed in this study. The reaction mixture (25 μl) for PCR consisted of PCR buffer II, 2.0 mM $MgCl_2$, 2.4 mM dNTPs, 2A6-int4-new sense primer (5′-GCT CCA ATC CAG CCT CGT TTA A-3′) and 2A6-int5R-new AS primer (5′-TCC AGC CCT TGC AGC AAC TG-3′) (0.4 μM), 2.0 U of AmpliTaq DNA polymerase and ~50 ng of the genomic DNA. PCR was carried out under the following conditions: initial denaturation at 94°C for 1.5 min followed by 30 cycles of reactions composed of cycle denaturation at 94°C for 20 s, annealing at 63°C for 30 s and extension at 72°C for 45 s. After amplification of a 344 bp fragment, restriction digestion using the restriction enzyme, *Mbo*II, was carried out. The digested fragments were analyzed on a 1.5% agarose gel. *CYP2A6*11/*11* yielded 300, 228, 72 and 44 bp fragments, and *CYP2A6*11/*11* yielded 300 and 44 bp fragments, while *CYP2A6*11/*1* yielded 228, 72 and 44 bp fragments.

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

Statistical analysis

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

Results

CYP2A6 genotypes and smoking behavior

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

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

Allele	Type of mutation	Detection of mutation (ref.)
*CYP2A6*1A*	Wild	*Acc*II-RFLP, *Eco*81I-RFLP (36)
*CYP2A6*1B*	Gene conversion with *CYP2A7* in the 3′-untranslated region	*Acc*II-RFLP, *Eco*81I-RFLP (36)
*CYP2A6*4B*	Whole deletion	*Bsp*HI and *Cla*I-RFLP
*CYP2A6*4C*	Whole deletion	*Acc*II-RFLP, *Eco*81I-RFLP (36)
*CYP2A6*7*	Point mutation in exon 9 (1412T>C, I471T) and gene conversion with *CYP2A7* in the 3′-untranslated region	Two-step allele-specific PCR (21)
*CYP2A6*9*	TATA box (-48T>G)	Hybridization probe (23)
*CYP2A6*10*	Point mutations in exon 9 (1412T>C, I471T and 1454G>T, R485L) and gene conversion with *CYP2A7* in the 3′-untranslated region	Two step allele-specific PCR (24)
*CYP2A6*11*	Point mutation in exon 5 (670T>C, S224P)	*Mbo*II-RFLP

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

CYP2A6 genotypes and tobacco-related lung cancer risk

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

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

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

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

Discussion

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

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

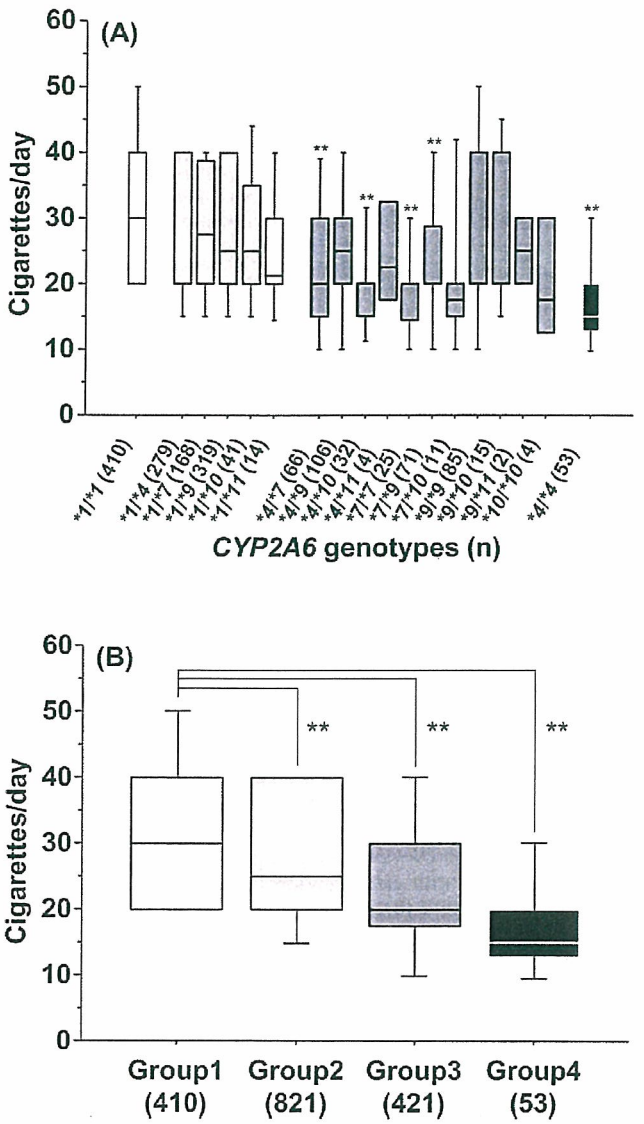

Fig. 1. Relationship between the *CYP2A6* genotypes and the amounts of daily cigarette consumption (**A**), and between CYP2A6 groups classified by *CYP2A6* genotypes and daily cigarette consumption (**B**). Cigarette consumption was investigated with all subjects employed in this study. *CYP2A6*1* consists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

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

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

Significant difference in the distribution of *CYP2A6* genotypes was found between lung cancer cases and control subjects (χ^2 value 42.6, $P = 0.0005$).
[a]*CYP2A6*1* consists of *CYP2A6*1A* and **1B* alleles. *CYP2A6*4* consists of *CYP2A6*4B* and **4C* alleles.
[b]To adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.
[e]Not applicable.

study and the present one may be explained by several possibilities. First, the sample size was small in the study reported by Yoshida *et al.* (40). They analyzed only 92 Japanese individuals, whereas we analyzed 611 controls. Secondly, the ethnic difference of the *CYP2A6*4* allele frequency might exist between Japanese and Chinese populations. In fact, the frequencies of *CYP2A6*4* in controls in their two different studies were almost the same (30,32), although the frequencies in lung cancer cases were largely different.

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

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

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

Significant difference in the distribution of *CYP2A6* genotypic groups was found between lung cancer cases and control subjects (χ^2 value 24.2, $P < 0.0001$).
[a]Groups 1, 2, 3 and 4 were classified according to the *CYP2A6* genotypes. Group 1 includes the subjects carrying *CYP2A6*1/*1* (wild-type). Group 2 contains subjects heterozygous for wild-type allele (*CYP2A6*1/*4*, *CYP2A6*1/*7*, *CYP2A6*1/*9*, *CYP2A6*1/*10*, *CYP2A6*1/*11*). Group 3 consists of subjects carrying *CYP2A6*4/*7*, *CYP2A6*4/*9*, *CYP2A6*4/*10*, *CYP2A6*4/*11*, *CYP2A6*7/*7*, *CYP2A6*7/*9*, *CYP2A6*7/*10*, *CYP2A6*9/*9*, *CYP2A6*9/*10*, *CYP2A6*9/*11* or *CYP2A6*10/*10*. Group 4 contains subjects homozygous for *CYP2A6* deletion allele (*CYP2A6*4/*4*).
[b]To adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.

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

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

Significant difference in the distribution of the six alleles between lung cancer cases and control subjects was found (χ^2 value 35.7, $P < 0.0001$).
[a]*CYP2A6*1* consists of *CYP2A6*1A* and **1B* alleles. *CYP2A6*4* consists of *CYP2A6*4B* and **4C* allele.
[b]Crude OR.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.

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

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

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

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

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

Significant association between the CYP2A6 groups and lung cancer risk with SqCC, SCC and Ad seen as χ^2 value 20.8 ($P = 0.0001$), χ^2 value 15.8 ($P < 0.01$) and χ^2 value 15.6 ($P < 0.01$), respectively.
[a]Groups 1, 2, 3 and 4 were classified according to the *CYP2A6* genotypes. See Table III for details.
[b]To adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.
[e]Not applicable.

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

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

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Establishment of ten strains of genetically engineered *Salmonella typhimurium* TA1538 each co-expressing a form of human cytochrome P450 with NADPH-cytochrome P450 reductase sensitive to various promutagens

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Abstract

We newly developed 10 *Salmonela typhimurium* TA1538 strains each co-expressing a form of human cytochrome P450s (P450 or CYP) together with NADPH-cytochrome P450 reductase (CPR) for highly sensitive detection of mutagenic activation of mycotoxins, polycyclic aromatic hydrocarbons, heterocyclic amines, and aromatic amines at low substrate concentrations. Each form of P450 (CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 or CYP3A5) expressed in the TA1538 cells efficiently catalyzed the oxidation of a representative substrate. Aflatoxin B_1 was mutagenically activated effectively by CYP1A1, CYP1A2, and CYP3A4 and weakly by CYP2A6 and CYP2C8 expressed in *S. typhimurium* TA1538. CYP1A1 and CYP1A2 were responsible for the mutagenic activation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-acetylaminofluorene. Benzo[*a*]pyrene was also activated efficiently by CYP1A1 and weakly by CYP1A2, CYP2C9, CYP2C19, and CYP3A4 expressed in TA1538. These results suggest that the newly developed *S. typhimurium* TA1538 strains are applicable for detecting the activation of promutagens of which mutagenic activation is not or weakly detectable with *N*-nitrosamine-sensitive YG7108 strains expressing human P450s.

Keywords: Metabolic activation; Genetically engineered *S. typhimurium*; Aflatoxin B_1; Benzo[*a*]pyrene; 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 2-Acetylaminofluorene

1. Introduction

Promutagens present in our environment require metabolic activation to exert their genotoxicity via formation of reactive intermediates. For example, mycotoxins, polycyclic aromatic hydrocarbons (PAHs), HCAs, aromatic amines and *N*-nitrosamines are known to be metabolically activated by drug-metabolizing enzymes such as P450 [1,2].

P450 is a family of heme-containing enzymes responsible for the metabolism of exogenous com-

Abbreviations: 2-AAF, 2-acetylaminofluorene; AFB_1, aflatoxin B_1; B[*a*]P, benzo[*a*]pyrene; CPR, NADPH-cytochrome P450 reductase (EC 1.6.2.4, NADPH:ferrihemoprotein reductase); CYP, individual forms of cytochrome P450 (EC 1.14.14.1); HCA, heterocyclic amine; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D(−)-thiogalactopyranoside; P450, general term for cytochrome P450; PAH, polycyclic aromatic hydrocarbon; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; S9, 9000 × *g* supernatant fraction

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pounds such as drugs, environmental pollutants and dietary chemicals, and endogenous compounds such as steroids, fatty acids and prostaglandins [3]. The hepatic P450 system consists of a number of P450 isoforms; each form has some substrate specificity. Some forms of P450 have been proven to activate promutagens to their mutagenic intermediates [4,5]. It is important to clarify the P450 form(s) involved in the activation of promutagens, since the catalytic properties and the population of P450s are sometimes considered as the determinants of the formation of reactive metabolites in the body.

In the classical Ames test with the histidine-requiring *Salmonella typhimurium* strains, S9 prepared from the liver is usually added to the reaction mixture to activate promutagens [6,7]. The information of tests with the rodent S9, however, is not sufficient to predict the capacity of human enzymes including P450 to activate promutagens, since the catalytic properties of P450 even in the same family vary among animal species [8]. Thus, the use of human S9 in the Ames test would be an advantage to investigate the mutagenicity of chemicals in humans. However, the use of human preparations is limited due to several factors such as ethical and medical reasons. In addition, the population of each form of P450 varies according to the medical background of donors. Furthermore, it is difficult to identify a form(s) of P450 involved in the metabolic activation of a certain promutagen, since the S9 contains various forms of P450 for routine testing to identify mutagens.

The use of human P450 isoforms in heterologous expression systems has become popular for the examination of the roles of human P450 in xenobiotic metabolism including metabolic activation of promutagens. Since Barnes et al. [9] succeeded in expressing bovine CYP17 in *Escherichia coli*, bacterial cells have been applied as host cells to express P450. The genetically engineered *S. typhimurium* strains each expressing a form of human P450 were expected to facilitate the examination of the metabolic activation of promutagens by human P450, the identification of a P450 form(s) involved in it and trans-species comparison of metabolism.

In human liver microsomes, CPR plays a role in the electron transfer from NADPH to P450 to support the catalytic activity of P450. The *S. typhimurium* cells may have an endogenous electron transport system to support the activity of P450 similar to *E. coli* cells [9,10]. However, the activity of the enzyme involved in the electron transport in the *S. typhimurium* cells is thought to be low, since the activity of the enzymes, flavodoxin and NADPH-flavodoxin reductase, in the *E. coli* cells is extremely low [10,11]. To support the P450 function, the expression of the CPR in addition to a form of human P450 in *S. typhimurium* cells was assumed to be needed.

Among the *S. typhimurium* strains, TA1535, TA1538 and the derivatives of these strains are frequently used in the Ames mutation test. Point mutations caused by mutagens formed from some alkylating agents such as *N*-nitrosamines and alkyl halides are detectable with the *S. typhimurium* TA1535 [12]. To test the mutagenicity of alkylating agents with a high sensitivity, *S. typhimurium* YG7108, which was a derivative of TA1535 lacking two O^6-methylguaine methyltransferase genes (ada_{ST} and ogt_{ST}), was developed by Yamada et al. [13,14]. Applying the ideas mentioned above, we recently developed strains of YG7108 cells each co-expressing a form of human P450 along with CPR [15,16]. By using the *S. typhimurium* cells, the metabolic activation of *N*-nitrosamines was detectable with a high sensitivity (at the micromolar level) [17]. However, the YG7108 strains expressing P450 were considered not to be suitable to detect the metabolic activation of promutagens such as PAHs, mycotoxins, HCAs and aromatic amines, since these chemicals mainly induced frame shift mutations which were known to be detectable with the TA1538 [6,12,18]. Therefore, in the present study, we developed 10 strains of TA1538 each co-expressing a form of human P450 (CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5) with the CPR to examine the activation of the promutagens.

We report herein that the genetically engineered TA1538 cells expressing human P450 are effective for human P450-mediacted activation of promutagens such as AFB_1, B[*a*]P, PhIP and 2-AAF as compared to the previously developed YG7108 cells expressing P450.

2. Materials and methods

2.1. Materials

2-AAF (CAS No. 53-96-3), δ-aminolevulinic acid, and *p*-aminophenol were obtained from Tokyo Chemical Industry (Tokyo, Japan), AFB_1 (CAS No. 1162-65-8), diclofenac sodium salt, 7-ethoxyresorufin, resorufin, and paclitaxel were from Sigma (St. Louis, MO, USA), and aniline hydrochloride, B[*a*]P (CAS No. 50-32-8), coumarin, cytochrome *c*, IPTG, and PhIP (CAS No. 105650-23-5) were from Wako Pure Chemicals (Osaka, Japan). Bufuralol, 1′-hydroxybufuralol, 4′-hydroxydiclofenac, 1′-hydroxymidazolam, 6α-hydroxypaclitaxel, and midazolam were purchased from Daiichi Pure Chemicals (Tokyo, Japan), 7-ethoxycoumarin and 7-hydroxycoumarin were from Aldrich Chemical (Milwaukee, WI, USA), and glucose 6-phosphate, glucose 6-phosphate dehydrogenase and $NADP^+$ were from Oriental Yeast (Tokyo, Japan). (*S*)-Mephenytoin and 4′-hydroxymephenytoin were the products of Sumitomo Chemicals (Osaka, Japan). All other chemicals and solvents were of the highest grade commercially available. cDNAs for CYP1A2, CYP2D6 and CYP2E1 were provided by Dr. Frank J. Gonzalez (National Cancer Institute, Bethesda, MD, USA). cDNAs for CYP1A1, CYP2A6, CYP2C19, CYP3A5 and CPR were obtained from human liver or lung total RNA by a reverse transcriptase-polymerase chain reaction method in our laboratory [19]. cDNA clones encoding CYP2C8, CYP2C9 and CYP3A4 were isolated from the human adult liver cDNA library prepared in our laboratory [20–22]. Sequences of P450 and the CPR cDNAs were identical to those of the wild-type cDNAs described elsewhere [23–29].

2.2. Expression plasmids

The construction of the expression plasmids carrying human CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 or CYP3A4 cDNA together with the CPR cDNA (pCYP/OR) was reported previously [19]. The expression plasmid carrying CYP3A5 and CPR cDNAs (pCYP3A5/OR) was constructed in our laboratory according to the method of Gillam et al. [30]. The pCYP/OR plasmids were first modified by introduction into *S. typhimurium* LB5000 (R^-M^+) cells [31] to prevent the digestion of the plasmid by restriction enzymes present in the TA1538.

2.3. Introduction of expression plasmid into S. typhimurium TA1538

The *S. typhimurium* LB5000 cells were transfected with the plasmid using an electroporation method. A cell porator system Gene-Pulser (Bio-Rad, Hercules, CA, USA) was used for electroporation. Twenty microliters of the suspension of the *S. typhimurium* LB5000 cells was mixed with 2 μL of the 50 ng/μL pCYP/OR plasmid solution. The mixture was loaded into an electroporation chamber (Bio-Rad), and then the electroporation was carried out at 1.5 kV/mm at 4 °C. The mixture was transferred into an SOC medium (1 mL) and incubated at 37 °C for 70 min with vigorous shaking. After the incubation, a 100 μL portion of the mixture was plated onto a Luria-Bertani plate supplemented with ampicillin (25 μg/mL). The pCYP/OR plasmids modified in the *S. typhimurium* LB5000 cells were extracted from the *S. typhimurium* cells and then introduced into the TA1538 cells by the electroporation. The electric field was 1.0 kV/mm for the transformation of the TA1538 competent cells.

2.4. Expression of P450 and CPR

Expression of each form of P450 and the CPR in the genetically engineered TA1538 cells was achieved as follows. The culture conditions varied depending on the form of P450 to achieve the high levels of expression. Bacterial stock solution (10 μL) was inoculated into 5 mL of a nutrient broth medium supplemented with ampicillin (100 μg/mL). Cultures were carried out overnight with shaking at 37 °C. An aliquot (200 L) was used to inoculate 200 mL of modified Terrific Broth medium [32] containing 0.5 mM δ-aminolevulinic acid in a 500 mL flask.

Optimal culture conditions to express each form of human P450 and the CPR in the genetically engineered TA1538 cells are summarized in Table 1. The bacteria were grown with shaking at 25 °C (to express CYP1A2, CYP2D6 and CYP2E1) or 30 °C (to express CYP1A1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP3A5) for 8 h prior to induction with 1.5 mM IPTG. The recombinant pro-

Table 1
Culture conditions of genetically engineered *S. typhimurium* TA1538 cells to express human P450 with CPR

P450	Incubation time (h)[a]	Temperature (°C)	pH[b]	0.4% (v/v) glycerol
CYP1A1	18	30	7.4	+
CYP1A2	18	25	7.4	+
CYP2A6	18	30	7.4	−
CYP2C8	18	30	7.4	+
CYP2C9	18	30	7.4	+
CYP2C19	24	30	7.4	+
CYP2D6	18	25	6.0	+
CYP2E1	18	25	5.5	−
CYP3A4	18	30	7.4	+
CYP3A5	18	30	7.4	+

[a] The number represents the incubation time of the established *S. typhimurium* TA1538 cells after the addition of IPTG.
[b] The number represents the pH of potassium phosphate buffer added to a modified Terrific Broth medium.

teins were expressed following a further incubation for 18 h or 24 h (for CYP2C19). The temperature of a culture was the same as that before the addition of IPTG. The modified Terrific Broth consisting of a potassium phosphate buffer (pH 5.5 or 6.0) was employed to express CYP2E1 or CYP2D6, respectively. Glycerol (0.4%, v/v) was omitted from the broth for the expression of CYP2A6 and CYP2E1.

The content of P450 holo-protein in the *S. typhimurium* cells was determined by $Fe^{2+}{}^{\bullet}CO$ versus Fe^{2+} difference spectra, according to the method of Omura and Sato [33]. The difference spectra were recorded by Hitachi UV–vis spectrophotometer, model U-3000 (Tokyo, Japan). The activity of the CPR in sonicated bacterial cells was measured with cytochrome *c* as an electron acceptor by measuring the absorbance change at 550 nm at 20 °C according to the method of Phillips and Longdon [34]. The unit of the CPR was defined as the amount of the enzyme that reduced 1 μmol of cytochrome *c*/min.

2.5. Assays for catalytic activity of human P450s

All assays were carried out with whole bacterial cells expressing each form of P450 with the CPR after a disruption by a freeze–thaw. The amount of bacterial preparation added to an incubation mixture will be shown as the amount of P450. A typical incubation mixture consisted of 100 mM sodium potassium phosphate buffer (pH 7.4), 50 μM EDTA, an NADPH-generating system (0.5 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate and 1 unit/mL glucose 6-phosphate dehydrogenase) and 5–50 pmol of P450 in a final volume of 1 mL. All reactions were initiated by the addition of a substrate. Metabolites of each reaction were produced linearly with time.

7-Ethoxycoumarin *O*-deethylase activity [35] and 7-ethoxyresorufin *O*-deethylase activity [36] were determined fluorometrically. The amount of P450 added to the incubation mixture was 50 pmol. Incubations were carried out at 37 °C for 15 min for the assay of 7-ethoxycoumarin *O*-deethylation by CYP1A1 and 10 min for the assay of 7-ethoxyresorufin *O*-deethylation by CYP1A2. To determine kinetic parameters for 7-ethoxycoumarin *O*-deethylation by CYP1A1 and 7-ethoxyresorufin *O*-deethylation by CYP1A2, the concentrations of 7-ethoxycoumarin and 7-ethoxyresorufin ranged from 5.0 to 40 μM and 0.19 to 1.5 μM, respectively.

Coumarin 7-hydroxylase activity was assayed by a fluorometric determination of 7-hydroxycoumarin [37]. An incubation mixture contained 10 pmol of P450. Incubations were performed at 37 °C for 10 min. The substrate concentrations ranged from 0.25 to 2.0 μM for the kinetic analysis of coumarin 7-hydroxylation.

Paclitaxel 6α-hydroxylation was assayed as described by Cresteil et al. [38] with minor modifications. Briefly, the reaction mixture consisted of 150 mM sodium potassium phosphate buffer (pH 7.4), the NADPH-generating system as described above and 30 pmol of P450 in a final volume of 0.5 mL. Reactions were carried out at 37 °C for 30 min. Analysis of the metabolite, 6α-hydroxypaclitaxel, was performed by HPLC using a computerized HPLC system (Hitachi model L-7000 series, Hitachi, Tokyo,

Japan) equipped with an analytical column Capcell Pak C18 (4.6 mm × 250 mm; SG120 Å; 5 μm; Shiseido, Tokyo, Japan). The metabolite was separated with 42% (v/v) acetonitrile as a solvent system at a flow rate of 1.0 mL/min. The metabolite was quantified by comparing the peak area of the metabolite in a chromatogram with that of an internal standard docetaxel. Incubations for kinetic studies for paclitaxel 6α-hydroxylation were carried out with substrate concentrations ranging from 6.3 to 50 μM.

The assay for diclofenac 4′-hydroxylation was performed according to the protocol by Crespi et al. [39] with minor modifications. Briefly, the incubation mixture contained the NADPH-generating system as described above and 5 pmol of P450 in a final volume of 0.25 mL. After incubation at 37 °C for 15 min, 50 μL of acetonitrile was added to terminate the reaction. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant fraction was subjected to HPLC equipped with an analytical column Capcell Pak C18. The mobile phase consisted of 12.5 mM Tris–HCl buffer (pH 7.4), methanol and acetonitrile (80:15:5, v/v) for solvent A and methanol for solvent B. The metabolite, 4′-hydroxydiclofenac, was separated using a solvent system: 100% solvent A to 0% linear gradient, 0–20 min at a flow rate of 1.0 mL/min. The reactions to determine the kinetic parameters for diclofenac 4′-hydroxylation were performed with the substrate concentrations ranging from 1.3 to 10 μM.

The assay of (*S*)-mephenytoin 4′-hydroxylation was performed as described by Yasumori et al. [40] with minor modifications. Briefly, the reaction mixture contained 10 pmol of P450 in a final volume of 0.25 mL. Incubations were carried out at 37 °C for 60 min. Analysis of the metabolite, 4′-hydroxymephenytoin, was performed by HPLC equipped with an analytical column Capcell Pak C18. The mobile phase consisting of acetonitrile and 2.5 mM sodium perchlorate (pH 2.5) (15:85, v/v) was delivered at a flow rate of 1.0 mL/min. The kinetic parameters for (*S*)-mephenytoin 4′-hydroxylation by CYP2C19 were calculated with the substrate concentrations ranging from 6.3 to 50 μM.

The assay of bufuralol 1′-hydroxylation was performed as described by Nakamura et al. [41], except that the incubation mixture contained 10 pmol of P450 in a final volume of 0.15 mL. The substrate concentrations ranging from 6.3 to 50 μM were used to estimate kinetic parameters for bufuralol 1′-hydroxylation.

Aniline *p*-hydroxylation was assayed by a colorimetric method as described by Imai et al. [42], except that the incubation mixture contained 25 pmol of P450, and that incubations were carried out at 37 °C for 15 min. The kinetic analysis for aniline *p*-hydroxylation was performed with the substrate concentrations ranging from 0.50 to 4.0 mM.

The assay of midazolam 1′-hydroxylation was performed by the method described by Li et al. [43] with minor modifications. The incubation mixture contained 10 pmol of P450. After incubation at 37 °C for 5 min, 5 mL of ethyl acetate was added to stop the reaction. One nanomole clonazepam was added to a tube as an internal standard. The mixture was extracted with ethyl acetate and centrifuged at 3000 rpm for 10 min. The organic layer was transferred to another tube and the solvent was evaporated. The residue was dissolved in 10 mM sodium acetate, methanol and acetonitrile (9:1:1, v/v) (200 μL), and was subjected to HPLC equipped with an analytical column TSK-gel ODS-120T (4.6 mm × 150 mm; 4 μm; TOSOH). The mobile phase consisted of the mixture of 10 mM sodium acetate, methanol and acetonitrile (9:1:1, v/v) for solvent A and the mixture of methanol and acetonitrile (2:1, v/v) for solvent B. The metabolite, 1′-hydroxymidazolam, was separated with a 40% solvent A at a flow rate of 0.8 mL/min. The concentration of midazolam ranged from 1.3 to 10 μM to determine kinetic parameters for midazolam 1′-hydroxylation catalyzed by CYP3A4 and CYP3A5.

All assays were performed in duplicate. Kinetic parameters were estimated using the nonlinear regression function in Origin version 6.1J (Origin Lab, Northampton, MA, USA), and the standard Michaelis–Menten velocity equation.

2.6. Mutation assay

The cultures expressing P450 and CPR were diluted by approximately two- to five-times to give 1–2 × 10^9 cells/mL with a nutrient broth. The assay was carried out as described [6,7] with minor modifications. The bacterial cells (0.1 mL) were pre-exposed to a promutagen (0.1 mL) in the presence of 0.5 mL of 0.1 M Na/K-phosphate buffer (pH 7.4) at 37 °C for 20 min (preincubation procedure).

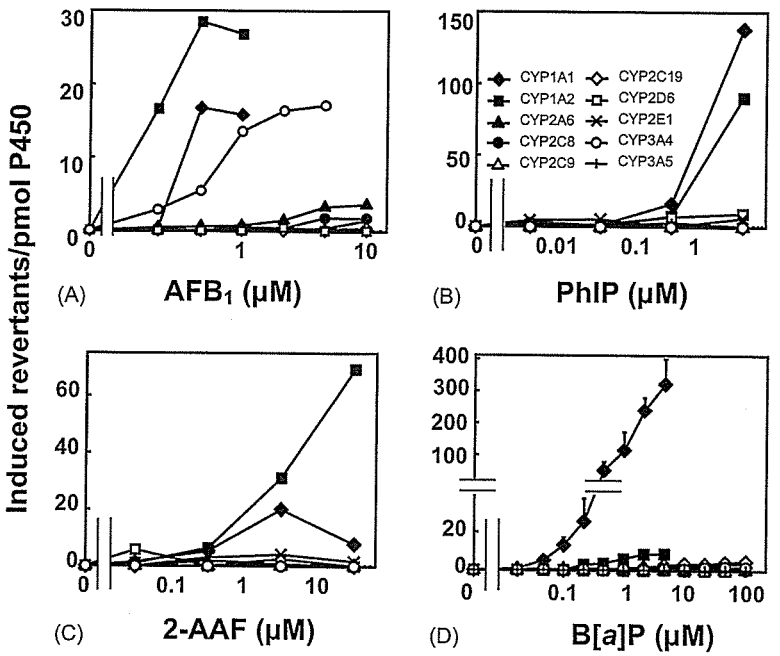

Fig. 1. Mutagenic activation of typical promutagens by human P450s expressed in a series of *S. typhimurium* TA1538 strains. Ten strains of *S. typhimurium* TA1538 each expressing a form of P450 were preincubated with the indicated concentrations of AFB_1 (A), PhIP (B), 2-AAF (C), and B[*a*]P (D) at 37 °C for 20 min. Induced revertants per P450 were calculated as the increased colonies per pmol of P450 content expressed in the tester strains, using the numbers of revertants per plate. Each point and bar indicates the mean of duplicate or triplicate determinations and S.D., respectively.

An NADPH-generating system was not added to a reaction mixture, since the mutagenic activation of promutagens was not affected by the addition of NADPH, probably because NADPH present in the viable bacterial cells was utilized as an electron donor. The plates were incubated at 37 °C for 2 days. Assays were carried out at least twice at each concentration of a promutagen. When the variability of the values of duplicate determinations was within ±20%, the results were adopted. The results were judged as positive when the number of colonies increased linearly with the concentration of promutagen and reached a level twice as high as that obtained with a vehicle alone. Whenever we performed the mutation assay using the genetically engineered *S. typhimurium* cells, the spontaneous revertant number per plate ranged from 20 to 40. Induced revertants per P450 were calculated as the increased colonies per pmol of P450 content expressed in the tester strains (Fig. 1).

3. Results

3.1. Expression of P450 and CPR in S. typhimurium TA1538

To develop new *S. typhimurim* strains sensitive to frameshift promutagens the TA1538 cells were transfected with a plasmid carrying cDNAs for a form of P450 and CPR. The Fe^{2+}•CO versus Fe^{2+} difference spectra of holo-P450 protein expressed in the TA1538 cells were observed (not shown). The peak wavelength in the difference spectra characteristics of each form of P450 was almost the same as that seen with purified preparations of P450 [44–47]. The expression levels of P450 and CPR in the genetically engineered cells are summarized in Table 2. The expression levels of holo-P450 protein ranged from 32 nmol/L culture (0.74×10^4 molecules/cell) for CYP2E1 to 300 nmol/L culture (13×10^4 molecules/cell) for CYP1A2. The expression level of CPR varied depend-

Table 2
Expression levels of human P450 and the CPR in *S. typhimurium* TA1538 cells

	P450 (nmol/L culture)	P450 ($\times 10^4$ molecules/cell)	CPR (units/L culture)	CPR ($\times 10^4$ molecules/cell)[a]	CPR/P450 ratio[b]
CYP1A1	54 ± 31	1.1 ± 0.6	290 ± 100	2.0 ± 0.7	1.8
CYP1A2	300 ± 70	13 ± 3.1	410 ± 44	6.1 ± 0.7	0.46
CYP2A6	200 ± 40	13 ± 2	630 ± 260	14 ± 5	1.0
CYP2C8	84 ± 39	3.1 ± 1.5	570 ± 150	7.1 ± 1.9	1.1
CYP2C9	170 ± 49	6.7 ± 1.9	590 ± 59	7.7 ± 0.8	2.3
CYP2C19	84 ± 40	3.1 ± 1.5	570 ± 320	6.9 ± 3.9	1.2
CYP2D6	120 ± 89	2.9 ± 2.2	430 ± 340	3.5 ± 2.7	2.3
CYP2E1	32 ± 5	0.74 ± 0.12	410 ± 260	3.2 ± 2.0	1.2
CYP3A4	69 ± 36	1.9 ± 1.0	310 ± 200	2.9 ± 1.7	4.3
CYP3A5	72 ± 61	2.9 ± 2.4	290 ± 27	3.9 ± 0.4	1.5

Data are shown as mean ± S.D. ($n = 3$).
[a] The numbers were calculated by assuming that 1 nmol of the CPR corresponds to three units [49].
[b] The ratio of the CPR to P450 was calculated on the molecular basis.

ing on the form of P450 co-expressed, and ranged from 290 units/L culture (2.0×10^4 molecules/cell) for CYP1A1 to 630 units/L culture (14×10^4 molecules/cell) for CYP2A6. The molar ratio of CPR to P450 varied from 0.46 for CYP1A2 to 4.3 for CYP2E1 (Table 2).

3.2. Catalytic property of human P450 expressed in S. typhimurium TA1538

The catalytic activities of human P450 expressed in the genetically engineered TA1538 were examined by using whole cells after a disruption by a freeze–thaw. Each form of P450 expressed in the TA1538 cells catalyzed the oxidation of a representative substrate at an efficient rate. The kinetic parameters for the catalytic reactions are summarized in Table 3. For example, CYP1A1 was found to catalyze 7-ethoxycoumarin *O*-demethylation with K_m and V_{max} values of 8.9 μM and 9.0 nmol/min/nmol CYP1A1, respectively (Table 3).

The catalytic property of P450 was examined using independent preparations of *S. typhimurium* cells obtained by different cultures. We obtained reproducible data about the catalytic activity of P450. For example, 7-ethoxycoumarin *O*-deethylase activities of human CYP1A1 expressed in the developed *S. typhimurium* cells obtained by two distinct cultures were comparable ($K_m = 8.9$ and 6.9 μM, $V_{max} = 9.0$ and 7.3 nmol/min/nmol CYP1A1).

3.3. Mutation assay with developed S. typhimurium TA1538 cells

It has been reported that AFB_1 is metabolically activated by human P450 to exert their mutagenicity

Table 3
Kinetic parameters for the reaction catalyzed by each form of human P450 expressed in *S. typhimurium* TA1538 cells

P450	Reaction	K_m (μM)	V_{max} (nmol/min/nmol P450)
CYP1A1	7-Ethoxycoumarin *O*-deethylation	8.9 ± 0.7	9.0 ± 0.2
CYP1A2	7-Ethoxyresorufin *O*-deethylation	4.3 ± 1.3	0.15 ± 0.03
CYP2A6	Coumarin 7-hydroxylation	0.63 ± 0.13	8.1 ± 0.7
CYP2C8	Paclitaxel 6α-hydroxylation	40 ± 6	0.98 ± 0.09
CYP2C9	Diclofenac 4′-hydroxylation	5.8 ± 1.7	49 ± 7
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylation	41 ± 17	6.1 ± 1.4
CYP2D6	Bufuralol 1′-hydroxylation	49 ± 6	6.1 ± 0.5
CYP2E1	Aniline *p*-hydroxylation	620 ± 170	11 ± 1
CYP3A4	Midazolam 1′-hydroxylation	8.0 ± 1.8	5.4 ± 1.7
CYP3A5	Midazolam 1′-hydroxylation	0.98 ± 0.23	6.1 ± 0.3

Kinetic parameters were calculated from the fitted curve by nonlinear regression (mean ± S.E.).

[48]. AFB_1 was metabolically activated by human CYP1A1, CYP1A2, CYP3A4, CYP2A6 and CYP2C8 expressed in the TA1538 cells (Fig. 1A). Among these P450s, CYP1A1, CYP1A2 and CYP3A4 were found as the major forms involved in the activation of AFB_1; the activation was seen at the nM level of the mycotoxin (Fig. 1A). These results indicate that the developed TA1538 cells expressing P450 are applicable to investigate the metabolic activation of AFB_1.The mutagenic activation of PhIP, 2-AAF, and B[*a*]P was also investigated with the genetically engineered TA1538 cells expressing P450 (Fig. 1). PhIP is known as one of the representative HCAs found in cooked foods, and 2-AAF is recognized as one of aromatic amine promutagens [50,51]. CYP1A1 and CYP1A2 showed the capacity to activate PhIP and 2-AAF (Fig. 1B and C). B[*a*]P is known to be one of the typical PAHs [52]. CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP3A4 were involved in the mutagenic activation of B[*a*]P (Fig. 1D). Among them, CYP1A1 and CYP1A2 activated B[*a*]P at the concentrations lower than 1 μM.

4. Discussion

Many promutagens such as mycotoxins, PAHs, HCAs and aromatic amines are considered as risk factors for cancer in humans. For example, exposure of humans to AFB_1 is regarded as one of the major factors for hepatocellular carcinoma [53]. Therefore, the metabolic activation of the promutagens by human P450 should be examined to predict the cancer risk of the chemicals in humans. Accordingly, we examined the activation of AFB_1 by human P450 using 10 strains of the *S. typhimurium* cells, each co-expressing a form of human P450 and the CPR.

The level of CYP1A2 expressed in 1 L of a culture of the TA1538 cells expressing P450 corresponded to that expressed in 160 g of human liver, assuming that 1.91 nmol of CYP1A2 was contained in the 1 g of human adult liver according to the report by Shimada et al. [54]. The levels of P450 in the TA1538 cells expressing P450 were comparable to those in the YG7108 cells expressing P450 developed in our laboratory [15], suggesting that the difference of the host *S. typhimurium* strains might not affect the expression level of P450.

Each form of P450 expressed in TA1538 cells catalyzed the oxidation of a representative substrate (Table 3). The kinetic parameters were comparable to or somewhat high to the reported activities of human P450 expressed in the other expression systems. The K_m values in the developed systems were also comparable to those reported in human liver microsomes (Table 4), although activities in the literatures were based on mg microsomal protein, not on each P450 form level. The 7-ethoxycoumarin *O*-deethylase activity of CYP1A1 expressed in the developed TA1538 expressing P450 was similar to that of human CYP1A1 expressed in insect cells (K_m = 10.0 μM, V_{max} = 33.0 nmol/min/nmol CYP1A1) [55]. On the other hand, drug oxidation activities of CYP1A2, CYP2A6, CYP2C8 and CYP2C9 were somewhat higher than those of human CYP1A2 expressed in yeast microsomes (K_m = 1.05 μM, V_{max} = 0.90 nmol/min/nmol CYP1A2) [56], CYP2A6 present in human lymphoblastoid cells (K_m =0.40 μM, V_{max} =0.56 nmol/min/nmol CYP2A6) [57], CYP2C8 expressed in yeast micro-

Table 4
Typical kinetic parameters of drug oxidation activities in human liver microsomes reported in the literatures

Drug oxidation reaction	K_m (μM)	V_{max} (nmol/min/mg protein)	References
7-Ethoxycoumarin *O*-deethylation	11	0.1	[68]
7-Ethoxyresorufin *O*-deethylation	0.5	0.6	[69]
Coumarin 7-hydroxylation	0.6	3.0	[70]
Paclitaxel 6α-hydroxylation	14	0.9	[71]
Diclofenac 4′-hydroxylation	5	6.0	[72]
(*S*)-Mephenytoin 4′-hydroxylation	50	3.0	[69]
Bufuralol 1′-hydroxylation	50	0.4	[73]
Aniline *p*-hydroxylation	1000	15	[74]
Midazolam 1′-hydroxylation	4.7	1.2	[75]