Review

Genetic Polymorphism of Drug Metabolizing Enzymes: New Mutations in *CYP2D6* and *CYP2A6* Genes in Japanese

Tsuyoshi Yokoi[1] and Tetsuya Kamataki[2,3]

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Genetic polymorphism of drug metabolizing enzymes, particularly cytochrome P450 (CYP), is an important cause of adverse drug reactions. Multiple gene mutations in CYP have been shown to be phenotype. The occurrence of genetic polymorphism has been seen in genes for *CYP1A1, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1* and *CYP3A5*. This review discusses the molecular mechanism of two genetic polymorphisms, debrisoquine/sparteine (CYP2D6) coumarin (CYP2A6) polymorphisms. In addition, elucidation of gene mutations of *CYP2D6* and *CYP2A6* in Japanese will be discussed.

KEY WORDS: genetic polymorphism; phenotype; drug metabolism; genotype; cytochrome P450; pharmacogenetics; clinical pharmacology.

INTRODUCTION

Interindividual variation of drug effects often occurs with a variety of drugs. The variation can be caused by many factors. Among the factors, the rate of drug metabolism has been regarded as the most important one. Although some of the variation in the enzyme activity will be attributable to the inhibition or induction of enzyme activity by co-administered drugs or to the nonphysiological conditions of patients, a genetic defect of enzymes involved in drug metabolism is believed to be one of the important causal factors.

CYP is a heme-containing enzyme which catalyzes the metabolism of a wide variety of exogenous compounds such as drugs, environmental pollutants and dietary chemicals (1). To date, the CYP superfamily has been known to consist of 74 families (CYP1 to CYP118). Among the forms of CYPs in humans, at least 18 different xenobiotic-metabolizing CYPs encoded by separate genes have been identified. Three families, CYP1, CYP2 and CYP3, are mainly involved in the metabolism of foreign compounds in humans. The CYP2 family consists of six distinct subfamilies. With respect to CYP2A, CYP2C and CYP2D are of pharmacogenetic interest and of clinical relevance. Recently, CYP2C19 which is responsible for the 4'-hydroxylation of *S*-mephenytoin was extensively studied (2–5). The genetic defects (*CYP2C19m1* and *CYP2C19m2*) have been reported which account for 100% of Oriental PM alleles (5).

This review concerns the human CYP2D subfamily, since CYP2D is representative and clinically important forms showing genetic polymorphism. More than 95% of PMs in Caucasians can be predicted by gene diagnosis with known genotypes of *CYP2D6*, while only about 30% of Japanese PMs can be accounted for by the mutations of the *CYP2D6* reported so far in Caucasians. Thus, new mutations in the *CYP2D6* gene were assumed to be present in Japanese. Recently, particular interest has been focused on genetic polymorphism of CYP2A6 in relation to the coumarin 7-hydroxylation and the metabolic activation of mutagens and carcinogens.

In this paper, new variant alleles in *CYP2D6* and *CYP2A6* genes in Japanese will be described. Ethnic differences in the genetic polymorphism of CYPs will also be discussed.

CYP2D6 POLYMORPHISM

Background and Molecular Mechanism

The DB/SP-type polymorphism is generally characterized by two phenotypes termed as EM and PM. Individuals with normal DB 4'-hydroxylase activity are classified as EM (6,7). The oxidation polymorphism is reportedly due to mutant or null alleles of the *CYP2D6* gene. The PM phenotype is incapable of metabolizing over 60 therapeutically important drugs including antidepressants, antiarrythmics, antihypertensive drugs and histamine H_1 antagonists (8,9). Some of these drugs also

[1] Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920 Japan.
[2] Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060 Japan.
[3] To whom all correspondence should be addressed. (e-mail: kamataki@pharm.hokudai.ac.jp)

ABBREVIATIONS: CYP, cytochrome P450; DB, debrisoquine; EM, extensive metabolizer; FMO, flavin-containing monooxygenase; HPLC, high-performance liquid chromatography; IM, intermediate metabolizer; *i.p.*, intraperitoneally; *i.v.*, intravenously; *K*m, Michaelis constant; LA-PCR, long and accurate polymerase chain reaction; NDEA, *N*-nitrodiethylamine; NDMA, *N*-nitrosodimethylamine; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN, *N*-nitrosonornicotine; PAF, platelet-activating factor; PCR, polymerase chain reaction; *p.o.*, perorally; PM, poor metabolizer; RFLP, restriction fragment length polymorphism; RT-PCR, reverse-transcriptase-polymerase chain reaction; SM-12502, (+)-*cis*-3,5-dimethyl-2-(3-pyridyl) thiazolidin-4-one hydrochloride; SP, sparteine; UM, ultrarapid metabolizer.

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undergo oxidative metabolism at a minor rate by other form(s) of CYP.

According to pedigree phenotyping studies of the DB/SP-type polymorphism, the PM phenotype is inherited as an autosomal homozygous recessive trait, and is caused by mutations in the *CYP2D6* gene, whereas the EM phenotype is homozygous or has a heterozygous dominant trait (10,11).

This polymorphism is prevalent in Caucasians in which 5–10% of the population are PMs, while less than 1% of Oriental populations are believed to be PMs. The frequency of PMs in the DB/SP phenotypes were reported to be 0% (0/100), 2.4% (2/82), 0.5% (1/200) and 0.7% (2/295) using sparteine, debrisoquine and metoprolol as probe drugs in Japanese, respectively (12–15). Some of the major defective *CYP2D6* alleles reported in Caucasians have not been observed in Japanese, suggesting a ethnic difference in genetic polymorphism.

Recently, two additional categories of phenotypes designated as IM and UM, were found by analysis of genes from patients (16). The IM (carrying *CYP2D6Ch1* or *CYP2D6Ch2* gene) and the UM (carrying repeats of *CYP2D6L* gene) were not clearly distinguished from EMs (carrying a heterozygous or a homozygous *CYP2D6* wild-type allele) in *in vivo* phenotyping analysis. Among EMs, the metabolic ratio of DB ranged from approximately 0.1 to 10 (17). The large interindividual variation in CYP2D6 enzyme activity in EMs may also be explained by genetic factors (18), but not by non-genetic factors such as smoking, age, sex and environmental chemicals.

Human CYP2D6 cDNA was cloned and its gene was characterized by Gonzalez and his collaborators (19). The *CYP2D6* gene has been mapped to chromosome 22 with two pseudogenes, *CYP2D8P* and *CYP2D7P*, located upstream of the *CYP2D6* gene (20). The *CYP2D6* gene mutation has been analyzed extensively and the mutations in the gene spread largely as summarized by Daly *et al.* (21). Many types of mutations associated with impaired metabolism have also been clarified recently. Among mutations, four variants, *CYP2D6A, CYP2D6B, CYP2D6D and CYP2D6T*, have been reported to account for over 95% of the *CYP2D6* variant alleles causing PMs in Caucasians (22). The intermediate metabolic capacity caused by other variant alleles, such as *CYP2D6C, CYP2D6Ch1, CYP2D6Ch2* and *CYP2DJ*, as well as the heterozygous factor as one of the inactivating mutations, has recently been described (23,24). The IM has been noted particularly in Orientals.

A variant of the *CYP2D6* gene, termed *CYP2D6L*, which was seen in association with ultrarapid DB hydroxylation capacity, was identified by Johansson *et al.* (25). The *CYP2D6L* allele contained two mutations causing amino acid exchanges (Arg 296 Cys and Ser 486 Thr). The low DB metabolic ratio of below 0.1 was due to the duplication or the amplification of the *CYP2D6L* gene, which could be identified by *Eco*R I-RFLP. Analyzing the *CYP2D6* gene from a subject who showed a DB metabolic ratio of below 0.1, a 12-fold repeat in the wild type *CYP2D6L* gene, was found in a Swedish family. Other alleles, *CYP2D6Ch1* and *CYP2D6Ch2*, yielding, decreased activity of CYP2D6 are characterized by an exchange of Pro 34 to Ser (26). This *CYP2D6Ch* variant, as well as the *CYP2D6L* variant, is seen relatively in common in Orientals but is very rare in Caucasians (27). Recently, the capacity of *CYP2D6Ch* and *CYP2D6L* variants on the metabolism of propranolol, dextromethorphan and morphine has been extensively studied (28–30).

New Probes and Mutant Alleles of *CYP2D6* Gene

As mentioned before, more than 95% of PM phenotypes can be predicted by gene diagnosis in Caucasians, while only a part of the PMs can be diagnosed by the known mutant alleles in Japanese. Thus, we initiated gene analysis to find out new variant alleles to establish a gene diagnosis for Japanese populations. Since the population of PMs was estimated to be considerably low (less than 1%) as compared to Caucasians (5–7%), it was also necessary to find out commonly used drugs to make it easy to estimate a person as a PM phenotype. Thus, we found that promethazine and some other histamine H_1 antagonists were metabolized mainly by CYP2D6 (8). The side effect of histamine H_1 antagonists seen in common in PMs is severe drowsiness which lasts for about two days. Referring to this evidence as an indicator of volunteers as PMs, we found eight PMs, from whom we could obtain blood samples. In other experiments, we found some PMs by an institutional examination to determine the population of PMs of CYP2D6 by a sparteine test.

The *CYP2D6* gene from a healthy Japanese, who was judged as a PM on the evidence that he showed a urinary sparteine metabolic ratio of 31.6 and severe drowsiness after taking promethazine (13), was analyzed. A heterozygous *CYP2D6D*, a deletional type which indicated 11.5 kb and 29 kb *Xba* I fragments, was found by RFLP analysis. The genotype of the PM could not be accounted for by any of the previously reported mutations. The PM should have the homozygous mutations in the *CYP2D6* gene to cause the loss of catalytic activity of the CYP2D6 enzyme at the time this study was initiated. A possible new allele(s) responsible for the PM phenotype was expected. Thus, the *CYP2D6* gene from the PM was analyzed by sequencing all of the nine exons and their exon/intron boundaries. The results indicated that the PM possessed a new 9-base insertion in exon 9, designated as *CYP2D6J9* (31, Fig. 1). The 9-bases (TCACCCGTG) encoding 3 amino acids, valine, proline and threonine, were inserted at the 4213 position of exon 9 as a repeat. The insertion of the tripeptide probably enhances the hydrophobicity of the protein near the heme-binding HR-2 region. The *CYP2D6J9* and *CYP2D6D* alleles were clarified to be inherited from the mother [*2D6W/2D6J9*] and the father [*2D6W/2D6D*], respectively. The apparent *K*m values for bufuralol 1′-hydroxylase activity in yeast transformed with *CYP2D6W* and *CYP2D6J9* were 4.2 and 990 μM, respectively, indicating that the *CYP2D6J9* gene transcript has a low affinity to metabolize bufuralol. The plasma clearance of bufuralol 1′-hydroxylation by the CYP2D6J9 enzyme was estimated to be 8 to 21 times lower than the wild CYP2D6 enzyme at concentrations of bufuralol ranging from 0.03 to 2 μM, assuming that these concentrations of bufuralol were present in human livers after treatment with this drug (32). Four of 300 Japanese carried a heterozygous *CYP2D6J9* allele (0.66%, 4/600 chromosomes) as determined by a PCR analysis.

Analyzing genomic DNA extracted from a peripheral blood sample from another healthy volunteer who showed severe drowsiness after taking histamine H_1 antagonists, we found an additional new mutation. In this case, one base pair of cytosine was inserted at position 2661 of exon 5 in the

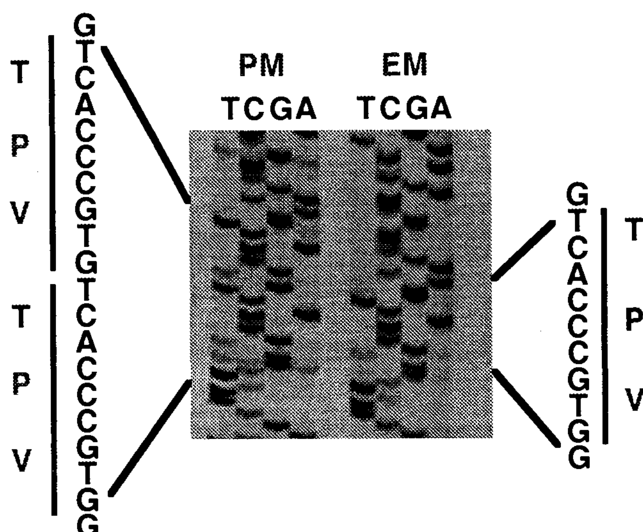

Fig. 1. Nucleotide sequence indicating a nine base insertion in exon 9 of *CYP2D6* gene in proband PM. Nucleotide sequence of part of exon 9 in *CYP2D6* allele from EM and *CYP2D6J9* allele from PM is shown. Note that nine bases encoding 3 amino acids, valine, proline and threonine, from 4213 to 4221 (TGACCCGTG), are inserted as a tendem repeat in exon 9 in PM. This figure was redrawn from Yokoi *et al.* (31).

CYP2D6 gene, leading the stop codon at the 11 bp downstream of the mutation site (Fig. 2).

Perspectives on Genetic Polymorphism of CYP2D6

The clinical significance of the CYP2D6 polymorphism relates mainly to drugs acting on the cardiovascular and central nervous systems. There are many reports showing that the PM phenotype suffered from the adverse effects of these drugs. Drugs, *e.g.* propafenone, possessing a high affinity to the CYP2D6 enzyme inhibit the metabolism of other drugs having a relatively low affinity. Consequently, drug interactions are expected to occur even in EM if these drugs are prescribed simultaneously. Drug interactions due to enzyme induction are

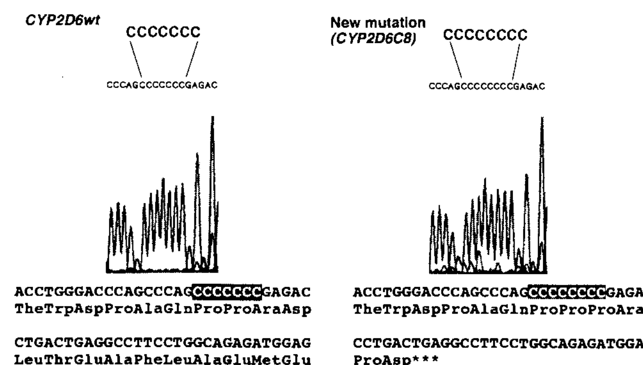

Fig. 2. Nucleotide sequence analysis of exon 5 of *CYP2D6*. Electropherograms of the nucleotide sequence of part of *CYP2D6/wt* (A) and *CYP2D6/C8* (B) and deduced amino acid sequence are shown. One cytosine was inserted at the position of 2661 in exon 5 in PM, leading a stop codon at 11 bp downstream of the insertion. ***; stop codon.

unlikely to occur since no evidence has been reported on the induction of the CYP2D6 enzyme by drugs (33).

In vitro experiments performed using human liver microsomes, liver slices and cDNA-expressed proteins allow the identification of enzymes involved in the metabolism of a drug. If a new drug is metabolized by CYP2D6, a phase I clinical study should be conducted considering the possible abnormal pharmacokinetic behavior and adverse side effects in PMs.

As mentioned above, Japanese PMs could not be fully predicted by gene diagnosis referring to mutant alleles reported so far in Caucasians. Our population studies have indicated that only 0.39% of the Japanese were judged to be PMs.[4]. This PM population is considerably lower than the predicted PM population (0.8%) in Japanese DB or SP test (12–15). Thus, other unknown mutant alleles responsible for the PM phenotype are expected to be present in Japanese. These are under current examination in our laboratory.

CYP2A6 POLYMORPHISM

Background

The involvement of CYP2A6 in coumarin 7-hydroxylation in humans was first evidenced by the results that the 7-hydroxylase activity in human liver microsomes was inhibited by antibodies raised to mouse Cyp2a5 (34). The catalytic function of CYP2A6 was then confirmed by the purified and cDNA-expressed human CYP2A6 enzymes (35). This enzyme was also reported to be involved in the metabolic activation of genotoxins including aflatoxin B_1 (36), NDEA (37) and the tobacco-specific nitrosamine, NNK (38) and NNAL (39).

A marked interindividual variation in the coumarin 7-hydroxylase activity was reported in *in vivo* and *in vitro* studies (40,41). Variables in the levels of coumarin 7-hydroxylase were first thought to be caused by factors other than genetic polymorphism since the *CYP2A6* gene was inducible in certain individuals by constituents in the diet or by drugs (42,43). A large individual variation was also found in the expression level of CYP2A6 mRNA. However, no association was found between the drug histories of liver donors and the microsomal levels of CYP2A6 mRNA, protein and coumarin 7-hydroxylase activity. These data and the results of human population studies using coumarin as a probe drug (40,41) supported the idea that genetic polymorphism existed in the *CYP2A6* gene.

Studies on the structural organization and chromosomal localization of the *CYP2A* gene family showed that the genes were assembled into a region 350 kb long and localized on the long arm of human chromosome 19 (44). Three complete *CYP2A* genes, *CYP2A6, CYP2A7* and *CYP2A13,* and two pseudogenes truncated after exon 5, were identified and sequenced (Fig. 3). A variant allele termed *CYP2A6v1,* identified by Yamano *et al.* (45), had a single amino acid substitution, Leu 160 His, resulting in an inactive enzymatic activity. This variant allele was found by cDNA cloning. However, this variant did not account for all PMs of coumarin. The other variant, termed *CYP2A6v2,* reported by Fernandez-Salguero *et al.* (46) was

[4] M. Chida, T. Yokoi, Y. Kosaka, K. Chiba, H. Nakamura, T. Ishizaki, J. Yokota, M. Kinoshita, K. Sato, M. Inaba, Y. Aoki and T. Kamataki. Genetic polymorphism of *CYP2D6* in Japanese population, submitted.

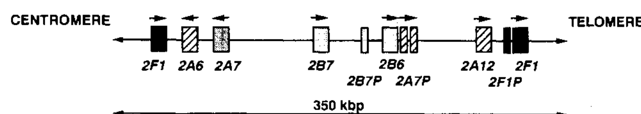

Fig. 3. Structural organization of CYP2 genefamily on human chromosome 19. A cluster spanning 350 kb is shown. The directions of transcription for the different genes are indicated by arrows. Telomere and cenrtromere directions are also indicated (Adapted from Fernandez-Salguero and Gonzales, 38).

caused by a gene-conversion in exon 3, 6 and 8 between the *CYP2A6* and the *CYP2A7* genes. Both allelic frequencies differed significantly among Caucasian, Oriental and African-American populations (46). Relatively high allelic frequencies of *CYP2A6v1* (20%) and *CYP2A6v2* (28%) are reported in Japanese, but these data were not reproducible in our laboratory.[5] Nevertheless, the two *CYP2A6v1* and *CYP2A6v2* variants did not seem to be the cause of all PMs of coumarin. In this paper, we introduce herein a new deletional *CYP2A6* allele found in Japanese.

A Novel CYP2A6 Substrate, SM-12502

SM-12502 is a newly synthesized PAF receptor antagonist currently under development. The *S*-oxide of SM-12502 was a sole metabolite seen in plasma or urine from healthy human volunteers given a single *i.v.* administration of SM-12502. Three of 28 Japanese volunteers given SM-12502 were judged to be PMs on the basis of *C*max, $T_{1/2}(\beta)$, AUC and urinary excretion of SM-12502 or the *S*-oxide metabolite (Fig. 4). In the three PMs, plasma AUC of SM-12502 were 13-, 14- and 11-fold higher, and $T_{1/2}(\beta)$ were 11-, 17- and 11-fold longer as compared with EMs who were given the same dose of the drugs. Eadie-Hofstee plots using human liver microsomes showed a biphasic pattern in the metabolism, suggesting that at least two enzymes were involved in the *S*-oxidation. Kinetic parameters (*K*m1, *V*max1) of the *S*-oxidation for a high-affinity enzyme were estimated to be 20.9 ± 4.4 μM and 0.111 ± 0.051 nmol/min/mg microsomal protein, respectively, while a low-affinity enzyme showed a 60-fold higher value of *K*m than did the high-affinity enzyme. Clinical studies in humans have been carried out at doses between 10 mg and 100 mg, with the mean *C*max value of SM-12502 at 1659.9 ng/ml (6.78 μM) in plasma. From these results, it is supposed that only the high-affinity enzyme may be responsible for the *S*-oxidation of SM-12502.

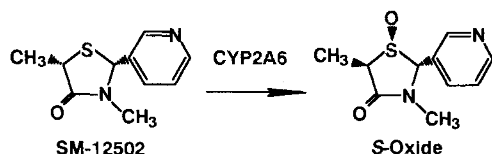

Fig. 4. Primary metabolic pathways of SM-12502 in humans. This figure was redrawn from Nunoya *et al.* (42).

[5] K. Nunoya, T. Yokoi, K. Kimura, T. Kainosho, K. Nagashima, N. Shimada, K. Sato, M. Kinoshita and T. Kamataki. *CYP2A6* gene deletion responsible for the deficiency of *in vivo* metabolism of (+)-*cis*-3,5-dimethyl-2-(3-pyridyl) thiazolidin-4-one hydrochloride (SM-12502) in humans, submitted.

FMO has been known to catalyze the *S*-oxidation of chemicals. FMO1, one of the FMOs expressed in yeast, was capable of catalyzing the *S*-oxidation of SM-12502, while the *K*m value of the reaction catalyzed by the FMO was higher than the expected value, and the value was close to that seen in the low-affinity enzyme as mentioned above. Thus, the *S*-oxidation was assumed to be catalyzed by an enzyme other than FMO (47). In another experiment to estimate the enzyme catalyzing the reaction, it was found that the *S*-oxidase was inhibited by coumarin and antibodies to rat CYP2A2 (48). The contents of CYP isoforms and the activity of each microsomes to *S*-oxidize SM-12502 in 20 human liver specimens were determined to learn the possible correlation between the content of a specific form of CYP and the amount of the *S*-oxidation (48). The results indicated that the content of the CYP2A6 protein correlated well with *S*-oxidase activity ($r = 0.808$, $P < 0.0005$). In addition, a closer correlation ($r = 0.908$, $P < 0.0001$) was observed between the activities of SM-12502 *S*-oxidase and coumarin 7-hydroxylase. Microsomes from genetically engineered human B-lymphoblastoid cells expressing CYP2A6 metabolized SM-12502 to the *S*-oxide efficiently (*K*m = 4.71 μM). Thus, it was highly possible that the CYP2A6 isozyme was an enzyme responsible for the *S*-oxidation of SM-12502 in human liver microsomes.

Role of Human CYP2A6 in the Metabolism of (*S*)-Nicotine and (*S*)-Cotinine

(*S*)-Nicotine is extensively metabolized in humans. The major pathway of metabolism is known to be *C*-oxidation to form (*S*)-cotinine (49, Fig. 5), and a major portion (70–80%) of (*S*)-nicotine ingested is metabolized to (*S*)-cotinine. The (*S*)-cotinine thus formed is subsequently converted to *trans*-3′-hydroxycotinine and excreted in urine in humans (50).

Small amounts of (*S*)-nicotine were detected in some volunteers who were judged as poor metabolizers of nicotine (51, 52). Cholerton *et al.* (51) reported that five out of 124 volunteers showed unusually high nicotine/cotinine ratios in the urine after *p.o.* administration of a nicotine capsule. These five subjects were found to be homozygous for *CYP2D6* deletion mutants, and the association with *CYP2D6* gene polymorphism was suggested. However, all PMs of CYP2D6 were not always poor

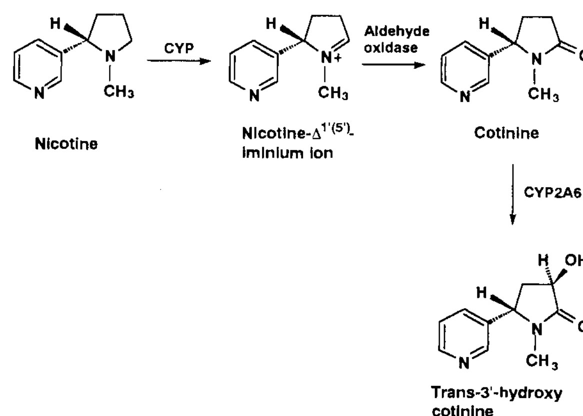

Fig. 5. Primary metabolic pathways of (*S*)-nicotine in humans. This figure was redrawn from Nakajima *et al.* (47).

metabolizers of nicotine. At present, it has not been accepted that CYP2D6 polymorphism is the cause of PMs (52).

The enzyme responsible for (*S*)-nicotine metabolism in humans was recently identified by Nakajima *et al.* (53). The capacity of liver microsomes from individual human livers to form (*S*)-cotinine from (*S*)-nicotine correlated significantly with the levels of the immunochemically determined levels of CYP2A6 protein ($r = 0.663$, $P < 0.05$) and the activities of coumarin 7-hydroxylase ($r = 0.831$, $P < 0.01$) (53). In addition, the formation of (*S*)-cotinine in human liver microsomes was inhibited by coumarin and rabbit antibodies to rat CYP2A1 specifically. When the capability of microsomes from B-lymphoblastoid cells expressing human CYPs to metabolize (*S*)-nicotine to (*S*)-cotinine was determined, the cDNA-expressed CYP2A6 enzyme exhibited the highest activity to form (*S*)-cotinine. They also demonstrated that the formation of *trans*-3′-hydroxycotinine from cotinine was catalyzed solely by CYP2A6 in humans. Thus, the series of studies by Nakajima *et al.* (53,54) confirmed that (*S*)-nicotine was oxidized to (*S*)-cotinine by CYP2A6, and (*S*)-cotinine was also metabolized to *trans*-3′-hydroxycotinine by the same enzyme in humans.

(*S*)-Nicotine itself is not carcinogenic, while the nitroso derivatives of (*S*)-nicotine and other (*S*)-nicotine metabolites have been known to be carcinogenic (56). It may be possible to assume that genetic polymorphism in the metabolism (*S*)-nicotine is linked to cancer risk.

Genetic Polymorphism of Human CYP2A6

As mentioned above, a large interindividual difference was found in the metabolism of SM-12502. The interindividual difference was closely associated with the genetic polymorphism of CYP2A6. These results led us to further investigate the genetic polymorphism of CYP2A6. The PMs in SM-12502 metabolism were not CYP2A6*v1* and CYP2A6*v2*, reported variants in the *CYP2A6* gene. We intended to clarify other variant alleles which would account for the large interindividual variation of CYP2A6 activity (55). Genomic DNA from three PMs and three EMs was analyzed to investigate the genetic polymorphism of the *CYP2A6* gene. DNAs from the three PMs digested with *Sac* I- or *Sph* I-restriction enzyme demonstrated novel RFLPs using cDNA encoding the entire sequence of CYP2A6 as a probe (Fig. 6). Novel RFLPs in three PMs were characterized by the absence of 2.6-, 4.0-, 4.5-, 4.9- and 6.4-kb *Sac* I-restriction fragments (E type in Fig. 6), and by the absence of 5.5- and 7.1-kb *Sph* I-restriction fragments (C′ type in Fig. 6), respectively. Judging from RFLP patterns in genomic DNA prepared from human livers and periferal blood, the frequency of the occurrence of the E type and C′ type mutations was 3.2% (4/125 subjects) and 3.2% (4/125 subjects), respectively. The sequence of the *CYP2A6* gene isolated from genomic libraries from the EMs and the PMs was analyzed for all exons and intron/exon boundaries. We obtained several clones for the *CYP2A6*-related gene(s) from the library from a PM. However, these clones were proven to be derived from unknown pseudogenes. Our further studies indicated that the RFLP pattern seen in the three PMs was derived from the lack of the whole coding region of the *CYP2A6* gene.[5]

Gaedigk *et al.* (57) proposed that the 3′-end break point of the *CYP2D6* gene deletion, termed as *CYP2D6D*, was located at 536 bp downstream from the exon 9 of the *CYP2D6* gene.

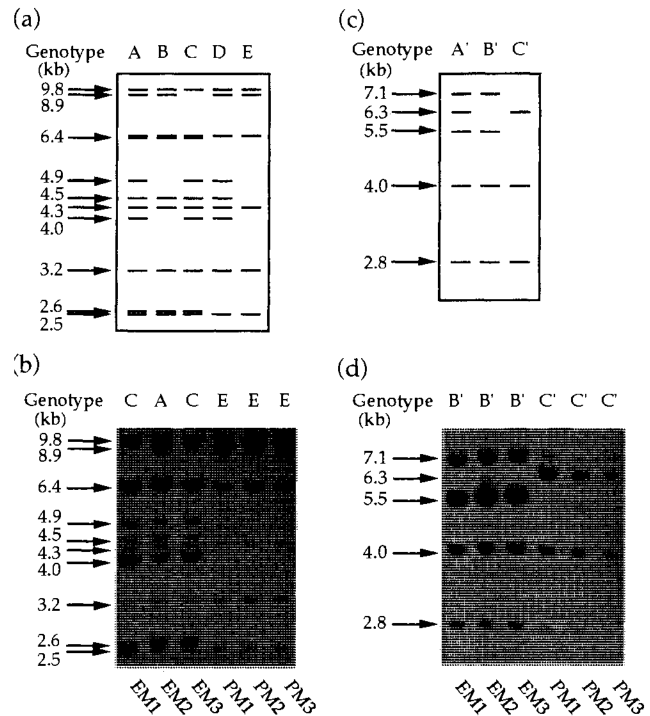

Fig. 6. RFLPs generated by *Sac* I and *Sph* I associated with PM phenotype of SM-12502 metabolism. (A) Schematic illustration of *Sac* I-RFLP pattern, A, B, C, D or E denotes each genotype. (C) Schematic illustrations *Sph* I-RFLP pattern. A', B' or C' denotes each genotype. Autoradiographs of (B) *Sac* I and (D) *Sph* I digestion of DNAs isolated from peripheral leukocytes are shown. DNAs from three EMs and three PMs were digested with *Sac* I or *Sph* I, fractionated by 0.6% agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with CYP2A6 cDNA as a probe. This figure was redrawn from Nunoya *et al.* (55).

Steen *et al.* (58) reported that a causal mechanism for the *CYP2D6D* was homologous unequal cross-over involving a 2.8 kb repeated region which flanks the 3′-downstream of *CYP2D7* and *CYP2D6* genes. We supposed that a similar mechanism as seen in *CYP2D6D* was possibly involved in the *CYP2A6* gene deletion. Thus, we compared the sequence of 3′-noncoding region of *CYP2A6* and *CYP2A7* genes between EMs and PMs, particularly the sequence of a breakpoint around the *CYP2A6* gene causing the PM phenotype (55). A chimeric *CYP2A7*/*CYP2A6* gene associated with homologous unequal cross-over between *CYP2A7* and *CYP2A6* genes was expected to cause the whole *CYP2A6* gene deletion. If this is the case, the 3′-downstream sequence located at the 325 bp downstream from *Sac* I site in exon 9 of *CYP2A7* gene must be identical to that of wild *CYP2A6* gene in the E type variant. We found that all seven individuals of the E type variant possessed the same breakpoint resulting in the whole *CYP2A6* gene deletion. Based on these results, we designed the PCR primers to identify the chimeric *CYP2A7*/*CYP2A6* gene associated with the deletion of whole *CYP2A6* gene (Fig. 7). E type subjects showed a 364 bp amplified fragment by PCR. The frequency of homozygous *CYP2A6* gene deletion in Japanese was 4.5% (12/264 chromosomes). According to the Hardy-Weinberg equation, the allelic frequency of *CYP2A6* gene deletion was calculated to be 21.2%.

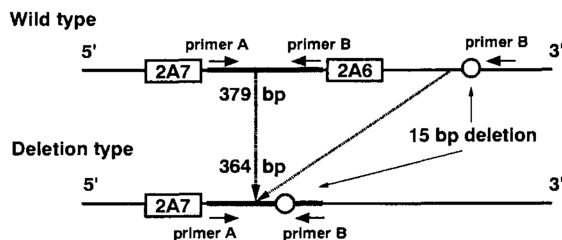

Fig. 7. Proposed mechanism for generating chimeric *CYP2A7/CYP2A6* gene associated with deletion of entire *CYP2A6* gene. The chimeric *CYP2A7/CYP2A6* gene was generated through unequal crossing over between two wild type alleles in 3'-flanking region of *CYP2A7* and *CYP2A6*.

Conclusions and Future Directions on CYP2A6

In 1954, coumarin was banned from use by the Food and Drug Administration for hepatotoxicity (59). Thus, coumarin may not be a good probe drug for *in vivo* phenotyping of CYP2A6 activity in humans. By contrast, SM-12502 has been shown to be relatively nontoxic in mice in acute toxicity studies. LD_{50} values were 1300 mg/kg and 460 mg/kg, when administered *p.o.* and *i.v.*, respectively (60). SM-12502 instead of coumarin will be expected to become an important probe drug that is metabolized to yield a single metabolite by CYP2A6, once permitted for clinical use. (*S*)-Nicotine and probably cotinine will also be interesting as new probes for CYP2A6 activity in humans, since (*S*)-nicotine can be applied to volunteers as chewing gum. CYP2A6 activity might be phenotyped by smoking. This is under investigation in our laboratories.

CYP2A6 is expressed in a tissue-specific manner. CYP2A6 protein is present in the liver, but the expression level of the enzyme protein has been reported to be below the detection limit (< 0.2 pmol/mg protein) in microsomes from the adult human lung, colon, breast, and kidney (61). On the other hand, fetal adrenal microsomes have been reported to possess coumarin 7-hydroxylase activity, but with a large interindividual variation (62). As noted in CYP2D6 genetic polymorphism, it is also important that there may be a large ethnic difference in the frequency of allelic variants of the *CYP2A6* gene. This should also be clarified in Caucasians.

CYP2A6 has been known to metabolically activate several procarcinogenic nitrosamines, *e.g.* NNK, NDMA, NDEA, NNAL and NNN. A large interindividual variation (10–90%) in the inhibition of NDEA metabolism by antibodies to Cyp2a5 has been reported using human liver microsomes (63). Therefore, genetic defects in the *CYP2A6* gene may affect susceptibility to these procarcinogens. The association between the CYP2A6 genetic polymorphism and cancer susceptibility in humans is another important issue to be clarified.

CONCLUSION

More than 95% Caucasian populations who show CYP2D6 poor metabolizer phenotype can be diagnosed by gene analysis, while about 20–30% of poor metabolizers can be diagnosed in Japanese population, suggesting that (a) new mutation(s) is present in Japanese. Thus, we found a new mutation, namely CYP2D6/J9 in Japanese. In addition to coumarin, we discovered a new probe drug, SM-12502 currently under development, which was mainly metabolized by CYP2A6. Using this drug as a probe, we found poor metabolizers and analyzed the genes for CYP2A6. Thus, we found a new mutation (CYP2A6 whole deletion) responsible for the poor metabolizer phenotype. Taken these results together, it should be considered that gene mutations occur dependent on the races and thus clarified for each race.

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