DMD Fast Forward. Published on January 31, 2007 as doi:10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

A NOVEL DUPLICATION TYPE OF *CYP2A6* GENE IN AFRICAN-AMERICAN POPULATION

Tatsuki Fukami, Miki Nakajima, Hiroyuki Yamanaka, Yasunari Fukushima, Howard L. McLeod, and Tsuyoshi Yokoi

Drug Metabolism and Toxicology, Division of Pharmaceutical Sciences, Graduate School of Medical Science, Kanazawa University, Kakuma-machi, Kanazawa, Japan (*T.F., M.N., H.Y., Y.F., T.Y.*); and Department of Medicine, Washington University School of Medicine, St. Louis, Missouri (*H.L.M.*)

Running title: A novel duplication type of *CYP2A6*

To whom all corresponding should be sent:

Miki Nakajima, PhD.

Drug Metabolism and Toxicology

Division of Pharmaceutical Sciences

Graduate School of Medical Science

Kanazawa University

Kakuma-machi

Kanazawa 920-1192, Japan

Tel/Fax +81-76-234-4407

E-mail: nmiki@kenroku.kanazawa-u.ac.jp

Number of text pages: 12

Number of tables: 1

Number of figures: 3

Number of references: 25

Number of words

Abstract: 169

Introduction: 340

Discussion: 783

Abbreviations: CYP, Cytochrome P450; SNP, single nucleotide polymorphism; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; AS-PCR, allele specific-polymerase chain reaction; bp, base pair(s).

DMD Fast Forward. Published on January 31, 2007 as DOI: 10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Human CYP2A6 is responsible for the metabolism of nicotine and the its genetic polymorphisms affect smoking behavior and risk of lung cancer. In the present study, we identified a novel type of *CYP2A6* gene duplication that is created through an unequal crossover event with the *CYP2A7* gene at 5.2 – 5.6 kb downstream from the stop codon. The novel duplication type of *CYP2A6* was found in African-Americans (n = 176) at an allele frequency of 1.7%, but not found in European-American (n = 187), Korean (n = 209), or Japanese (n = 184) populations. The plasma cotinine/nicotine ratio in subjects having the novel *CYP2A6* gene duplication with the *CYP2A6*1* allele (10.8 ± 7.0, n = 4) was 1.4 fold higher than that in homozygotes of the wild type (8.0 ± 5.0, n = 87), although the difference was not statistically significant. The findings in the present study suggested that the novel duplicated *CYP2A6* allele, which is specific for African-Americans, would increase nicotine metabolism and may affect the smoking behavior.

DMD Fast Forward. Published on January 31, 2007 as DOI: 10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cytochrome P450 (CYP) is a superfamily of hemoproteins, many of which can metabolize xenobiotics such as clinically used drugs, procarcinogens, and environmental pollutants. The *CYP2A* gene subfamily comprises three genes, *CYP2A6*, *CYP2A7*, and *CYP2A13*, as well as a split pseudogene, *CYP2A18*. Among them, the genes coding functional enzymes are *CYP2A6* and *CYP2A13* (Yamano et al. 1990; Su et al. 2000). CYP2A6 is mainly expressed in liver and is responsible for nicotine metabolism (Nakajima et al. 1996). In addition, it can metabolize some pharmaceutical agents such as losigamone, letrozole, halothane, valproic acid, and disulfiram, and activates some procarcinogens such as 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone and *N*-nitrosodiethylamine (Nakajima et al. 2002).

Genetic polymorphisms in the *CYP2A6* gene are associated with large interindividual variability in nicotine metabolism, smoking behavior, and the risk of lung cancer (Fujieda et al. 2004). Several *CYP2A6* alleles possessing a single nucleotide polymorphism (SNP) which leads to an amino acid change have decreased or no enzymatic activity. Other *CYP2A6* alleles such as *CYP2A6*1B* or *CYP2A6*12* are created by gene conversion or unequal crossover event with the *CYP2A7* gene (Ariyoshi et al. 2000; Oscarson et al. 2002). The *CYP2A7* gene is located approximately 25 kb upstream of the *CYP2A6* gene and shows an identity of 96.5% in the coding sequences (Yamano et al. 1990). Rao et al (2000) reported a duplicated *CYP2A6* allele, *CYP2A6*1X2*. This allele is created through an unequal crossover with the *CYP2A7* gene at intron 8 – exon 9 and the *CYP2A6*4D* allele is produced as the reciprocal product (Oscarson et al. 1999a). *CYP2A6*4A* and *CYP2A6*4B* alleles have also been reported as entire gene deleted alleles (Oscarson et al. 1999b; Ariyoshi et al. 2004).

In a previous study (Nakajima et al. 2006), we found an African-American subject possessing the *CYP2A6*1A*, *CYP2A6*1D* (g.-1013A>G), and *CYP2A6*1H* (g.-745A>G) alleles, envisaging the existence of three copies of the *CYP2A6* gene. However, genotyping analysis did not assigned the *CYP2A6*1X2* allele reported by Rao et al (2000). In the present study, we investigated the *CYP2A* locus and identified the novel duplication type of *CYP2A6*

DMD Fast Forward. Published on January 31, 2007 as DOI: 10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

gene.

Materials and methods

Chemicals and reagents. LA Taq DNA polymerase and Blend Taq DNA polymerase were obtained from Takara (Shiga, Japan) and Toyobo (Osaka, Japan), respectively. Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). The restriction enzymes were purchased from Takara, Toyobo, New England Biolabs (Beverly, MA), and Fermentas (Hanover, MD). Nicorette® (nicotine gum containing 2 mg of nicotine) was obtained from Pfizer Japan (Tokyo, Japan). All other chemicals and solvents were of analytical or the highest grade commercially available.

Genomic DNA. This study was approved by the Human Studies Committee of Washington University School of Medicine (St. Louis, MO) and the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Soonchunhyang University Hospital (Chonan, Korea). Written informed consent was obtained from 187 European-American, 176 African-American, 209 Korean, and 184 Japanese subjects. Blood samples were collected from a cubital vein. Genomic DNA was extracted from peripheral lymphocytes using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

Genotyping of *CYP2A6* alleles. The genotyping of *CYP2A6*1A* (Nakajima et al. 2006), *CYP2A6*1B1* (Nakajima et al. 2006), *CYP2A6*1B2* (Nakajima et al. 2006), *CYP2A6*1B13* (Nakajima et al. 2006), *CYP2A6*1D* (Nakajima et al. 2006), *CYP2A6*1F* (Nakajima et al. 2004), *CYP2A6*1G* (Nakajima et al. 2004), *CYP2A6*1H* (Nakajima et al. 2006), *CYP2A6*1J* (Nakajima et al. 2006), conventional *CYP2A6*1X2* (Yoshida et al. 2002), *CYP2A6*2* (Nakajima et al. 2000), *CYP2A6*3* (Nakajima et al. 2000), *CYP2A6*4A* (Fukami et al. 2006), *CYP2A6*4D* (Fukami et al. 2006), *CYP2A6*5* (Nakajima et al. 2001), *CYP2A6*6* (Yoshida et al. 2002), *CYP2A6*7* (Yoshida et al. 2002), *CYP2A6*8* (Yoshida et al. 2002), *CYP2A6*9*

(Nakajima et al. 2006), *CYP2A6*10* (Yoshida et al. 2002), *CYP2A6*11* (Yoshida et al. 2002), *CYP2A6*12* (Nakajima et al. 2004), *CYP2A6*13* (Fukami et al. 2005b), *CYP2A6*14* (Fukami et al. 2005b), *CYP2A6*15* (Fukami et al. 2005b), *CYP2A6*16* (Fukami et al. 2005b), *CYP2A6*17* (Fukami et al. 2004), *CYP2A6*18* (Fukami et al. 2005b), *CYP2A6*19* (Fukami et al. 2005b), *CYP2A6*20* (Fukami et al. 2005a), *CYP2A6*21* (Nakajima et al. 2006), and *CYP2A6*22* (Nakajima et al. 2006) were performed as described previously.

Determination of the relative gene copy ratio of *CYP2A6*/*CYP2A7*. To determine the copy number of the *CYP2A6* gene, PCR analyses were performed as described previously (Fukami et al. 2006). DNA samples from the subjects who were genotyped as *CYP2A6*4A*/*CYP2A6*4A* (n = 2, Japanese), *CYP2A6*1*/*CYP2A6*4A* (n = 4, Japanese), *CYP2A6*1*/*CYP2A6*1* (n = 4, Japanese), and *CYP2A6*1*/conventional *CYP2A6*1X2* (n = 1, Korean) were used for the standard curve.

PCR-RFLP to identify the crossover region. To determine the crossover region of the novel duplicated *CYP2A6* allele with the *CYP2A7* gene, Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed at the 3'-flanking region. The primer sets used were 2A6FR-S and 2A6FR13.8kR, 2A7FR-S and 2A7FR16.9kR, or 2A6FR-S and 2A7FR16.9kR (Table 1). The reaction mixture contained genomic DNA (200 ng), 1 X LA-PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM each primer, and 1 U of LA Taq DNA polymerase in a final volume of 25 μl. After an initial denaturation at 94˚C for 1 min, the amplification was performed by denaturation at 98˚C for 20 sec, annealing and extension at 68˚C for 12 min (with prolongation for 15 sec per 1 cycle during 13 to 26 cycles) for 26 cycles, following by a final extension at 72˚C for 10 min. The LA-PCR product was digested with *Ale* I, *Eco*R I, *Eco*R V, or *Fsp* I at 37˚C for 3 hr. The digestion patterns were determined by electrophoresis in a 0.8% agarose gel.

Sequence analysis of the 3'-flanking region of the *CYP2A6* gene. Sequence analysis was

performed to examine the nucleotide sequences of the crossover region of the novel duplicated *CYP2A6* allele. The PCR reaction mixture contained genomic DNA (100 ng), 1 X PCR buffer, 0.2 mM dNTPs, 0.4 µM 2A6FR4.2kS and 2A7FR7.8kR primers (Table 1), and 0.5 U of Blend Taq DNA polymerase in a final volume of 25 µl. After an initial denaturation at 94˚C for 3 min, the amplification was performed by denaturation at 94˚C for 25 sec, annealing at 57˚C for 25 sec, and extension at 72˚C for 3 min for 28 cycles, following by a final extension at 72˚C for 5 min. The PCR product was submitted to DNA sequencing using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (GE Healthcare Bio-Science, NJ) with a Long-Read Tower DNA sequencer (GE Healthcare Bio-Science).

Genotyping method for the novel duplicated *CYP2A6* allele. Allele specific (AS)-PCR was applied for the genotyping with the primer sets 2A6FR4.2kS and 2A6FR6.8kR or 2A7FR7.8kR (Table 1). The reaction mixture and PCR condition were the same as described above except for the primers. The *CYP2A6*1* allele was amplified with the primer set 2A6FR4.2kS and 2A6FR6.8kR (2,681 bp) and the novel duplicated *CYP2A6* allele was amplified with the primer set 2A6FR4.2kS and 2A7FR7.8kR (2,387 bp).

***In vivo* phenotyping of nicotine metabolism.** Since 16 out of 176 African-Americans were judged as smokers based on the baseline presence of nicotine and cotinine, phenotyping of *in vivo* nicotine metabolism was not performed in these subjects. Phenotyping analysis was performed in 160 African-Americans in our previous study (Nakajima et al. 2006). Briefly, the subjects chewed one piece of nicotine gum (Nicorette®) for 30 min, chewing for 10 sec per 30 sec. Blood samples were collected from a cubital vein just before and 2 hr after the start of chewing. The concentrations of nicotine and cotinine in the plasma samples were determined by HPLC as described previously (Nakajima et al. 2000). The cotinine/nicotine ratio of the plasma concentration was calculated as an index of nicotine metabolism. Statistical analysis of the cotinine/nicotine ratios between the different genotypes was performed by Mann-Whitney *U*-test. A value of $P < 0.05$ was considered statistically significant.

DMD Fast Forward. Published on January 31, 2007 as DOI: 10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

Results

A subject possessing three *CYP2A6* alleles. In our recent study (Nakajima et al. 2006), we encountered an African-American subject who possessed the *CYP2A6*1A*, *CYP2A6*1D*, and *CYP2A6*1H* alleles. The subject was suspected to have three copies of the *CYP2A6* gene. We determined the copy number of the *CYP2A6* gene according to a PCR method described previously (Fukami et al. 2006). The ratios of the PCR products (exon 3 to intron 3) corrected by the fragment lengths of *CYP2A6*/*CYP2A7* for the standard samples were as follows: 0.38 to 0.40 for *CYP2A6*4A*/*CYP2A6*4A* (n = 2), 1.67 to 1.80 for *CYP2A6*1*/*CYP2A6*4A* (n = 4), 2.55 to 2.95 for *CYP2A6*1*/*CYP2A6*1* (n = 4), and 4.01 for *CYP2A6*1*/conventional *CYP2A6*1X2* reported by Rao et al (2000) (n = 1). With the standard curve, the ratio of the copy numbers of *CYP2A6*/*CYP2A7* in the subject was 1.43. The ratios of the PCR products (exon 5 to intron 5) corrected by the fragment lengths of *CYP2A6*/*CYP2A7* for the standard samples were as follows: 0.00 for *CYP2A6*4A*/*CYP2A6*4A* (n = 2), 0.75 to 0.83 for *CYP2A6*1*/*CYP2A6*4A* (n = 4), 1.47 to 1.61 for *CYP2A6*1*/*CYP2A6*1* (n = 4), and 2.28 for *CYP2A6*1*/conventional *CYP2A6*1X2* (n = 1). With the standard curve, the ratio of the copy numbers of *CYP2A6*/*CYP2A7* in the subject was 1.40. These results suggested that the subject had three copies of the *CYP2A6* gene.

Identification of the unequal crossover region of the novel duplicated *CYP2A6* allele. Genotyping analysis revealed that the subject did not have the conventional *CYP2A6*1X2* allele reported by Rao et al (2000). Thus, the crossover region was considered to be located downstream of exon 9 of the *CYP2A6* gene. When the primer set 2A6FR-S and 2A6FR13.8kR was used, the 3'-flanking region of the *CYP2A6* gene (13,507 bp, referring to the genomic sequences NG_000008.5) was amplified (Fig. 1). When the primer set 2A7FR-S and 2A7FR16.9kR was used, the 3'-flanking region of the *CYP2A7* gene (16,471 bp) was amplified. When the primer set 2A6FR-S and 2A7FR16.9kR was used, the chimeric region of the novel

duplicated *CYP2A6* allele (15,300 bp by estimation) was amplified. PCR-RFLP analyses were performed to determine the crossover region. Based on the comparison of the RFLP patterns with 4 restriction enzymes, the crossover region was suspected to be approximately 6 kb downstream from the stop codon of the *CYP2A6* gene. Sequence analysis demonstrated that the novel duplicated *CYP2A6* allele has the *CYP2A6* sequences up to 5.2 kb downstream of the stop codon, and the *CYP2A7* sequences 5.6 kb downstream (Fig. 2). Thus, the crossover region was considered to be 5.2 – 5.6 kb downstream from the stop codon of the *CYP2A6* gene.

Allele frequency of the novel duplicated *CYP2A6* allele. We performed the genotyping analysis for the novel duplicated *CYP2A6* allele in 187 European-Americans, 176 African-Americans, 209 Koreans, and 184 Japanese. Six out of 176 African-Americans were judged as carriers of the novel duplicated *CYP2A6* allele, resulting in a prevalence of 3.4%. They were originally genotyped as *CYP2A6*1*/*CYP2A6*1* (n = 4) and *CYP2A6*1*/*CYP2A6*9* (n = 2). The genotyping method could not determine whether they have the duplicated *CYP2A6* allele heterozygously or homozygously. However, it was suggested that they have three copies of the *CYP2A6* gene, since the ratios of the copy numbers of *CYP2A6*/*CYP2A7* in these 6 subjects were 1.41 – 1.49 (exon 3 – intron 3) and 1.40 – 1.47 (exon 5 – intron 5). Accordingly, the allele frequency of the novel duplicated *CYP2A6* allele was determined as 1.7%. It was confirmed that the sequences around the crossover region of the novel duplicated allele in 6 subjects were identical. The novel duplicated *CYP2A6* allele was not found in European-Americans, Koreans, or Japanese.

***In vivo* nicotine metabolism in the subjects possessing the novel duplicated *CYP2A6* allele.** The cotinine/nicotine ratios in plasma were calculated as an index of nicotine metabolism in the African-Americans. Since there was no association between the *CYP2A6*1* suballeles (such as *CYP2A6*1A, CYP2A6*1B, CYP2A6*1D* etc) and the phenotype, the suballeles were combined as *CYP2A6*1* alleles (Nakajima et al. 2006). Among 6 subjects possessing the novel duplicated *CYP2A6* allele, the phenotype of one subject with the *CYP2A6*9* allele could not be determined,

because she was a smoker. Another subject possessing the novel duplicated *CYP2A6* allele with the *CYP2A6*9* allele showed a cotinine/nicotine ratio 6.5. However, the ratio was excluded from the data analysis because we could not determine whether his genotype was *CYP2A6*1*/*CYP2A6*1*/*CYP2A6*9* or *CYP2A6*1*/*CYP2A6*9*/*CYP2A6*9*. Interestingly, we found that the ratio in the subjects possessing the novel duplicated *CYP2A6* allele (10.8 ± 7.0, n = 4) was 1.4 fold higher than that in homozygotes of the *CYP2A6*1* allele (8.0 ± 5.0, n = 87), although the difference was not statistically significant. Further characterization with a much larger sample size may be required, but our data demonstrated that the novel duplicated *CYP2A6* allele likely increased nicotine metabolism.

Discussion

Several years ago, Rao et al (2000) found a duplication allele, *CYP2A6*1X2*. This allele is considered to be created through an unequal crossover event at intron 8 - exon 9, with the gene deleted allele *CYP2A6*4D* as the reciprocal product. Although other deleted alleles such as *CYP2A6*4A* and *CYP2A6*4B* have been reported, the reciprocal products have not been identified. In the present study, we found a novel duplicated *CYP2A6* allele that is considered to be created by an unequal crossover with the *CYP2A7* gene at 5.2 – 5.6 kb downstream from the stop codon. Interestingly, the Alu elements were found 4.7 - 6.4 kb downstream from the stop codon of the *CYP2A6* gene (Fig. 2). Generally, homologous recombination between Alu elements might result in various genetic exchanges, including duplications, deletions, and translocations (Deininger et al. 1999). For example, duplicated or multiplicated alleles for *CYP2D6* are well known, and it has been reported that repetitive sequences containing Alu elements (CYP-REP units) would be involved in the generation of these alleles (Steen et al. 1995; Lundqvist et al. 1999).

Previously, Ariyoshi et al. (2004) reported that the entire *CYP2A6* gene deletion allele *CYP2A6*4B* would be caused by unequal crossover at approximately 5.0 kb downstream from the stop codon of *CYP2A6* gene. We could also confirm the crossover region in the *CYP2A6*4B*

DMD Fast Forward. Published on January 31, 2007 as DOI: 10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

gene (data not shown). Since the crossover site is very close to that in the novel duplicated *CYP2A6* allele found in this study, the possibility is suggested that the novel duplicated *CYP2A6* allele may be the reciprocal product of the *CYP2A6*4B* allele (Fig. 3). So far, the *CYP2A6*4B* allele was analyzed only in the Japanese population and found with an allele frequency of 0.24% (Ariyoshi et al. 2004). In order to investigate whether the *CYP2A6*4B* allele would also be found in African-Americans, we performed a genotyping analysis according to the PCR-RFLP method by Ariyoshi et al (2004). However, the genotyping was not accomplished owing to the presence of a SNP preventing the RFLP using *Bsp*H I. Therefore, new genotyping method for *CYP2A6*4B* allele with AS-PCR was developed in the present study. The used primer sets were 2A6FR4.2kS or 2A7FR5.4kS and 2A6FR6.8kR (Table 1; Fig. 2). The *CYP2A6*1* allele was amplified with the primer set 2A6FR4.2kS and 2A6FR6.8kR (2,681 bp) and the *CYP2A6*4B* allele was amplified with the primer set 2A7FR5.4kS and 2A6FR6.8kR (2,669 bp by estimation). Finally, the *CYP2A6*4B* allele was not found in African-Americans as well as European-Americans. The allele was found in a Korean subject and a Japanese subject, resulting in the allele frequency with 0.24% and 0.27%, respectively. Thus, the allele frequencies of the novel duplicated *CYP2A6* allele and the *CYP2A6*4B* allele were not in concordance. Moreover, the duplicated *CYP2A6* allele was not found in Japanese population although the frequency of the *CYP2A6*4A* allele was prominently high as 20% (Schoedel et al. 2004; Nakajima et al., 2006). Although we have no plausible explanation, these results may suggest the possibility that reciprocal products caused by an unequal crossover event might not be equally inherited in the same populations.

Interestingly, all subjects with the novel duplicated *CYP2A6* allele in the present study had the *CYP2A6*1H* allele, suggesting linkage of the duplication and the SNP g.-745A>G. Direct sequencing analyses demonstrated the presence of SNPs g.144G>A (exon 1), g.1620T>C (intron 2), g.3492C>T (exon 5), g.3570C>G (intron 5), g.5738C>T (exon 8), g.5843G>C (intron 8), and g.6692C>G (3'-untranslated region) in the novel *CYP2A6* allele in 6 subjects. The SNPs found in the exons are synonymous. An allele possessing these 8 SNPs would be very similar to the haplotype #30 or #31 reported by Haberl et al (2005).

Until now, we have evaluated *in vivo* nicotine metabolism using nicotine gum in non-smokers (Nakajima et al. 2006). There is now much evidence that the genetic polymorphisms of the *CYP2A6* gene are major determinants of the interindividual variability in nicotine metabolism. In the present study, we found that African-American subjects possessing the novel duplicated *CYP2A6* allele showed increased (1.4 fold) nicotine metabolism compared with homozygotes of the wild type, consistent with the presence of three copies of the *CYP2A6* gene. Our results are in agreement with a previous report by Rao et al (2000) showing that individuals with the duplication allele *CYP2A6*1X2* had a 1.4 fold higher plasma cotinine level than homozygotes of the wild type.

In conclusion, we found a novel duplicated *CYP2A6* allele that is created by unequal crossover with the *CYP2A7* gene at 5.2 – 5.6 kb downstream of the stop codon. The duplication allele was specific for African-American subjects at an allele frequency of 1.7%. Since the duplication allele likely increase nicotine metabolism, it might affect the smoking behavior or risk of lung cancer.

Acknowledgements

The enthusiasm and research support of Tracy Jones, Arnita Pitts, Phyllis Klein, and Ladonna Gaines (Washington University Center for Clinical Studies), and Margaret Ameyaw are greatly appreciated. We acknowledge Brent Bell for reviewing the manuscript.

References

Ariyoshi N, Sekine H, Nakayama K, Saito K, Miyamoto A, and Kamataki T (2004) Identification of deletion-junction site of *CYP2A6*4B* allele lacking entire coding region of *CYP2A6* in Japanese. *Pharmacogenetics* **14**:701-705.

Ariyoshi N, Takahashi Y, Miyamoto M, Umetsu Y, Daigo S, Tateishi T, Kobayashi S, Mizorogi Y, Loriot MA, Stucker I, Beaune P, Kinoshita M, and Kamataki T (2000) Structural characterization of a new variant of the *CYP2A6* gene (*CYP2A6*1B*) apparently diagnosed as heterozygotes of *CYP2A6*1A* and *CYP2A6*4C*. *Pharmacogenetics* **10**:687-693.

Deininger PL and Batzer MA (1999) Alu repeats and human disease. *Mol Genet Metab* **67**:183-193.

Fujieda M, Yamazaki H, Saito T, Kiyotani K, Gyamfi MA, Sakurai M, Dosaka-Akita H, Sawamura Y, Yokota J, Kunitoh H, and Kamataki T (2004) Evaluation of *CYP2A6* genetic polymorphisms as determinants of smoking behavior and tobacco-related lung cancer risk in male Japanese smokers. *Carcinogenesis* **25**:2451-2458.

Fukami T, Nakajima M, Higashi E, Yamanaka H, McLeod HL, and Yokoi T (2005a) A novel *CYP2A6*20* allele found in African-American population produces a truncated protein lacking enzymatic activity. *Biochem Pharmacol* **70**:801-808.

Fukami T, Nakajima M, Higashi E, Yamanaka H, Sakai H, McLeod HL, and Yokoi T (2005b) Characterization of novel *CYP2A6* polymorphic alleles (*CYP2A6*18* and *CYP2A6*19*) that affect enzymatic activity. *Drug Metab Dispos* **33**:1202-1210.

Fukami T, Nakajima M, Sakai H, McLeod HL, and Yokoi T (2006) *CYP2A7* polymorphic alleles confound the genotyping of *CYP2A6*4A* allele. *Pharmacogenomics J* **6**:401-412.

Fukami T, Nakajima M, Yoshida R, Tsuchiya Y, Fujiki Y, Katoh M, McLeod HL, and Yokoi T (2004) A novel polymorphism of human *CYP2A6* gene *CYP2A6*17* has an amino acid substitution (V365M) that decreases enzymatic activity *in vitro* and *in vivo*. *Clin*

Pharmacol Ther **76**:519-527.

Haberl M, Anwald B, Klein K, Weil R, Fuß C, Gepdiremen A, Zanger UM, Meyer UA, and Wojnowski L (2005) Three haplotypes associated with CYP2A6 phenotypes in Caucasians. *Pharmacogenetics Pharmacogenomics* **15**:609-624.

Lundqvist E, Johansson I, and Ingelman-Sundberg M (1999) Genetic mechanisms for duplication and multiplication of the human *CYP2D6* gene and methods for detection of duplicated *CYP2D6* genes. *Gene* **226**:327-338.

Nakajima M, Fukami T, Yamanaka H, Higashi E, Sakai H, Yoshida R, Kwon JT, McLeod HL, and Yokoi T (2006) Comprehensive evaluation of variability in nicotine metabolism and *CYP2A6* polymorphic alleles in four ethnic populations. *Clin Pharmacol Ther* **80**:282-297.

Nakajima M, Kuroiwa Y, and Yokoi T (2002) Interindividual differences in nicotine metabolism and genetic polymorphisms of human CYP2A6. *Drug Metab Rev* **34**:865-877.

Nakajima M, Kwon JT, Tanaka N, Zenta T, Yamamoto Y, Yamamoto H, Yamazaki H, Yamamoto T, Kuroiwa Y, and Yokoi T (2001) Relationship between interindividual differences in nicotine metabolism and *CYP2A6* genetic polymorphism in humans. *Clin Pharmacol Ther* **68**:72-78.

Nakajima M, Yamagishi S, Yamamoto H, Yamamoto T, Kuroiwa Y, and Yokoi T (2000) Deficient cotinine formation from nicotine is attributed to the whole deletion of the *CYP2A6* gene in humans. *Clin Pharmacol Ther* **67**:57-69.

Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T, and Kuroiwa Y (1996) Role of human cytochrome P4502A6 in *C*-oxidation of nicotine. *Drug Metab Dispos* **24**:1212-1217.

Nakajima M, Yoshida R, Fukami T, McLeod HL, and Yokoi T (2004) Novel human *CYP2A6* alleles confound gene deletion analysis. *FEBS Lett* **569**:75-81.

Oscarson M, McLellan RA, Asp V, Ledesma M, Ruiz MLB, Sinues B, Rautio A, and Ingelman-Sundberg M (2002) Characterization of a novel *CYP2A7*/*CYP2A6* hybrid

allele (*CYP2A6*12*) that causes reduced CYP2A6 activity. *Hum Mutat* **20**:275-283.

Oscarson M, McLellan RA, Gullstén H, Agúndez JAG, Benítez J, Rautio A, Raunio H, Pelkonen O, and Ingelman-Sundberg M (1999a) Identification and characterization of novel polymorphisms in the *CYP2A* locus: implications for nicotine metabolism. *FEBS Lett* **460**:321-327.

Oscarson M, McLellan RA, Gullstén H, Yue QY, Lang MA, Bernal ML, Sinues B, Hirvonen A, Raunio H, Pelkonen O, and Ingelman-Sundberg M (1999b) Characterization and PCR-based detection of a *CYP2A6* gene deletion found at a high frequency in a Chinese population. *FEBS Lett* **448**:105-110.

Rao Y, Hoffmann E, Zia M, Bodin L, Zeman M, Sellers EM, and Tyndale RF (2000) Duplications and defects in the *CYP2A6* gene: identification, genotyping, and in vivo effects on smoking. *Mol Pharmacol* **58**:747-755.

Schoedel KA, Hoffmann EB, Rao Y, Sellers EM, and Tyndale RF (2004) Ethnic variation in *CYP2A6* and association of genetically slow nicotine metabolism and smoking in adult Caucasians. *Pharmacogenetics* **14**:615-626.

Steen VM, Molven A, Aarskog NK, and Gulbrandsen AK (1995) Homologous unequal cross-over involving a 2.8 kb direct repeat as a mechanism for the generation of allelic variants of the human cytochrome P450 *CYP2D6* gene. *Hum Mol Genet* **4**:2251-2257.

Su T, Bao Z, Zhang QY, Smith TJ, Hong JY, and Ding X (2000) Human cytochrome P450 CYP2A13: predominant expression in the respiratory tract and its high efficiency metabolic activation of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res* **60**:5074-5079.

Yamano S, Tatsuno J, and Gonzalez FJ (1990) The *CYP2A3* gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* **29**:1322-1329.

Yoshida R, Nakajima M, Watanabe Y, Kwon JT, and Yokoi T (2002) Genetic polymorphisms in human *CYP2A6* gene causing impaired nicotine metabolism. *Br J Clin Pharmacol* **54**:511-517.

Footnotes

This study was supported in part by a grant from Japan Health Sciences Foundation with Research on Health Science Focusing on Drug Innovation, by a grant from the Smoking Research Foundation in Japan. Tatsuki Fukami was supported as a Research Fellow of the Japan Society for the Promotion of Science.

Address correspondence to: Dr. Miki Nakajima, Drug Metabolism and Toxicology, Division of Pharmaceutical Sciences, Graduate School of Medical Science, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. E-mail: nmiki@kenroku.kanazawa-u.ac.jp

Figure legends

Fig. 1. Amplification of the 3'-flanking regions of the *CYP2A6*, *CYP2A7*, and the chimeric genes in the novel duplicated *CYP2A6* allele and their RFLP patterns. A, Schematic diagram of the wild type and the novel duplicated *CYP2A6* allele. Closed and open regions represent *CYP2A7* and *CYP2A6* genes, respectively. *CYP2A7* is located approximately 25 kb upstream of the *CYP2A6* gene. Arrows indicate location and direction of the primer sets for PCR amplification. B, Schematic digestion pattern of the PCR product with *Ale* I, *Eco*R I, *Eco*R V, and *Fsp* I. The restriction sites of each restriction enzyme are indicated by vertical lines. Dashed lines indicate the *CYP2A7* specific region that lacks the *CYP2A6* gene. Fragment lengths were estimated based on the reference sequences NG_000008.5. C**,** Representative photograph of PCR-RFLP patterns for the *CYP2A6*, *CYP2A7*, and the chimeric genes in the novel duplicated *CYP2A6* allele.

Fig. 2. Sequence alignments of the 3'-flanking region of the *CYP2A6* gene, the *CYP2A7* gene, and the novel duplicated *CYP2A6* allele. Upper and lower nucleotide numbering refers to the next nucleotide of the stop codon as 1 for the *CYP2A6* and *CYP2A7* genes, respectively with the reference sequence NG_000008.5. Deletions are denoted by dashes. Open boxes represent whether the sequences in the novel duplicated *CYP2A6* allele are the same as the *CYP2A6* gene or the *CYP2A7* gene. Black and gray boxes represent the Alu elements and the AG-rich region, respectively.

Fig. 3. Proposed unequal crossover mechanism for generation of the *CYP2A6* duplication and deletion alleles. Closed and open regions represent the *CYP2A7* and *CYP2A6* genes, respectively. A, The duplication allele *CYP2A6*1X2* reported by Rao et al (2000) was proposed to be created by an unequal crossover event at the region from intron 8 to exon 9, with *CYP2A6*4D* allele as the reciprocal product. B, The novel duplicated *CYP2A6* allele (*CYP2A6*1X2*) found in this study may be created by an unequal crossover event at 5.2 – 5.6 kb

downstream from the stop codon, with the *CYP2A6*4B* allele as the reciprocal product.

DMD Fast Forward. Published on January 31, 2007 as DOI: 10.1124/dmd.106.013557
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Primers used in the present study

Primer	Sequence
2A6FR-S	5’-AAACCTCTGCGAAGGGCGT-3’
2A7FR-S	5’-AAACCTCTGCGAAGGGGAA-3’
2A6FR4.2kS	5’-GATAAAGAAAATGTGGATAGAATGG-3’
2A7FR5.4kS	5’-GATAAAGAAAATGTGGTACATTC-3’
2A6FR6.8kR	5’-GGACCATAGGTGCGCACGA-3’
2A7FR7.8kR	5’-GGACCATAGGCGCACACTT-3’
2A6FR13.8kR	5’-TCACAGTGGCTCTTGGCTT-3’
2A7FR16.9kR	5’-CTGCCCTTGAAGGAATGAATC-3’

Figure 1

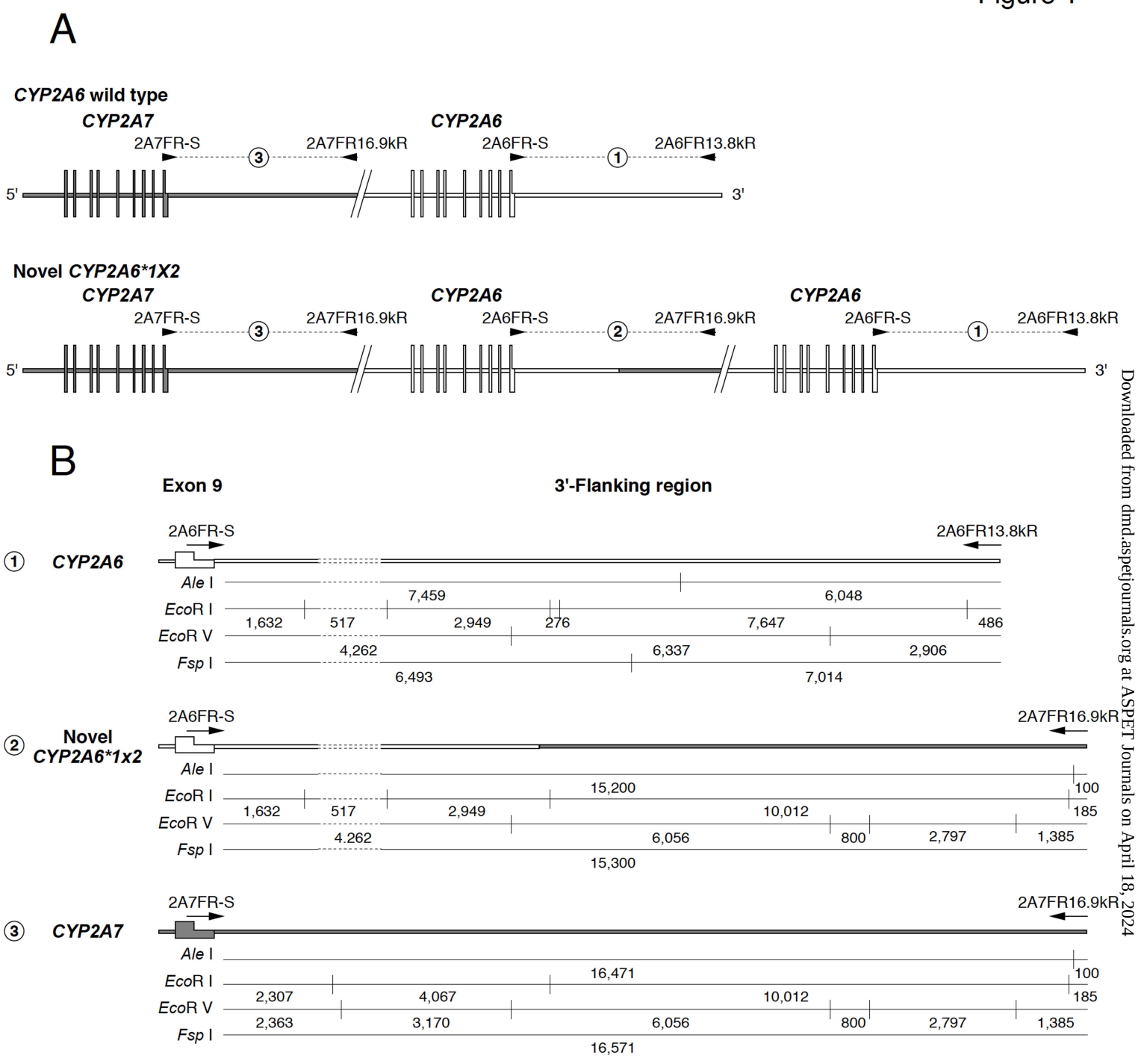

Figure 3