Short Communication

A Novel Duplication Type of CYP2A6 Gene in African-American Population

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ABSTRACT:

Human CYP2A6 is responsible for the metabolism of nicotine and 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 to 5.6 kilobases 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 was not found in European-American (n = 187), Korean (n = 209), or Japanese (n = 184) populations. The plasma cotinine/nicotine ratio in subjects possessing 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 smoking behavior.

Cytochrome P450 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, lortotoloxe, 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). Among them, the 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, lortotoloxe, 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).

Materials and Methods

Chemicals and Reagents. Long and accurate (LA) TaqDNA polymerase and Blend TaqDNA 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 (Ipswich, MA), and MBI Fermentas (Hanover, MD). Nicorette (nicotine gum containing 2 mg of nicotine) was obtained from Pfizer (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*IA (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*1*2 (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),...
TABLE 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>2A6FR-S</td>
<td>5'-AACATCTTCGCAAGGGCTC-3'</td>
</tr>
<tr>
<td>2A7FR-S</td>
<td>5'-AACATCTTCGCAAGGGCTC-3'</td>
</tr>
<tr>
<td>2A6FR4.2kS</td>
<td>5'-GATAAGAAGGATGTTAGAAGTTG-3'</td>
</tr>
<tr>
<td>2A7FR5.4kS</td>
<td>5'-GATAAGAAGGATGTTAGAAGTTG-3'</td>
</tr>
<tr>
<td>2A6FR6.8kR</td>
<td>5'-AGACATGATGCCGAGCAAC-3'</td>
</tr>
<tr>
<td>2A7FR7.8kR</td>
<td>5'-AGACATGATGCCGAGCAAC-3'</td>
</tr>
<tr>
<td>2A6FR13.8kR</td>
<td>5'-GCGAGGCTTGGCTTGGCT-3'</td>
</tr>
<tr>
<td>2A7FR16.9kR</td>
<td>5'-CTCCCTCTTGGAGAAGGATAC-3'</td>
</tr>
</tbody>
</table>

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 (Nakajima et al., 2004), 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) was performed as described previously.

**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*4A/CYP2A6*4A (n = 4), 2.55 to 2.95 for CYP2A6*1/CYP2A6*1 (n = 4), and 4.01 for CYP2A6*1/ conventional CYP2A6*1×2 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*1×2 (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*1×2 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 four 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 to 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 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 geno-
typing 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 six subjects were 1.41 to 1.49 (exon 3–intron 3) and 1.40 to 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 six 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 six 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 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*1×2. This allele is considered to be created through an unequal crossover event at intron 8 to 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
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.
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 to 5.6 kb downstream from the stop codon. Interestingly, the Alu elements were found 4.7 to 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 and Batzer, 1999). For example, duplicated or multiplicated alleles for CYP2A6 are well known, and it has been reported that repetitive sequences containing Alu elements 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 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 has been analyzed only in the Japanese population and found with an allele frequency of 0.24% (Ariyoshi et al., 2004). 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 BspHI. Therefore, a new genotyping method for CYP2A6*4B allele with allele-specific 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 (2681 bp), and the CYP2A6*4B allele was amplified with the primer set 2A7FR5.4kS and 2A6FR6.8kR (2669 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 of 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 the Japanese population, although the frequency of the CYP2A6*4A allele was prominently high, as much 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.

It is interesting that 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 six subjects. The SNPs found in the exons are synonymous. An allele possessing these eight 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 nonsmokers (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*4I*2 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 to 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 increases nicotine metabolism, it might affect the smoking behavior or risk of lung cancer.

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References
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