Comprehensive evaluation of variability in nicotine metabolism and CYP2A6 polymorphic alleles in four ethnic populations

Comprehensive evaluation of variability in nicotine metabolism and *CYP2A6* polymorphic alleles in four world populations

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ABSTRACT

Human cytochrome P450 (CYP) 2A6 metabolizes nicotine to cotinine and is a possible modulator of nicotine addiction. Quantitative and qualitative differences in nicotine addiction have been observed between ethnic groups. However, there is little data on the ethnic influences of the CYP2A6-nicotine metabolism relationship particularly as regards African-Americans. We determined the nicotine metabolism and *CYP2A6* genotype in 176 European-Americans and 160 African-Americans, comparing them with our previous data from 209 Koreans and 92 Japanese. Large interindividual differences were observed in the cotinine/nicotine ratios in plasma calculated as an index of nicotine metabolism in European-Americans (0.6 – 36.5) and in African-Americans (0.9 – 30.4). No ethnic difference was observed between European-Americans (7.2 ± 5.0), African-Americans (7.1 ± 4.7), and Koreans (8.7 ± 11.9), whereas Japanese showed a significantly ($P < 0.005$) lower metabolic ratio (3.8 ± 3.1) than the other populations. Females showed significantly ($P < 0.05$) higher metabolic ratios than males in African-Americans (8.0 ± 5.3 vs 6.0 ± 3.7). Obvious ethnic differences in the *CYP2A6* alleles were observed between these four populations. The combined frequencies of the alleles lacking or showing reduced enzymatic activity (*CYP2A6*2*, *CYP2A6*4*, *CYP2A6*5*, *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10*, *CYP2A6*11*, *CYP2A6*17*, *CYP2A6*19*, and *CYP2A6*20*) were 9.1, 21.9, 42.9, 50.5% in European-Americans, African-Americans, Koreans, and Japanese, respectively. These *CYP2A6* alleles were associated with reduced nicotine metabolism. Among the homozygotes of *CYP2A6*1*, interindividual and ethnic differences in the metabolic ratio were still observed. Thus, some factors other than genetic ones might also contribute to the interindividual and ethnic differences. This comprehensive study of four populations extends our understanding of nicotine metabolism and the impact of genetic polymorphisms of the *CYP2A6* gene.

INTRODUCTION

Cytochrome P450 (CYP)s, a superfamily of heme-containing monooxygenases, are involved in the metabolism of drugs, environmental pollutants, dietary chemicals and endogenous compounds.[1] CYP2A6 is responsible for a major metabolic pathway of nicotine.[2] Since metabolism is the primary route for the elimination of nicotine, variability in the metabolism is a determinant of the clearance of nicotine. CYP2A6 is also a possible modulator of nicotine addiction.[3] We previously evaluated the interindividual differences in nicotine metabolism in Japanese and Korean non-smokers.[4,5] We found that the cotinine/nicotine ratios calculated as a metabolic index in Korean subjects were significantly higher than those in Japanese subjects. Many research groups reported that the plasma cotinine levels in black smokers were higher than those in white smokers.[6-11] The plasma cotinine level greatly depends on the depth of inhalation, the volume of each puff, the force of drawing.[12] Thus, even if the consumption of cigarettes might be taken into consideration, the absolute values of cotinine concentration could not be used as an index of nicotine metabolism. In the present study, a phenotyping method using nicotine gum established in our previous study was applied for non-smokers, in order to determine the interindividual and interethnic differences in nicotine metabolism between European-Americans and African-Americans.

The large interindividual differences in nicotine metabolism are associated with genetic polymorphisms of the *CYP2A6* gene.[4,5,13,14] Among a variety of alleles, the *CYP2A6*2*,[15,16] *CYP2A6*4*,[17-19] *CYP2A6*5*[20] alleles are known to cause a lack of enzymatic activity. The alleles of *CYP2A6*6*,[21] *CYP2A6*7*,[22] *CYP2A6*9*,[14] *CYP2A6*10*,[13] *CYP2A6*11*,[23] *CYP2A6*12*[24] are known to decrease enzymatic activity. Recently, we found *CYP2A6*17*,[25] *CYP2A6*18*,[26] *CYP2A6*19*,[26] and *CYP2A6*20*[27] alleles. The *CYP2A6*17* and *CYP2A6*19* alleles decrease the enzymatic activity and the *CYP2A6*20* allele produces a truncated protein with no activity. Furthermore, several single nucleotide polymorphisms (SNPs) were reported in the 5'-flanking region of the *CYP2A6* gene.[28,29] In the present study, we exhaustively analyzed that

the interindividual, ethnic, and sex differences in nicotine metabolism and the genetic polymorphisms of the *CYP2A6* gene in European-Americans, African-Americans, Koreans, and Japanese. Since we had previously determined through *CYP2A6*11* for Koreans and Japanese, [4,5,13,14] we expanded the genotyping analyses through *CYP2A6*22* and *CYP2A6*1* sub-alleles in the present study. This is the first study to determine the CYP2A6 phenotype and genotype in African-Americans. It has been reported that there were systematic differences in the absolute values of nicotine and cotinine concentrations between laboratories.[30] Therefore, the strength of the present study is that we used a unified phenotyping protocol, analytical method and apparatus to measure the plasma concentrations of nicotine and cotinine, and the same genotyping method for all populations.

METHODS

Chemicals and Regents.

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

Phenotyping of In Vivo Nicotine Metabolism.

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 all subjects. Healthy non-smokers were recruited. No subjects were taking any medications. Exclusion criteria included pregnancy, drug or alcohol

abuse, and abnormal liver, renal, or cardiac function. In our previous studies,[4,5] 209 Korean subjects (18 – 47 years old, 58.3 ± 9.7 kg, 78 males and 131 females) and 92 Japanese subjects (19 – 39 years old, 56.3 ± 10.7 kg, 37 males and 55 females) were already phenotyped for *in vivo* nicotine metabolism. In the present study, 187 European-American subjects (19 – 47 years old, 78.7 ± 20.7 kg, 87 males and 100 females) and 176 African-American subjects (18 – 45 years old, 92.5 ± 28.1 kg, 87 males and 89 females) were recruited. 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. Separated plasma and buffy coat samples were stored at –20°C until analyzed. It has been confirmed that nicotine and cotinine in plasma are stable for >1 year under the condition. All samples with dry ice were shipped to Kanazawa University (Kanazawa, Japan) and the concentrations of nicotine and cotinine in the plasma samples were determined by HPLC as described previously.[31] The cotinine/nicotine ratio of the plasma concentration was calculated as an index of the nicotine metabolism. We previously confirmed that the intraindividual changes in the cotinine/nicotine ratio were at most 6%. [32] Probit transformations of the data were conducted as described previously.[4] Plasma concentrations of nicotine and cotinine before chewing one piece of nicotine gum were measured to confirm non-smoking. Since 11 out of 187 European-Americans and 16 out of 176 African-Americans were judged as smokers based on the baseline presence of nicotine and cotinine, the phenotyping was not performed in these subjects.

Genotyping of CYP2A6 Alleles.

Genomic DNA from all subjects was extracted from peripheral lymphocytes using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). The primers used in the present study are shown in Table I. The genotyping of *CYP2A6*1X2* (duplication),[13] *CYP2A6*2* (L160H),[12] *CYP2A6*3* (*CYP2A6/CYP2A7* hybrid),[12] *CYP2A6*4A* (entire gene deletion),[33] *CYP2A6*4D* (entire gene deletion),[33] *CYP2A6*5* (G479V),[4] *CYP2A6*6* (R128Q),[13] *CYP2A6*7* (I471T),[13] *CYP2A6*8* (R485L),[13] *CYP2A6*10* (I471T and R485L),[13]

*CYP2A6*11* (S224P),[13] *CYP2A6*12* (10 amino acid substitutions),[34] *CYP2A6*13* (g.-48T>G and G5R),[26] *CYP2A6*14* (S29N),[26] *CYP2A6*15* (g.-48T>G and K194E),[26] *CYP2A6*16* (R203S),[26] *CYP2A6*17* (V365M),[25] *CYP2A6*18* (Y392F),[26] *CYP2A6*19* (Y392F and I471T),[26] and *CYP2A6*20* (frameshift)[27] were performed as described previously.

An allele specific (AS)-PCR method for the *CYP2A6*9* allele (g.-48T>G and g.-1013A>G) targeting the SNP of g.-48T>G was modified in the present study. The sense primers were 2A6*9-wt-S or 2A6*9-mut-S and the antisense primer was 2A6int1AS (Table I). The PCR product (385 bp) was analyzed by electrophoresis with 2% agarose gel. The genotyping method for the *CYP2A6*21* allele (K476R) with AS-PCR was established in the present study. The sense primers were 2A6*21-wt or 2A6*21-mut and the antisense primer was 2A6R2 (Table I). The PCR product (421 bp) was analyzed by electrophoresis with 2% agarose gel. A PCR-RFLP method was developed for the genotyping of the *CYP2A6*22* allele (D158E and L160I) targeting the SNP of g.1794C>G. Primers were 2A6int2F and 2A6ex3R1 (Table I). The PCR product was digested with *Eco*N I. The *CYP2A6*1* allele yields a 270-bp fragment and the *CYP2A6*22* allele yields 233- and 37-bp fragments.

The SNPs of g.-1013A>G and g.-745A>G were genotyped by PCR-RFLP and AS-PCR in combination. In the first PCR (PCR I), the sense primer was 2A6-1188F and the antisense primers were 2A6-745wt-AS or 2A6-745mut-AS (Table I). The PCR product was digested with *Bgl* II. The digestion patterns were determined by electrophoresis in a 2% agarose gel (Fig 1). The alleles possessing g.-1013A and g.-745A were assigned to type I. In this type, the alleles were classified as *CYP2A6*1A* or *CYP2A6*1B1*, based on the sequences in 3'-UTR that are derived from the *CYP2A6* or *CYP2A7* sequences, respectively. The genotyping of 3'-UTR was performed by the method that we previously reported.[33] The alleles possessing g.-1013G and g.-745A were assigned to type II. In this type, the alleles were classified as *CYP2A6*1D* or *CYP2A6*1B2*, based on the sequences in 3'-UTR that are derived from the *CYP2A6* or *CYP2A7* sequences, respectively. The alleles possessing g.-1013A and g.-745G were assigned to type III. The allele in which the 3'-UTR has the *CYP2A6* sequence is the *CYP2A6*1H* allele. In this study, we found a novel allele possessing g.-1013A, g.-745G and

gene conversion with *CYP2A7* in 3'-UTR. This allele was termed *CYP2A6*1B13* by the Human CYP Allele Nomenclature Committee. The allele possessing g.-1013G and g.-745G was assigned to type IV. In this type, only the *CYP2A6*1J* allele was found. Thus, the *CYP2A6*1A*, *CYP2A6*1B1*, *CYP2A6*1B2*, *CYP2A6*1B13*, *CYP2A6*1D*, *CYP2A6*1H*, and *CYP2A6*1J* alleles were genotyped by the SNPs of g.-1013A>G and g.-745A>G as well as the sequences in 3'-UTR.

If the subjects are typed as I/II, the genotypes of *CYP2A6*1A*/*CYP2A6*1B2* or *CYP2A6*1B1*/*CYP2A6*1D* are possible. In addition, for the type I/III, the genotypes of *CYP2A6*1A*/*CYP2A6*1B13* or *CYP2A6*1B1*/*CYP2A6*1H* are possible. For the type II/III, the genotypes of *CYP2A6*1B2*/*CYP2A6*1H* or *CYP2A6*1D*/*CYP2A6*1B13* are possible. To determine the genotype, a second PCR (PCR II) was performed. Sense primers were 2A6-1013A-S or 2A6-1013G-S and the antisense primer was 2A7UTR-RV (Table I). Using the LA-PCR product (7753 bp) as the template, the PCR I was performed as described above.

Data Analysis.

Fisher's exact test was used to compare the observed and calculated genotype frequencies. The expected genotype frequencies were calculated using the Hardy-Weinberg equation. The Kruskal-Wallis test or Mann-Whitney *U*-test was used to investigate the ethnic and sex differences in metabolic ratios. $P < 0.05$ was considered statistically significant.

RESULTS

Interindividual and Interethnic Differences in Nicotine Metabolism.

Phenotyping data from 176 European-Americans (82 males and 94 females) and 160 African-Americans (75 males and 85 females) were analyzed. In European-Americans (n = 176), the plasma concentrations of nicotine and cotinine were 2.3 ± 1.2 ng/ml and 13.3 ± 6.0 ng/ml, respectively. The cotinine/nicotine ratios of the plasma concentration calculated as an index of nicotine metabolism ranged from 0.6 – 36.5 (7.2 ± 5.0) (Table II). In African-

Americans (n = 160), the plasma concentrations of nicotine and cotinine were 2.2 ± 1.1 ng/ml and 13.2 ± 6.6 ng/ml, respectively. The cotinine/nicotine ratios ranged from 0.9 – 30.4 (7.1 ± 4.7). Thus, large interindividual differences in nicotine metabolism were observed. Among the present four populations, the Japanese revealed a significantly ($P < 0.005$) lower metabolic ratio than the other populations. The ethnic differences were also obvious in the probit plots of the cotinine/nicotine ratios (Fig 2), since the plots of the Japanese were shifted to the left from the plots of the other populations. These results indicated that Japanese have significantly lower capability for nicotine metabolism than other populations.

Sex Differences in Nicotine Metabolism.

The probit plots of cotinine/nicotine ratios were separately made for males and females (Fig 3). In European-Americans, the plots of the cotinine/nicotine ratios in females shifted to the right from those in males up to the ratio of 10. It was reversed over the ratio of 10. Overall, there was no statistical difference between females and males (Table II). In African-Americans, Koreans, and Japanese, the probit plots of the ratio in females shifted to the right from those in males on the whole. Thus, the ratios in females were higher than in males (Table II), although a statistical difference was observed only in African-Americans ($P < 0.05$). These results suggested that the capability for nicotine metabolism might be higher in females than in males.

Allele Frequencies of CYP2A6 Alleles.

The genotyping of the *CYP2A6* gene was performed for 187 European-Americans, 176 African-Americans, 209 Koreans, and 92 Japanese (Table III). The genotype frequencies were in accordance with the Hardy-Weinberg equation. No sex difference was observed in the allele frequencies in any population (data not shown). The only allele found in all populations was *CYP2A6*9*, although the frequencies could be divided roughly into two groups: Americans and Asians. In European-Americans, the alleles of *CYP2A6*2*, *CYP2A6*14*, *CYP2A6*16*, and *CYP2A6*21* were found. These alleles were also found in African-

Americans, but not in Koreans and Japanese. In addition, in African-Americans, *CYP2A6*4A*, *CYP2A6*4D*, *CYP2A6*17*, and *CYP2A6*20* were found, whereas *CYP2A6*18* was found in European-Americans.

In Koreans and Japanese, the alleles of *CYP2A6*4A*, *CYP2A6*7*, *CYP2A6*8*, *CYP2A6*10*, *CYP2A6*11*, *CYP2A6*13*, *CYP2A6*15* were found in common. It should be emphasized that the allele frequency of *CYP2A6*4A* was prominently higher in Japanese than in the other populations. In Koreans, the alleles of *CYP2A6*1X2*, *CYP2A6*18*, and *CYP2A6*19* were also found.

SNPs in the 5'-Flanking Region and Gene Conversion in 3'-Untranslated Region.

The homozygotes of *CYP2A6*1* (126 European-Americans, 91 African-Americans, 64 Koreans, and 22 Japanese) were next subjected to the genotyping of the *CYP2A6*1A*, *CYP2A6*1B1*, *CYP2A6*1B2*, *CYP2A6*1B13*, *CYP2A6*1D*, *CYP2A6*1H*, and *CYP2A6*1J* alleles. In this analysis, we found an African-American subject who possessed three alleles of *CYP2A6*1A*, *CYP2A6*1D*, and *CYP2A6*1H*. If the subject had been genotyped for the duplication allele of *CYP2A6*1X2*, it would mean that he has three *CYP2A6* alleles. However, he was not genotyped for the *CYP2A6*1X2* allele. We confirmed that the relative gene copy number ratio of *CYP2A6*/*CYP2A7* in exon 3 or exon 5 in this subject was 1.5 (data not shown).[33] These results suggested that he would have a novel duplication allele other than the *CYP2A6*1X2* allele. Therefore, the number of alleles in 91 African-Americans was suspected to be 183 as shown in Table IV. The *CYP2A6* gene structure in this subject is now being analyzed in detail in our laboratory.

The frequency of the *CYP2A6*1A* allele was higher than that of the *CYP2A6*1B1* allele in all populations. In European-Americans and African-Americans, the frequencies of *CYP2A6*1D* were higher than those of *CYP2A6*1B2*, but the frequencies of *CYP2A6*1B2* were higher than those of *CYP2A6*1D* in Koreans and Japanese. The frequencies of *CYP2A6*1H* were higher than those of *CYP2A6*1B13* in European-Americans and African-Americans, but the frequencies of *CYP2A6*1B13* were higher than those of *CYP2A6*1H* in

Koreans and Japanese. The *CYP2A6*1J* allele was not found in any population in this study.

Effects of Genetic Polymorphisms of CYP2A6 on Nicotine Metabolism.

The cotinine/nicotine ratios in homozygotes of *CYP2A6*1* were compared among different sub-genotypes (Table V). We could not find any association between the *CYP2A6*1* sub-genotypes and the phenotype. Therefore, the *CYP2A6*1* sub-genotypes were regarded as *CYP2A6*1* in further analyses. In addition, the *CYP2A6*4A* and *CYP2A6*4D* alleles were regarded as *CYP2A6*4*, since both alleles delete the entire *CYP2A6* gene.

In European-Americans, the mean cotinine/nicotine ratio in homozygotes of *CYP2A6*1* was 7.7 ± 5.5 (Table VI). The heterozygotes of *CYP2A6*1* appeared to show ratios similar to those of homozygotes of *CYP2A6*1*. Although statistical analysis could not be performed because of the limited number of samples, 2 subjects with *CYP2A6*2*/*CYP2A6*9* revealed lower nicotine metabolic ratios. In contrast, 2 subjects with *CYP2A6*9*/*CYP2A6*18* revealed moderate ratios, indicating that the effects of mutation of *CYP2A6*18* would be weak on nicotine metabolism. A homozygote of *CYP2A6*14* revealed a relatively low ratio.

In African-Americans, the mean cotinine/nicotine ratio in homozygotes of *CYP2A6*1* was 8.1 ± 5.1. The subjects with *CYP2A6*1*/*CYP2A6*14* revealed significantly higher nicotine metabolic ratios compared with homozygotes of *CYP2A6*1*. Since a European-American *CYP2A6*14* homozygote revealed a relatively low ratio, we could not find the effects of the *CYP2A6*14* allele on *in vivo* nicotine metabolism. As we previously reported,[25] the subjects with *CYP2A6*1*/*CYP2A6*17* or *CYP2A6*17*/*CYP2A6*17* revealed significantly ($P < 0.05$) lower metabolic ratios compared with homozygotes of *CYP2A6*1*. Furthermore, the subjects with *CYP2A6*4*/*CYP2A6*9*, *CYP2A6*9*/*CYP2A6*9*, *CYP2A6*9*/*CYP2A6*17* , *CYP2A6*14*/*CYP2A6*17* , *CYP2A6*17*/*CYP2A6*20* , and *CYP2A6*20*/*CYP2A6*21* also revealed relatively low metabolic ratios, although we could not perform statistical analysis because of the limited number of subjects. In addition, the subjects with *CYP2A6*1/CYP2A6*2*, *CYP2A6*1/CYP2A6*4*, or *CYP2A6*1/CYP2A6*20* also revealed relatively low metabolic ratios. Thus, the subjects with *CYP2A6*2*, *CYP2A6*4*, *CYP2A6*17*,

*CYP2A6*20* alleles revealed low nicotine metabolism activity. Subjects with *CYP2A6*16/CYP2A6*16* or *CYP2A6*16*/*CYP2A6*17* revealed a similar nicotine metabolic ratio compared to the homozygotes of *CYP2A6*1*, indicating that the *CYP2A6*16* allele might not affect the enzymatic activity.

In Koreans, the mean cotinine/nicotine ratio in homozygotes of *CYP2A6*1* was 13.7 ± 18.7. The subjects with *CYP2A6*1*/*CYP2A6*7* or *CYP2A6*1*/*CYP2A6*15* revealed a significantly ($P < 0.05$) lower nicotine metabolic ratio compared with homozygotes of *CYP2A6*1*. As we previously reported, the subjects possessing two alleles of *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10*, and *CYP2A6*19* in combination revealed low nicotine metabolic ratios. Especially, the metabolic ratios of the homozygotes of *CYP2A6*4* were zero. Our result that a subject with *CYP2A6*7*/*CYP2A6*11* revealed a low nicotine metabolic ratio (2.9) was consistent with a previous *in vitro* study showing that CYP2A6.11 had decreased enzymatic activity.[23] A subject with *CYP2A6*11*/*CYP2A6*13* revealed a relatively low nicotine metabolic ratio. The subjects with *CYP2A6*1*/*CYP2A6*15* revealed significantly ($P < 0.05$) lower nicotine metabolic ratios than the subjects with *CYP2A6*1*/*CYP2A6*9*.

In Japanese, the mean cotinine/nicotine ratio in homozygotes of *CYP2A6*1* was 5.7 ± 4.0. Similar to Koreans, the subjects possessing two alleles of *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, and *CYP2A6*10* in combination revealed low nicotine metabolic ratios. Especially, the metabolic ratios of the homozygotes of *CYP2A6*4* were zero. A subject with *CYP2A6*7*/*CYP2A6*13* revealed a relatively low nicotine metabolic ratio, suggesting that the *CYP2A6*13* allele might cause the decreased activity. A subject with *CYP2A6*4*/*CYP2A6*15* revealed a similar nicotine metabolic ratio to that in the subjects with *CYP2A6*4*/*CYP2A6*9*, indicating that the *CYP2A6*15* allele also might cause the decreased activity. The *CYP2A6*13* and *CYP2A6*15* alleles have the SNP in the TATA box (g.-48T>G) found in the *CYP2A6*9* allele as well as the SNPs leading to amino acid changes of G5R and K194E, respectively.[35] Although the effects of the amino acid changes on the enzymatic activity remain to be investigated *in vitro*, we clarified that *CYP2A6*13* and *CYP2A6*15* decreased the enzymatic activity *in vivo*.

If a probit plot produces curved or broken lines, it indicates that each data point is not normally distributed. Although the plots of Koreans and Japanese had antimode of approximately 0.6, those of European-Americans and African-Americans did not show a clear antimode. This would be due to the fact that there were no subjects possessing two alleles with dramatically decreased or lacking enzymatic activity among European-Americans and African-Americans.

Interindividual, Ethnic, and Sex Differences in Nicotine Metabolism in Homozygotes of CYP2A6*1.

Among the homozygotes of *CYP2A6*1*, the interindividual differences in the metabolic ratio were still large in European-Americans (0.6 – 36.5, 7.7 ± 5.5), African-Americans (0.9 – 30.4, 8.1 ± 5.1), Koreans (1.8 – 143.9, 13.7 ± 18.7), and Japanese (0.9 – 14.7, 5.7 ± 4.0) (Fig 4, Table VI). The mean metabolic ratio in Japanese was significantly ($P < 0.05$) lower than those in African-Americans and Koreans. On the other hand, the mean metabolic ratio in Koreans was significantly ($P < 0.005$) higher than in the other populations (Table VI).

In European-Americans, the mean metabolic ratios in females (n = 72) tended to be lower than that in males (n = 54) (7.1 ± 3.4 vs 8.4 ± 7.4). In contrast, in African-Americans (42 females and 48 males), Koreans (45 females and 19 males), and Japanese (10 females and 12 males), the mean metabolic ratios in females tended to be higher than that in males (9.3 ± 6.0 vs 7.1 ± 3.9, 14.1 ± 20.9 vs 13.0 ± 12.7, and 7.5 ± 4.7 vs 4.2 ± 2.6, respectively). However, no statistical difference was observed.

DISCUSSION

Nicotine is responsible for the addiction to smoking which is associated with a higher incidence of various types of cancers, respiratory and cardiovascular disease, gastrointestinal disorders as well as many other medical complications.[36] Nicotine has roles in replacement therapy for smoking cessation and has been studied as an experimental therapy for several diseases such as Parkinson's disease, Alzheimer's disease, and ulcerative colitis.[37-39] Therefore,

the variability of nicotine metabolism would have an impact on various clinical outcomes. Furthermore, several research groups have reported that smoking behavior and lung cancer risk are related to genetic polymorphisms of the *CYP2A6* gene.[3,40] In the present study, we determined the interindividual, ethnic, and sex differences in nicotine metabolism and genetic polymorphisms of the *CYP2A6* gene in European-Americans, African-Americans, Koreans, and Japanese. We found that there was no significant difference in the nicotine metabolic ratio between European-Americans and African-Americans. Therefore, the differences in plasma cotinine levels between white and black people that were reported previously would not be due to differences in the nicotine metabolism activity. As previously reported, it may be that blacks take in significantly more nicotine per cigarette compared with whites,[10] or blacks have significantly lower clearance of cotinine than whites.[41] Since cotinine is metabolized to *trans*-3'-hydroxycotinine by CYP2A6[42] and *N*-glucuronide by UGT1A4,[43] Benowitz et al.[41] considered that the observed slow metabolism of cotinine in blacks may be due to reduced CYP2A6 activity and glucuronidation activity. However, in the present study, we were able to exclude ethnic differences in the CYP2A6 activity between European-Americans and African-Americans. CYP2A6 catalyzes the metabolic activation of tobacco-specific nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).[44] The incidence of lung cancer has been reported to be greater in blacks compared with whites.[45] Since we found no ethnic difference in CYP2A6 activity between blacks and whites, the difference in the incidence of lung cancer might be because blacks inhale more deeply, which could be a reason for smoking mentholated cigarettes,[46] resulting in greater carcinogen exposure.

We found that the nicotine metabolism potency in Japanese was significantly lower than that in other populations. This result was consistent with a previous *in vitro* study to showing that the coumarin 7-hydroxylation activity in liver microsomes from Japanese was lower than that in liver microsomes from Caucasians.[47] One of the reasons for the low CYP2A6 activity in Japanese would be a prominently high frequency of *CYP2A6*4* alleles (19.0%). There were ethnic differences in the allele frequencies of the *CYP2A6* gene. The combined frequencies of the alleles lacking or showing reduced enzymatic activity

(*CYP2A6*2*, *CYP2A6*4*, *CYP2A6*5*, *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10*, *CYP2A6*11*, *CYP2A6*17*, *CYP2A6*19*, and *CYP2A6*20*) were 9.1, 21.9, 42.9, 50.5% in European-Americans, African-Americans, Koreans, and Japanese, respectively. It should be noted that the interindividual differences in the metabolic ratio were still large among the homozygotes of *CYP2A6*1*. Furthermore, among the homozygotes of *CYP2A6*1*, Japanese revealed significantly lower nicotine metabolism than African-Americans and Koreans, whereas Koreans revealed significantly higher nicotine metabolism than the other populations. Therefore, some factors other than genetic ones, such as diet and/or environmental factors as well as unknown or uncharacterized alleles might contribute to the interindividual and ethnic differences. In addition, we may have to consider differences in the post-transcriptional or post-translational regulation of CYP2A6.

Concerning the SNPs in the 5'-flanking region of the *CYP2A6* gene, we analyzed three SNPs of g.-1013A>G, g.-745A>G, and g.-48T>G. It has been reported that these SNPs decrease the transcriptional activity in luciferase assays.[28,29,48] The CYP2A6 mRNA level, protein level, and enzymatic activity have been reported to be decreased by the SNP of g.-48T>G in human livers,[14,49] but not by g.-1013A>G.[50] For the SNP of g.-745A>G, increased mRNA level in human livers has been reported.[50] In the present study, we found no significant effects of the SNPs of g.-1013A>G and g.-745A>G on *in vivo* nicotine metabolism (Table V).

Sex differences (female > male) in nicotine metabolism were observed in the present study. Previously, Zeman et al.[51] have reported that the ratio of nicotine/(cotinine + 3'-hydroxycotinine) in 24-hr urine was significantly lower in females compared with males. Benowitz et al.[52] has clearly shown that nicotine and cotinine clearances are higher in females compared with males after intravenous infusion of both nicotine and cotinine. In addition, it has been reported that the urinary excretion of 7-hydroxycoumarin was higher in females compared with males.[53,54] An *in vitro* study revealed that the coumarin 7-hydroxylation activity in liver microsomes obtained from females was higher than in those obtained from males.[55] Our results and these previous papers suggested that CYP2A6 activity in females is higher than in males. It has been reported that nicotine and cotinine clearance is higher in

pregnancy compared with postpartum[56] and is accelerated by oral contraceptive use in females.[52] Females have higher concentrations of estrogens and progesterone than males; the concentrations of these sex hormones are increased with the use of oral contraceptives or during pregnancy. Thus, these hormones might possibly induce CYP2A6. Further study is needed to clarify the mechanism of the sex differences in CYP2A6 activity.

In conclusion, we comprehensively determined the interindividual, ethnic, and sex differences in nicotine metabolism and genetic polymorphisms of the *CYP2A6* gene in European-Americans, African-Americans, Koreans, and Japanese. The findings in this study extend our understanding of nicotine metabolism and the impact of genetic polymorphisms of the *CYP2A6* gene.

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References

1. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, et al. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 1996;6:1-42.
2. Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, et al. Role of human cytochrome P4502A6 in *C*-oxidation of nicotine. Drug Metab Dispos 1996;24:1212-7.
3. Malaiyandi V, Sellers EM, Tyndale RF. Implications of *CYP2A6* genetic variation for smoking behaviors and nicotine dependence. Clin Pharmacol Ther 2005;77:145-58.
4. Nakajima M, Kwon J-T, Tanaka N, Zenta T, Yamamoto Y, Yamamoto H, et al. Relationship between interindividual differences in nicotine metabolism and *CYP2A6* genetic polymorphism in humans. Clin Pharmacol Ther 2001;69:72-8.
5. Kwon J-T, Nakajima M, Chai S, Yom Y-K, Kim H-K, Yamazaki H, et al. Nicotine metabolism and *CYP2A6* allele frequencies in Koreans. Pharmacogenetics 2001;11:317-23.
6. Wagenknecht LE, Cutter GR, Haley NJ, Sidney S, Manolio TA, Hughes GH, et al. Racial differences in serum cotinine levels among smokers in the coronary artery risk development in (young) adult study. Am J Public Health 1990;80:1053-6.
7. English PB, Eskenazi B, Christianson RE. Black-White differences in serum cotinine levels among pregnant women and subsequent effect on infant birthweight. Am J Public Health 1994;84:1439-43.
8. Clark PI, Gautam S, Gerson LW. Effect of menthol cigarettes on biochemical markers of smoke exposure among Black and White smokers. CHEST 1996;110:1194-8.
9. Caraballo RS, Giovino GA, Pechacek TF, Mowery PD, Richter PA, Strauss WJ, et al. Racial and ethnic differences in serum cotinine levels of cigarette smokers. J Am Med Assoc 1998;280:135-9.
10. Pérez-Stable EJ, Herrera B, Jacob P III, Benowitz NL. Nicotine metabolism and intake in black and white smokers. J Am Med Assoc 1998;280:152-6.

11. Ahijevych KL, Tyndale RF, Dhatt RK, Weed HG, Browning KK. Factors influencing cotinine half-life during smoking abstinence in African-American and Caucasian women. Nicotine Tob Res 2002;4:423-31.
12. Nakajima M, Yamagishi S, Yamamoto H, Yamamoto T, Kuroiwa Y, Yokoi T. Deficient cotinine formation from nicotine is attributed to the whole deletion of the *CYP2A6* gene in humans. Clin Pharmacol Ther 2000;67:57-69.
13. Yoshida R, Nakajima M, Watanabe Y, Kwon J-T, Yokoi T. Genetic polymorphisms in human *CYP2A6* gene causing impaired nicotine metabolism. Br J Clin Pharmacol 2002;54:511-7.
14. Yoshida R, Nakajima R, Nishimura K, Tokudome S, Kwon J-T, Yokoi T. Effects of polymorphism in promoter region of human *CYP2A6* gene (*CYP2A6*9*) on expression level of messenger ribonucleic acid and enzymatic activity in vivo and in vitro. Clin Pharmacol Ther 2003;74:69-76.
15. Yamano S, Tatsuno J, Gonzalez FJ. The *CYP2A3* gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. Biochemistry 1990;29:1322-9.
16. Oscarson M, Gullustén H, Raunio A, Bernal ML, Sinues B, Dahl ML, et al. Genotyping of human cytochrome P450 2A6 (CYP2A6), a nicotine *C*-oxidase. FEBS Lett 1998;438:201-5.
17. Oscarson M, McLellan RA, Gullstén H, Yue QY, Lang MA, Bernal ML, et al. Characterization and PCR-based detection of a *CYP2A6* gene deletion found at a high frequency in a Chinese population. FEBS Lett 1999;448:105-10.
18. Nunoya K-I, Yokoi T, Kimura K, Kainuma T, Saito K, Kinoshita M, et al. A new *CYP2A6* gene deletion responsible for the in vivo polymorphic metabolism of (+)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride in humans. J Pharmacol Exp Ther 1999;289:437-42.
19. Nunoya K-I, Yokoi T, Takahashi Y, Kimura K, Kinoshita M, Kamataki T. Homologous unequal cross-over within the human *CYP2A* gene cluster as a mechanism for the deletion of the entire *CYP2A6* gene associated with the poor metabolizer phenotype. J Biochem 1999;126:402-7.

20. Oscarson M, McLellan RA, Gullstén H, Agúndez JAG, Benítez J, Raunio A, et al. Identification and characterization of novel polymorphisms in the *CYP2A* locus: implications for nicotine metabolism. FEBS Lett 1999;460:321-7.
21. Kitagawa K, Kunugita N, Kitagawa M, Kawamoto T. *CYP2A6*6*, a novel polymorphism in cytochrome P450 2A6, has a single amino acid substitution (R128Q) that inactivates enzymatic activity. J Biol Chem 2001;276:17830-5.
22. Ariyoshi N, Sawamura Y, Kamataki T. A novel single nucleotide polymorphism altering stability and activity of CYP2A6. Biochem Biophys Res Commun 2001;281:810-4.
23. Daigo S, Takahashi Y, Fujieda M, Ariyoshi N, Yamazaki H, Koizumi W, et al. A novel mutant allele of the *CYP2A6* gene (*CYP2A6*11*) found in a cancer patient who showed poor metabolic phenotype towards tegafur. Pharmacogenetics 2002;12:299-306.
24. Oscarson M, McLellan RA, Asp V, Ledesma M, Ruiz MLB, Sinues B, et al. Characterization of a novel *CYP2A7*/*CYP2A6* hybrid allele (*CYP2A6*12*) that causes reduced CYP2A6 activity. Hum Mutat 2002;20:275-83.
25. Fukami T, Nakajima M, Yoshida R, Tsuchiya Y, Fujiki Y, Katoh M, et al. A novel polymorphism of *CYP2A6* gene *CYP2A6*17* has an amino acid substitution (V365M) that decreases enzymatic activity in vitro and in vivo. Clin Pharmacol Ther 2004;76:519-27.
26. Fukami T, Nakajima M, Higashi E, Yamanaka H, Sakai H, McLeod HL, et al. Characterization of novel *CYP2A6* polymorphic alleles (*CYP2A6*18* and *CYP2A6*19*) that affect enzymatic activity. Drug Metab Dispos 2005;33:1202-10.
27. Fukami T, Nakajima M, Higashi E, Yamanaka H, McLeod HL, Yokoi T. A novel *CYP2A6*20* allele found in African-American population produces a truncated protein lacking enzymatic activity. Biochem Pharmacol 2005;70:801-8.
28. Pitarque M, von Richter O, Rodriguez-Antona C, Wang J, Oscarson M, Ingelman-Sundberg M. A nicotine *C*-oxidase gene (*CYP2A6*) polymorphism important for promoter activity. Hum Mutat 2004;23:258-66.
29. von Richter O, Pitarque M, Rodriguez-Antona C, Testa A, Mantovani R, Oscarson M, et al. Polymorphic NF-Y dependent regulation of human nicotine *C*-oxidase (CYP2A6).

Pharmacogenetics 2004;14:369-79.

30. Biber A, Scherer G, Hoepfner I, Adlkofer F, Heller WD, Haddow JE, et al. Determination of nicotine and cotinine in human serum and urine: an interlaboratory study. Toxicol Lett 1987;35:45-52.
31. Nakajima M, Yamamoto T, Kuroiwa Y, Yokoi T. Improved highly sensitive method for determination of nicotine and cotinine in human plasma by high-performance liquid chromatography. J Chromatogr B 2000;742:211-5.
32. Nakajima M, Itoh M, Yamanaka H, Fukami T, Tokudome S, Yamamoto Y, et al. Isoflavones inhibit nicotine *C*-oxidation catalyzed by human CYP2A6. J Clin Pharmacol 2006;46:337-44.
33. Fukami T, Nakajima M, Sakai H, McLeod HL, Yokoi T. *CYP2A7* polymorphic alleles confound the genotyping of *CYP2A6*4A* allele. Pharmacogenomics J in press.
34. Nakajima M, Yoshida R, Fukami T, McLeod HL, Yokoi T. Novel human *CYP2A6* alleles confound gene deletion analysis. FEBS Lett 2004;569:75-81.
35. Kiyotani K, Fujieda M, Yamazaki H, Shimada T, Guengerich FP, Parkinson A, et al. Twenty one novel single nucleotide polymorphisms (SNPs) of the *CYP2A6* gene in Japanese and Caucasians. Drug Metab Pharmacokin 2002;17:482-7.
36. Lee EW, D'Alonzo GE. Cigarette smoking, nicotine addiction, and its pharmacologic treatment. Arch Inter Med 1993;153:34-48.
37. Jani N, Regueiro MD. Medical therapy for ulcerative colitis. Gastroenterol Clin North Am 2002;31:147-66.
38. Quik M. Kulak JM. Nicotine and nicotinic receptors; relevance to Parkinson's disease. Neurotoxicology 2002;23:581-94.
39. Sabbagh MN, Lukas RJ, Sparks DL, Reid RT. The nicotinic acetylcholine receptor, smoking, and Alzheimer's disease. J Alzheimers Dis 2002;4:317-25.
40. Kamataki T, Fujieda M, Kiyotani K, Iwano S, Kunitoh H. Genetic polymorphism of CYP2A6 as one of the potential determinants of tobacco-related cancer risk. Biochem Biophys Res Commun 2005;338:306-10.
41. Benowitz NL, Perez-Stable EJ, Fong I, Modin G, Herrera B, Jacob P III. Ethnic

differences in *N*-glucuronidation of nicotine and cotinine. J Pharmacol Exp Ther 1999;291:1196-203.

42. Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, et al. Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. J Pharmacol Exp Ther 1996;277:1010-5.
43. Nakajima M, Tanaka E, Kwon J-T, Yokoi T. Characterization of nicotine and cotinine *N*-glucuronidations in human liver microsomes. Drug Metab Dispos 2002;30:1484-90.
44. Yamazaki H, Inui Y, Yun CH, Guengerich FP, Shimada T. Cytochrome P450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of *N*-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes. Carcinogenesis 1992;13:1789-94.
45. Harris RE, Zang EA, Anderson JI, Wynder EL. Race and sex differences in lung cancer risk associated with cigarette smoking. Int J Epidemiol 1993;22:592-9.
46. Hymowitz N, Mouton C, Edkholdt H. Menthol cigarette smoking in African Americans and Whites. Tob Control 1995;4:194-7.
47. Shimada T, Yamazaki H, Guengerich FP. Ethnic-related differences in coumarin 7-hydroxylation activities catalyzed by cytochrome P4502A6 in liver microsomes of Japanese and Caucasian populations. Xenobiotica 1996;26:395-403.
48. Pitarque M, von Richter O, Oke B, Berkkan H, Oscarson M, Ingelman-Sundberg M. Identification of a single nucleotide polymorphism in the TATA box of the *CYP2A6* gene: impairment of its promoter activity. Biochem Biophys Res Commun 2001;284:455-60.
49. Kiyotani K, Yamazaki H, Fujieda M, Iwano S, Matsumura K, Satarug S, et al. Decreased coumarin 7-hydroxylase activities and CYP2A6 expression levels in humans caused by genetic polymorphism in *CYP2A6* promoter region (*CYP2A6**9*). Pharmacogenetics 2003;13:689-95.
50. Haberl M, Anwald B, Klein K, Weil R, Fuss C, Gepdiremen A, et al. Three haplotypes associated with *CYP2A6* phenotypes in Caucasians. Pharmacogenet Genomics 2005;15:609-24.
51. Zeman MV, Hiraki L, Sellers EM. Gender differences in tobacco smoking: higher relative

exposure to smoke than nicotine in women. J Womens Health Gend Based Med 2002;11:147-53.

52. Benowitz NL, Swan GE, Lessov CN, Jacob P III. Oral contraceptives induce CYP2A6 activity and accelerate nicotine metabolism (abstract). Clin Pharmacol Ther 2004;75:36.
53. Ujjin P, Satarug S, Vabavanitkun Y, Daigo S, Ariyoshi N, Yamazaki H, et al. Variation in coumarin 7-hydroxylase activity associated with genetic polymorphism of cytochrome P450 2A6 and the body status of iron stores in adult Thai males and females. Pharmacogenetics 2002;12:241-9.
54. Iscan M, Rostami H, Iscan M, Guray T, Pelkonen O, Raunio A. Interindividual variability of coumarin 7-hydroxylation in a Turkish population. Eur J Clin Pharmacol 1994;47:315-8.
55. Parkinson A, Mudra DR, Johnson C, Dwyer A, Carroll KM. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. Toxicol Appl Pharmacol 2004;199:193-209.
56. Dempsey D, Jacob P III, Benowitz NL. Accelerated metabolism of nicotine and cotinine in pregnant smokers. J Pharmacol Exp Ther 2002;301:594-8.

Figure legend

Fig 1. Genotyping of *CYP2A6*1* sub-alleles by PCR-RFLP and AS-PCR in combination. **A**, Schematic structures of *CYP2A6* gene. Open and dotted boxes represent exons of *CYP2A6* and *CYP2A7,* respectively. Lines represent 5'-flanking regions or introns. PCR amplification was performed with the primer pairs indicated by horizontal arrows. The primer 2A6-745wt-AS specifically anneals to the *CYP2A6*1A, CYP2A6*1B1, CYP2A6*1B2, CYP2A6*1D* alleles, whereas the primer 2A6-745mut-AS specifically anneals to the *CYP2A6*1H, CYP2A6*1J,* and *CYP2A6*1B13* alleles. The amplified product was digested with *Bgl* II. The restriction sites of *Bgl* II are indicated by vertical arrows. **B**, Schematic PCR-RFLP patterns for different *CYP2A6* genotypes. After digestion with *Bgl* II, *CYP2A6*1A* and *CYP2A6*1B1* alleles yield 286-bp and 195-bp fragments in the primer pair of 2A6-1188F and 2A6-745wt-AS; *CYP2A6*1D* and *CYP2A6*1B2* alleles yield 481-bp fragment in the primer pair of 2A6-1188F and 2A6-745wt-AS; *CYP2A6*1H* and *CYP2A6*1B13* alleles yield 286-bp and 195-bp fragments in the primer pair of 2A6-1188F and 2A6-745mut-AS; *CYP2A6*1J* allele yields 481-bp fragment in the primer pair of 2A6-1188F and 2A6-745mut-AS.

Fig 2. Probit analysis for cotinine/nicotine ratios of the plasma concentration 2 hr after chewing one piece of nicotine gum in 176 European-Americans, 160 African-Americans, 209 Koreans and 92 Japanese. The abscissa denotes the cotinine/nicotine ratio of the plasma concentration in different individuals. The ordinate represents the percent area under the normal probability curve for each data point. The plots for Koreans and Japanese were quoted from our previous study.[4,5]

Fig 3. Probit analysis for cotinine/nicotine ratios of the plasma concentration 2 hr after chewing one piece of nicotine gum. **A**, 94 Female and 82 male European-Americans, **B**, 85 female and 75 male African-Americans, **C**, 131 female and 78 male Koreans and **D**, 55 female and 37 male Japanese. The abscissa denotes the cotinine/nicotine ratio of the plasma

concentration in different individuals. The ordinate represents the percent area under the normal probability curve for each data point.

Fig 4. The cotinine/nicotine ratios in subjects with different *CYP2A6* genotypes. **A**, 176 European-Americans, **B**, 160 African-Americans, **C**, 209 Koreans, and **D**, 92 Japanese. Circles show each subject and bars show the mean of each group. *$P < 0.05$, compared with the homozygotes of *CYP2A6*1*.

Table I. Primers used in the present study

Primer	*Sequence*	*Location*
2A6-1188F	5’-CTGACAAAGCAGGAATCATT-3’	5’-flanking region
2A6-1013A-S	5’-GTCTGTTTTCTGTCCTCTGTA-3’	5’-flanking region
2A6-1013G-S	5’-GTCTGTTTTCTGTCCTCTGTG-3’	5’-flanking region
2A6-745wt-AS	5’-TCCACTGCCCATCTCTGAT-3’	5’-flanking region
2A6-745mut-AS	5’-TCCACTGCCCATCTCTGAC-3’	5’-flanking region
2A6*9-wt-S	5’-TCCCTCTTTTTCAGGCAGGCAGTAT-3’	5’-flanking region
2A6*9-mut-S	5’-TCCCTCTTTTTCAGGCAGGCAGTAG-3’	5’-flanking region
2A6int1AS[a]	5’-TCCTGTCTTTCTGATGCTGA-3’	intron 1
2A6int2F	5’-TGTCTCCATTCCCGCGTTC-3’	intron 2
2A6ex3R1[b]	5’-GTCCCCTGCTCACCGCCA-3’	exon 3
2A6*21-wt	5’-CATTGACGTGTCCCCCAA-3’	exon 9
2A6*21-mut	5’-CATTGACGTGTCCCCCAG-3’	exon 9
2A7UTR-RV	5’-ATTCTTATACCCGCCTCTTCCGCGAA-3’	3’-untranslated region
2A6R2[c]	5’-AAAATGGGCATGAACGCCC-3’	3’-flanking region

[a]Data from Fukami et al.[25]

[b]Data from Oscarson et al.[24]

[c]Data from Oscarson et al.[17]

Table II. The cotinine/nicotine ratios in four populations

Population	*Overall*	*Female*	*Male*
European-Americans	7.2 ± 5.0 (n = 176)	7.1 ± 3.6 (n = 94)	7.2 ± 6.3 (n = 82)
African-Americans	7.1 ± 4.7 (n = 160)	8.0 ± 5.3[†] (n = 85)	6.0 ± 3.7 (n = 75)
Koreans	8.7 ± 11.9 (n = 209)	9.4 ± 13.6 (n = 131)	7.7 ± 8.2 (n = 78)
Japanese	3.8 ± 3.1[*] (n = 92)	4.1 ± 3.5 [*] (n = 55)	3.3 ± 2.4 [*] (n = 37)

n: number of subjects.

[*] $P < 0.005$, Japanese showed significantly lower nicotine metabolic ratio than the other populations.

[†] $P < 0.05$, In African-Americans, females showed significantly higher nicotine metabolic ratio than males.

Table III. Allele frequencies (%) of *CYP2A6* in four populations

Allele	*Effects*	*European-Americans*	*African-Americans*	*Koreans*	*Japanese*
*CYP2A6*1*		84.5 (n = 316)	74.4 (n = 262)	53.3 (n = 223)	45.1 (n = 83)
*CYP2A6*1X2*	duplication	0	0	0.2 (n = 1)	0
*CYP2A6*2*	L160H	1.1 (n = 4)	0.3 (n = 1)	0	0
*CYP2A6*3*	*CYP2A6*/*CYP2A7* hybrid	0	0	0	0
*CYP2A6*4A*	*CYP2A6* deleted	0	0.6 (n = 2)	10.8 (n = 45)	19.0 (n = 35)
*CYP2A6*4D*	*CYP2A6* deleted	0	0.3 (n = 1)	0	0
*CYP2A6*5*	G479V	0	0	0.5 (n = 2)	0
*CYP2A6*6*	R128Q	0	0	0	0
*CYP2A6*7*	I471T	0	0	9.8 (n = 41)	9.8 (n = 18)
*CYP2A6*8*	R485L	0	0	1.2 (n = 5)	1.1 (n = 2)
*CYP2A6*9*	SNPs of A-1013G and T-48G	8.0 (n = 30)	8.5 (n = 30)	19.6 (n = 82)	19.0 (n = 35)
*CYP2A6*10*	I471T; R485L	0	0	1.0 (n = 4)	2.2 (n = 4)
*CYP2A6*11*	S224P	0	0	0.7 (n = 3)	0.5 (n = 1)
*CYP2A6*12*	10 amino acid substitutions	0	0	0	0
*CYP2A6*13*	SNP of T-48G; G5R	0	0	0.2 (n = 1)	1.1 (n = 2)
*CYP2A6*14*	S29N	3.5 (n = 13)	1.4 (n = 5)	0	0
*CYP2A6*15*	SNP of T-48G; K194E	0	0	1.2 (n = 5)	2.2 (n = 4)
*CYP2A6*16*	R203S	0.3 (n = 1)	1.7 (n = 6)	0	0
*CYP2A6*17*	V365M	0	10.5 (n = 37)	0	0
*CYP2A6*18*	Y392F	2.1 (n = 8)	0	0.5 (n = 2)	0
*CYP2A6*19*	Y392F; I471T	0	0	1.0 (n = 4)	0
*CYP2A6*20*	Frameshift	0	1.7 (n = 6)	0	0
*CYP2A6*21*	K476R	0.5 (n = 2)	0.6 (n = 2)	0	0
*CYP2A6*22*	D158E; L160I	0	0	0	0
Total (number of alleles)		(n = 374)	(n = 352)	(n = 418)	(n = 184)

Table IV. Allele frequencies (%) of *CYP2A6*1* sub-alleles in homozygotes of *CYP2A6*1* allele in four populations

Allele	*Mutations*	*European-Americans*	*African-Americans*	*Koreans*	*Japanese*
*CYP2A6*1A*	-1013A, -745A	17.1 (n = 43)	26.2 (n = 48)	32.8 (n = 42)	22.7 (n = 10)
*CYP2A6*1B1*	-1013A, -745A, *CYP2A7* in 3'-UTR	0.4 (n = 1)	2.2 (n = 4)	0	2.3 (n = 1)
*CYP2A6*1D*	-1013G, -745A	38.5 (n = 97)	48.1 (n = 88)	8.6 (n = 11)	18.2 (n = 8)
*CYP2A6*1B2*	-1013G, -745A, *CYP2A7* in 3'-UTR	31.7 (n = 80)	14.2 (n = 26)	40.6 (n = 52)	36.4 (n = 16)
*CYP2A6*1H*	-1013A, -745G	11.1 (n = 28)	9.3 (n = 17)	1.6 (n = 2)	4.5 (n = 2)
*CYP2A6*1B13*	-1013A, -745G, *CYP2A7* in 3'-UTR	1.2 (n = 3)	0	16.4 (n = 21)	15.9 (n = 7)
*CYP2A6*1J*	-1013G, -745G	0	0	0	0
Total (number of alleles)		(n = 252)	(n = 183)	(n = 128)	(n = 44)

Table V. The cotinine/nicotine ratios in homozygotes of *CYP2A6*1* alleles

CYP2A6 genotype	*European-Americans*	*African-Americans*	*Koreans*	*Japanese*
**1A/*1A*	2.5 (n = 2)	7.2 ± 3.2 (n = 9)	8.2 ± 2.1 (n = 5)	5.3 (n = 1)
**1A/*1B1*		6.6 (n = 2)		4.3 (n = 1)
**1A/*1D*	7.2 ± 3.6 (n = 15)	7.8 ± 3.8 (n = 15)	12.4 ± 14.4 (n = 5)	6.7 ± 4.4 (n = 4)
**1A/*1B2*	6.6 ± 2.5 (n = 17)	6.9 ± 3.7 (n = 8)	18.3 ± 31.7 (n = 18)	10.1 (n = 2)
**1A/*1H*	5.5 ± 2.5 (n = 7)	7.7 ± 5.3 (n = 4)	15.8 (n = 1)	
**1A/*1B13*			10.4 ± 12.2 (n = 8)	12.8 (n = 1)
**1B1/*1D*	10.0 (n = 1)	5.8 (n = 1)		
**1B1/*1H*		6.9 (n = 1)		
**1D/*1D*	6.3 ± 3.7 (n = 21)	9.2 ± 7.3 (n = 25)		0.9 (n = 1)
**1D/*1B2*	9.7 ± 7.9 (n = 27)	7.7 ± 3.8 (n = 14)	13.4 ± 7.8 (n = 4)	
**1D/*1H*	11.3 ± 8.4 (n = 10)	8.2 ± 4.8 (n = 7)	6.5 (n = 1)	1.7 (n = 1)
**1D/*1B13*	5.3 (n = 2)		3.3 (n = 1)	4.2 (n = 2)
**1B2/*1B2*	8.6 ± 5.5 (n = 15)		14.4 ± 14.3 (n = 9)	4.1 ± 1.3 (n = 5)
**1B2/*1H*	5.3 ± 2.9 (n = 5)	10.0 ± 7.0 (n = 4)		6.3 (n = 1)
**1B2/*1B13*	7.6 (n = 1)		12.8 ± 7.1 (n = 12)	7.1 ± 6.9 (n = 3)
**1H/*1H*	4.4 ± 0.7 (n = 3)			
**1B13/*1B13*				1.7 (n = 1)
*Unknown (*1A/*1D/*1H)*		4.6 (n = 1)		
Total (number of subjects)	7.7 ± 5.5 (n = 126)	8.1 ± 5.1 (n = 91)	13.7 ± 18.7 (n = 64)	5.7 ± 4.0 (n = 22)

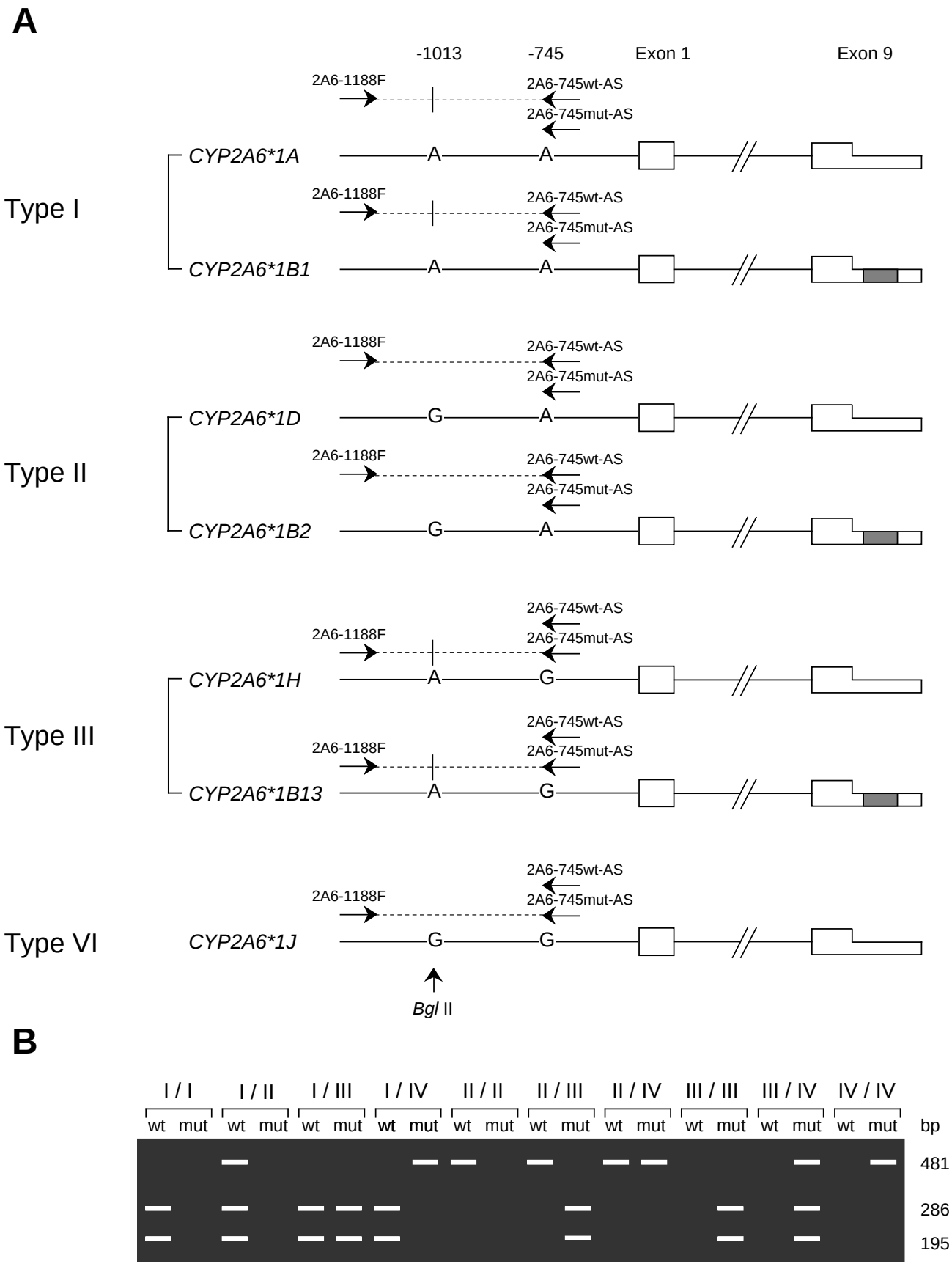
Table VI. The cotinine/nicotine ratios in four populations

CYP2A6 genotype (number of subjects)	*European-Americans* (n = 176)	*African-Americans* (n = 160)	*Koreans* (n = 209)	*Japanese* (n = 92)
*CYP2A6*1/CYP2A6*1*	7.7 ± 5.5 (n = 126)	8.1 ± 5.1 (n = 90)	13.7 ± 18.7 § (n = 64)	5.7 ± 4.0 † (n = 22)
*CYP2A6*1/CYP2A6*1X2*			12.5 (n = 1)	
*CYP2A6*1/unknown*		4.6 (n = 1)		
*CYP2A6*1/CYP2A6*2*	4.7 (n = 2)	1.3 (n = 1)		
*CYP2A6*1/CYP2A6*4*		3.4 (n = 2)	7.9 ± 4.2 (n = 24)	3.9 ± 2.0 (n = 16)
*CYP2A6*1/CYP2A6*5*			10.3 (n = 2)	
*CYP2A6*1/CYP2A6*7*			7.1 ± 4.2 * (n = 19)	4.9 ± 1.5 (n = 4)
*CYP2A6*1/CYP2A6*8*			11.6 ± 7.6 (n = 3)	5.6 (n = 1)
*CYP2A6*1/CYP2A6*9*	5.8 ± 3.1 (n = 25)	7.4 ± 4.0 (n = 22)	9.5 ± 8.3 (n = 37)	5.0 ± 3.5 (n = 14)
*CYP2A6*1/CYP2A6*10*				4.4 (n = 2)
*CYP2A6*1/CYP2A6*11*			5.2 (n = 1)	
*CYP2A6*1/CYP2A6*13*				3.0 (n = 1)
*CYP2A6*1/CYP2A6*14*	6.4 ± 4.4 (n = 11)	13.7 ± 3.4 * (n = 3)		
*CYP2A6*1/CYP2A6*15*			3.4 ± 2.0 * (n = 4)	2.5 (n = 1)
*CYP2A6*1/CYP2A6*16*	7.1 (n = 1)	5.2 ± 1.0 (n = 3)		
*CYP2A6*1/CYP2A6*17*		5.2 ± 3.0 * (n = 22)		
*CYP2A6*1/CYP2A6*18*	5.7 ± 3.3 (n = 5)		5.2 (n = 2)	
*CYP2A6*1/CYP2A6*19*			6.8 (n = 2)	
*CYP2A6*1/CYP2A6*20*		3.1 (n = 2)		
*CYP2A6*1/CYP2A6*21*	8.9 (n = 1)	4.0 (n = 1)		
*CYP2A6*2/CYP2A6*9*	1.8 (n = 2)			
*CYP2A6*4/CYP2A6*4*			0.0 (n = 4)	0.0 (n = 3)
*CYP2A6*4/CYP2A6*7*			1.8 ± 1.2 * (n = 4)	0.8 ± 0.6 (n = 5)
*CYP2A6*4/CYP2A6*9*		0.9 (n = 1)	2.6 ± 0.8 * (n = 8)	1.8 ± 0.9 * (n = 7)
*CYP2A6*4/CYP2A6*10*			0.1 (n = 1)	
*CYP2A6*4/CYP2A6*15*				2.4 (n = 1)
*CYP2A6*7/CYP2A6*7*			1.0 (n = 1)	1.1 (n = 1)
*CYP2A6*7/CYP2A6*9*			5.0 ± 3.8 * (n = 10)	1.7 ± 0.7 * (n = 4)
*CYP2A6*7/CYP2A6*10*			2.2 ± 1.6 * (n = 3)	0.4 (n = 2)
*CYP2A6*7/CYP2A6*11*			2.9 (n = 1)	
*CYP2A6*7/CYP2A6*13*				0.9 (n = 1)
*CYP2A6*7/CYP2A6*15*			6.0 (n = 1)	
*CYP2A6*7/CYP2A6*19*			0.8 (n = 1)	
*CYP2A6*8/CYP2A6*9*			2.8 (n = 2)	1.2 (n = 1)
*CYP2A6*9/CYP2A6*9*		3.0 (n = 2)	4.1 ± 2.4 * (n = 12)	2.7 ± 0.4 (n = 4)
*CYP2A6*9/CYP2A6*11*				4.2 (n = 1)
*CYP2A6*9/CYP2A6*17*		3.5 (n = 1)		
*CYP2A6*9/CYP2A6*18*	6.4 (n = 2)			
*CYP2A6*9/CYP2A6*19*			2.7 (n = 1)	
*CYP2A6*11/CYP2A6*13*			2.5 (n = 1)	
*CYP2A6*14/CYP2A6*14*	3.4 (n = 1)			
*CYP2A6*14/CYP2A6*17*		3.9 (n = 1)		
*CYP2A6*15/CYP2A6*15*				4.4 (n = 1)
*CYP2A6*16/CYP2A6*16*		6.8 (n = 1)		
*CYP2A6*16/CYP2A6*17*		9.2 (n = 1)		
*CYP2A6*17/CYP2A6*17*		2.3 ± 0.5 * (n = 4)		
*CYP2A6*17/CYP2A6*20*		1.9 (n = 1)		
*CYP2A6*20/CYP2A6*21*		3.4 (n = 1)		

*$P < 0.05$, compared with *CYP2A6*1/CYP2A6*1* by Mann-Whitney *U*-test.

† $P < 0.05$, Japanese showed significantly lower nicotine metabolic ratio than African-Americans and Koreans.

§ $P < 0.005$, Koreans showed significantly higher nicotine metabolic ratio than the other populations.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Exon 1
Exon 9
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A
A
CYP2A6*1B1
Type II
CYP2A6*1D
G
A
CYP2A6*1B2
Type III
CYP2A6*1H
CYP2A6*1B13
Type VI
CYP2A6*1J
G
Bgl II
B
I / I
I / II
I / III
I / IV
II / II
II / III
II / IV
III / III
III / IV
IV / IV
wt mut
bp
481
286
195

Fig. 2. Nakajima et al.

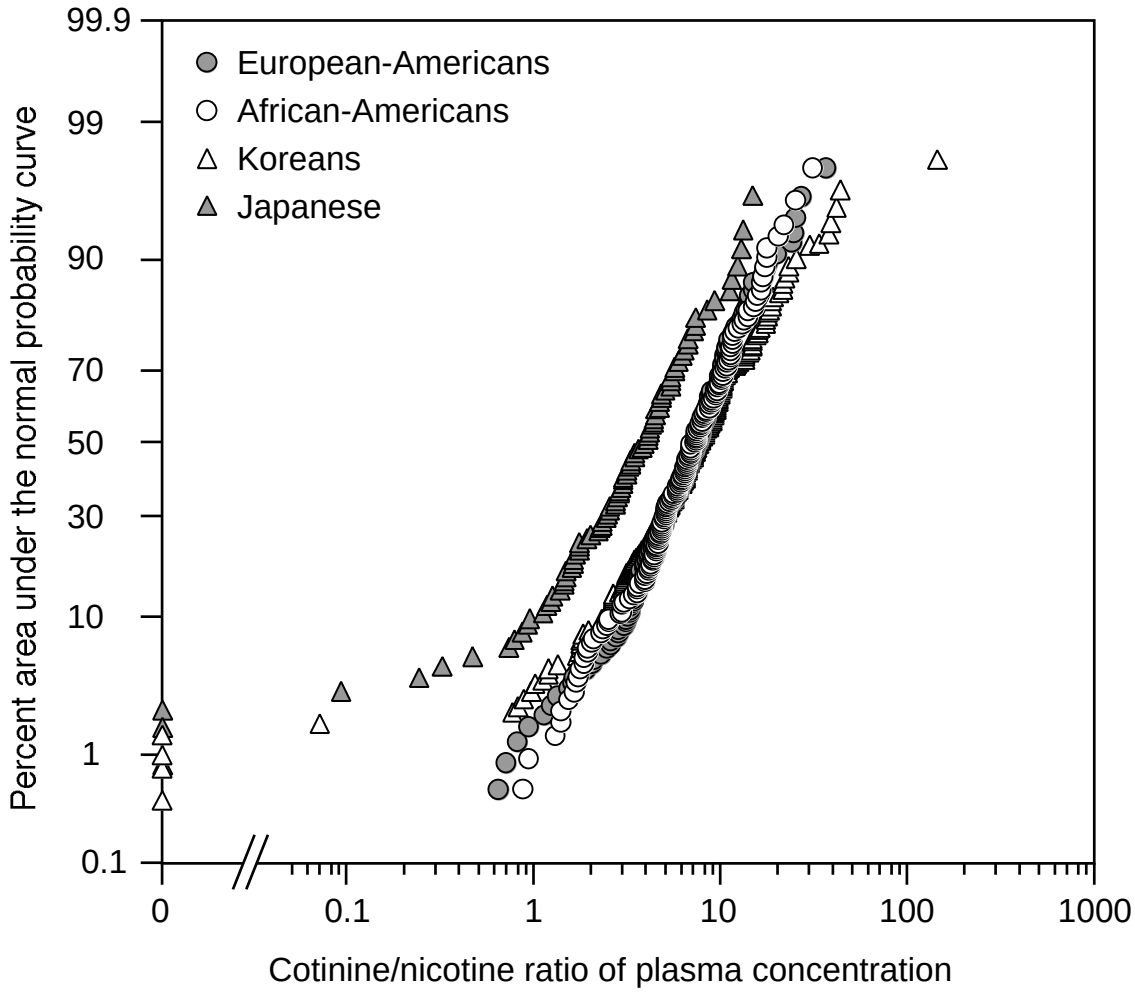

Fig. 3. Nakajima et al.

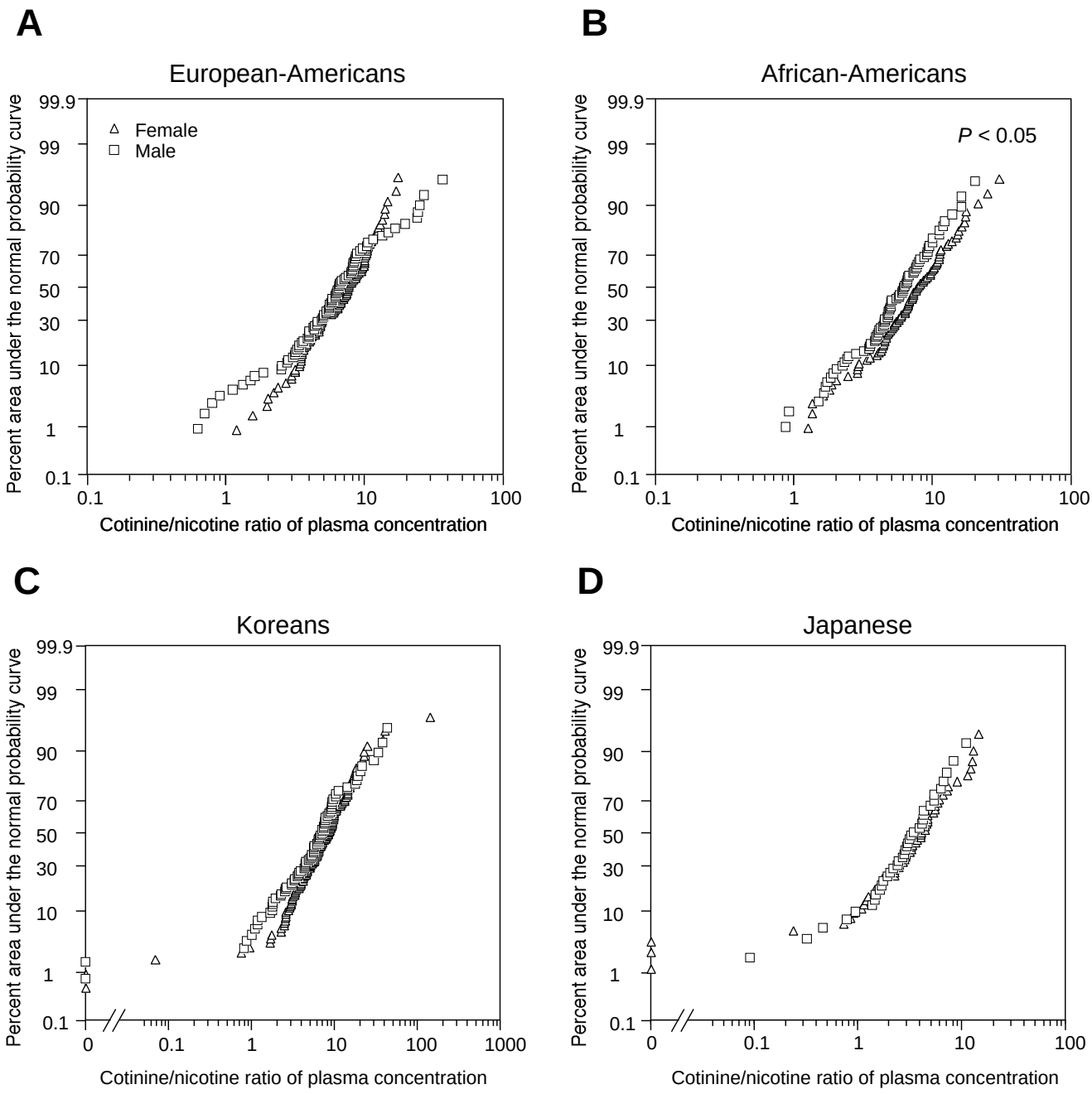

Fig. 4. Nakajima et al.