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RESEARCH ARTICLE

Do *CYP2A6* and *GSTM1* Genotypes have any Impact on Genotoxicity in Healthy Turkish Smokers?

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Summary

Cigarette smoking is the major cause of mortality and morbidity. There are more than 3000 compounds including 30 carcinogens in cigarette smoke. Genetic susceptibility to cigarette smoke-induced cancers may result from polymorphisms in carcinogen metabolism and DNA repair. Nicotine, the primary compound in tobacco, is mainly metabolized to cotinine by polymorphically expressed CYP2A6. GSTM1 and CYP2A6 were reported to be associated with risk of cigarette-related cancers. In addition, GSTM1 was associated with increased frequency of chromosomal aberration (CA). In this study, we aimed to evaluate whether cigarette smoking causes damage to DNA and increases CA frequency and to investigate the relation with CYP2A6 and GSTM1 genotypes in 24 healthy subjects in a Turkish population. We also investigated the consumption of cigarettes by measuring nicotine and cotinine with gas chromatography (GC) and its correlate with the CYP2A6 genotype. Our results showed that there was a significant increase in CA frequency in smoker subjects compared to non-smoker subjects. We found that the ratio of nicotine/cotinine excretion tended to rise in CYP2A6-mutant subjects compared to CYP2A6-wild subjects. CA frequencies were not significantly different in GSTM1-positive and GSTM1-null subjects ($p>0.05$). However, there was a significant association between GSTM1-null genotype and cigarette smoking (>15 cigarettes/day). Our results suggest an association between smoking and the CYP2A6 polymorphism and also indicate that genotypes play a pivotal role in smoking-related DNA damage.

Key Words: *CYP2A6, GSTM1, chromosomal aberration, cigarette smoking, nicotine, genetic polymorphism.*

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Sağlıklı Sigara İçen Türk Popülasyonunda CYP2A6 ve GSTM1 Genatiplerinin Genotoksisite Üzerine Herhangi Bir Etkisi Var mıdır?

Özet

Sigara içimi mortalite ve morbiditenin önemli sebebidir. Sigara dumanında 30 karsinojen maddeyi de içeren 3000'den fazla kimyasal madde bulunmaktadır. Bunun neden olduğu kanserlerde genetik duyarlılık karsinojen maddenin metabolizması ve DNA onarımındaki polimorfizmlerden kaynaklanmaktadır.Tütünde bulunan birincil bileşik olan nikotin polimorfik CYP2A6 ile kotinine metabolize olmaktadır. GSTM1 ve CYP2A6 genleri ile sigara içiminin neden olduğu kanser riski arasında ilişki olduğuna dair çalışmalar mevcuttur. Ayrıca, GSTM1 ile kromozom aberasyon sıklığı arasında ilişki olduğunu gösteren çalışmalar vardır. Bu çalışmada Türk populasyonunda 24 sağlıklı bireyde sigara içiminin DNA üzerinde hasar meydana getirmesi ve kromozom aberasyon sıklığında artışa neden olup-olmadığı değerlendirilip, CYP2A6 ve GSTM1 genotipleri arasındaki ilişki araştırılmıştır. Ayrıca, Gaz Kromatografisi (GC) ile nikotin ve kotinin seviyeleri ölçülmüş ve bu değerlerin CYP2A6 genotipi ile korelasyonu araştırılmıştır.

Anahtar kelimeler: *CYP2A6, GSTM1, kromozom aberasyonu, sigara içimi, nikotin, genetik polimorfizm.*

INTRODUCTION

Cigarette smoking has long been recognized as a major cause of mortality and morbidity, responsible for an estimated 434,000 deaths per year in the United States. Tobacco use is known to cause cancer at various

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sites, in particular the lung[1]. Cigarette smoking is a major risk factor for heart disease and can also cause respiratory diseases[2].

Based on the weight of the available scientific evidence, the U.S. Environmental Protection Agency (EPA) has concluded that widespread exposure to environmental tobacco smoke (ETS) in the United States presents a serious and substantial public health impact[2].

Cigarette smoke is a rich source of chemical carcinogens and reactive oxygen species (ROS) that can induce a variety of DNA damage, some of which is repaired by the base excision repair (BER) pathway. Chemical carcinogens in tobacco include polycyclic aromatic hydrocarbons (PAH), aromatic amines and *N*-nitroso compounds like NNK. Among xenobiotic metabolizing enzymes, the *CYP2A* family is characteristic in its catalytic properties to nitrosamines, which can produce DNA bulky adducts that may lead to DNA damage[3]. ROS are present in both the gas-phase and the particulate matter and include oxygen radicals such as superoxide and hydroxyl radicals. Furthermore, through endogenous enzymatic reactions mediated by bacteria and inflammatory cells, *N*-nitroso compounds, such as those in cigarette smoke, can generate nitric oxide radicals that can induce oxidative damage[4].

The cytogenetic endpoints in peripheral blood lymphocytes (chromosome aberration, CA; sister chromatid exchange, SCE; and micronuclei, MN) have been used for over 30 years to assess DNA damage, and they are established as biomarkers of exposure for mutagens and carcinogens. Without doubt, these cytogenetic endpoints may serve as biomarkers for at least certain genotoxic exposures. CA especially serves as biomarker of an early mutagen effect indicating increased cancer risk[5].

Variability in the genome occurs at the levels of genes (mutation / deletions / insertions / DNA adducts) or chromosomes (CA, SCE). Some of these changes may be associated with interindividual differences in susceptibility to toxicity and the tumor initiation[6,7]. Individuals are known to differ in their susceptibility to different types of cancers. Genetic polymorphisms are often the reason for this phenomenon. The available studies have revealed a higher level of DNA adducts and chromosome damage in lymphocytes of cigarette smokers and other subjects exposed to PAH, who lack glutathione S-transferase M1 *(GSTM1)* due to a homozygous deletion (null) affecting the *GSTM1* gene. *GSTM1* is an important detoxification enzyme which is commonly (in about 50% of Caucasians) lacking in the human population. Other polymorphic GSTs include *GSTM3*, *GSTP1*, and *GSTT1*. The *GSTT1* null genotype (10-20% of Caucasians) has been associated with an increased "baseline" level of SCE in peripheral blood lymphocytes, possibly reflecting an interaction between the genotype and some common endogenous or exogenous exposure[6].

Nicotine is known to be metabolized to its major and active metabolite cotinine by members of the cytochrome P450 (CYP) monooxygenase superfamily. Metabolization of nicotine to cotinine by the enzyme *CYP2A6* influences the interindividual differences in smoking behavior as lung cancer susceptibility8. Although *CYP2A6* has now been identified as the principal enzyme which catalyzes this biotransformation, *CYP2D6* is also an active nicotine C-oxidase. One of the common known alleles of *CYP2A6*, *CYP2A6*1*, is responsible for the c-oxidation of nicotine to cotinine, and *CYP2A6*2* and *CYP2A6*3* alleles encode for catalytically inactive enzymes. Some 8% of the Caucasian population has reduced or absent *CYP2A6* activity; *CYP2D6* may play a significant role in nicotine metabolism in these individuals[9]. In this preliminary study, we aimed to evaluate whether *CYP2A6* and *GSTM1* genetic polymorphisms could be related to individual susceptibility to smoking-induced DNA damage and to investigate the association between *CYP2A6* genotype and phenotype in healthy smokers in a Turkish population.

MATERIALS AND METHODS

Subjects

Unrelated healthy smoker and non-smoker subjects were voluntarily recruited from Gazi University staff. Each subject was interviewed using a standardized questionnaire with questions on smoking habit, drug

intake, contraception, diseases during the previous three months, and diagnostic and therapeutic X-rays. All gave their full informed consent to the study, granting the appropriate ethical approval. None of them was taking any medication at the time of the study.

In the present study, genomic DNA for genotyping studies and chromosomes for CA assay were prepared from peripheral blood samples of 24 volunteers (28-56 years; 13 female and 11 male; 14 cigarette smokers and 10 non-smokers). The levels of nicotine and cotinine were analyzed from urine samples of six volunteers with *CYP2A6* carrying three different types of alleles (W=Wild; H= Heterozygous; M=Mutant).

GSTM1 and CYP2A6 genotyping

DNA was extracted from whole blood using a sodium perchlorate / chloroform extraction method 10. The GSTM1 null allele was detected by minor modification of the method described by Comstock et al. and Zhong et al.[11, 12]. The sequences of the GSTM1 primers were: Primer 1 (AA1): 5′-CGCCATCTTGTGCTA CATTGCCCG-3′; Primer 2 (AA2); control primer 5′ ATCTTCTCCTCTTCT-GTCTC-3′ and Primer 3 (AA3); diagnostic primer: 5′-TTCTGGATTGTAGCAGATCA-3′; common primer. The primers were obtained from Operon Technologies, Inc., Alameda, CA, USA. A 550 µl polymerase chain reaction (PCR) was performed using 1 µl genomic DNA, 25 µM each primer (5.5 µl AA1, AA2, AA3), 16.5 µl 10 mM dNTPmix (dTTP, dATP, dGTP, dCTP), 2.75 Unit Taq DNA polymerase (Bioline) using 16.5 µl 50 µM $MgCl_2$ and 55 ml 10 x NH_4 buffer supplied by the manufacturer and 444 µl 5% sterile water with DMSO. The reaction consisted of 35 cycles of 1 min at 93°C, 1.5 min at 52°C, 2 min at 70°C and 1 cycle of final extension for 10 min at 70°C. The specific size of *GSTM1* gene PCR product (230 bp) was initially assessed at 100V by electrophoresis with 1% tris-borate-EDTA electrophoresis buffer (TBE buffer) in 2% agarose gel. Internal standard was used in all the samples to detect failure of the amplification reaction. A CYP2A6-specific PCR reaction was performed according to the method of Fernandez-Salguero et al.[13]. The PCR was accomplished with the primer pair: F4 (forward): 5′ CCT CCC TTG CTG GCT GTG TCC CAA GCT AGG C 3′ and R4 (reverse): 5′ CGC CCC TTC CTT TCC GCC ATC CTG CCC CCA G 3′, yielding a 7.8 kb product. The amplification was performed after an initial denaturation at 94°C for 1 min for 1 cycle by denaturing at 94°C for 1 min, annealing at 65°C for 1 min 30 s, extending at 68°C for 6 min for 30 cycles, and final extending at 68°C for 10 min for 1 cycle (Perkin-Elmer). Amplification of the diagnostic exon (exon 3) was done with the following primer pair: E3F (forward): 5′ GCG TGG TAT TCA GCA ACG GG3′ and E3R (reverse): 5′ TCG TCC TGG GTG TTT TCC TTC 3′. The reaction mixture (500µl) contained 2 µl of the dilution of 7.8 kb PCR reaction (1:10) as template, 0.25 MM of each primer, 200 mM dNTPs, 1.5 mM of $MgCl_2$ and 1.25 U of Taq DNA polymerase supplemented with NH_4Cl as reaction buffer (Perkin-Elmer). Amplification was carried out for 35 cycles by denaturing at 93°C for 30 s, annealing at 66°C for 30 s extending at 70°C for 45 s and final extending at 70°C for 10 min for 1 cycle. PCR products were then digested without further purification by restriction enzymes that detect the presence of the *CYP2A6*1* (wild type-no digestion), *CYP2A6*2* (T to A mutation, creating an Xcm I restriction site), and *CYP2A6*3* (C to A mutation, creating a Dde I restriction site) alleles. The restriction products were electrophoresed in 3.5% Nusieve agarose gels, 10% polyacrylamide (PAGE) gels and stained with ethidium bromide[13].

CA assay

Venous blood samples (5 ml) were drawn into a heparinized tube. Fourteen drops of venous blood were added to 5 ml TC 199 medium (Seromed, Germany) supplemented with 20% fetal calf serum (Seromed, Germany) and 2% phytohemagglutinin (PHA-L, Seromed). The cultures were incubated for 48 h at 37°C three hours before the harvest colchicine (10 mg/ml, Sigma) was added to the culture media. The cells were collected by centrifugation, re-suspended in hypotonic solution (0.075 M KCl, Merck) for 20 min and fixed in acetic acid/methanol (1/3:v/v). The slides were prepared by an air-dried method and stained with a 4% Giemsa solution (pH=6.8) (Mer-

ck)[14,15]. One hundred well-spread metaphases were scored for CA in each sample.
One or more of the following types of aberrations were counted for each 100 metaphases: (1) Chromosome break (2) Chromosome gap (3) Chromatid break (4) Chromatid gap (5) Acentric fragment, i.e. a pair of chromatids without a centromere (6) Minute (7) Dicentric.

Measurement of urinary nicotine and cotinine by gas chromatography (GC)

Morning spot urine specimens were collected from subjects. Cotinine in urine samples was extracted according to the method by Beckett and Triggs[16] with slight modification. The stored urine samples were left to defrost, homogenized and kept 30 min at room temperature. After adding quinoline as an internal standard (50 µg/ml), 5 ml of urine was mixed with 0.5 ml 5N ammonium hydroxide in a glass tube. The solution was extracted 4X5 ml of dichloromethane. After centrifugation, the organic phase was concentrated to 100 µl and 1µl sample was injected into gas chromatograph.
Gas chromatographic analyses were carried out on a Hewlett-Packard Model 5890 Gs chromatograph equipped with a flame ionization detector (FID) and a HP 3396 integrator. Chromatographic determination of nicotine and cotinine was carried out using a 25mx0.2mm fused silica capillary column HP-Ultra 1 from Hewlett-Packard. The operating conditions were as follows: injector temperature 250°C; detector 250°C; column 110°C hold 13°C/min to 220°C; 1/30 split ratio. Peak areas were used as the basis for quantification. A spiked quality control sample was used. Urinary cotinine concentrations were expressed after correction for creatinine concentrations and presented as µg/g creatinine.

Statistical analysis

After data acquisition, comparison of the arithmetic mean (±SD) values among groups was calculated by non-parametric t-test. A p value < 0.05 was considered statistically significant. All p values were two-tailed.

RESULTS

Table 1. General characteristics of subjects

	Total number of subjects (24)	
	Smoker	Non-smoker
N	14	10
Age (mean±SD)	38.21±6.54	40.1±9.50
Gender (F/M)	4F/10M	9F/1M
Smoking Status		
Cigarettes/day (mean±SD)	19.64±8.87	-
Years (mean±SD)	14.43±5.40	-

Table 2. Data for 24 Turkish individuals: *CYP2A6* and *GSTM1* genotypes, chromosomal aberrations and levels of nicotine and cotinine

No	CYP2A6 genotype		GSTM 1 genotype	CA frequency (%)	Excretion of nicotine (µg/g creatinine)	Excretion of cotinine (µg/g creatinine)
	2A6*2	2A6*3				
1	W	W	-	2	-	-
2	W	W	+	0	-	-
3	W	W	+	1	-	-
4	W	H	+	0	-	-
5	W	W	+	1	-	-
6	W	H	-	1	1945	1860
7	W	W	+	1	-	-
8	W	W	-	1	-	-
9	W	H	+	2	-	-
10	W	W	-	0	-	-
11	W	W	+	1	3240	4989
12	W	W	-	0	-	-
13	W	M	+	4	4639	2248
14	W	W	+	1	-	-
15	W	W	-	0	-	-
16	W	W	-	0	-	-
17	W	H	-	4	-	-
18	W	W	+	3	-	-
19	W	H	+	1	-	-
20	W	W	+	1	-	-
21	W	W	-	2	2466	2363
22	W	H	+	2	2181	1362
23	W	W	+	2	2130	2096
24	W	H	+	1	-	-

W = *Wild allele.* ***H=*** *Heterozygous allele.* ***M=*** *Mutant allele.*

Table 3. Mean number of CA (including gap) uncultured lymphocytes according to sex and smoking status

Subjects (Sex/Smoking status)	CA Frequency (%) (mean±SD)
SEX	
Female (n=13)	1.00±1.08[A]
Smoker (n=4)	2.00±1.41
Non-smoker (n=9)	0.96±0.53
Male (n=11)	1.60±1.21[A]
Smoker (n=10)	-
Non-smoker (n=1)	-
SMOKING STATUS*	
Smoker (n=14)	1.86±1.17[B]
Non-smoker (n=10)	0.50±0.53[B]

*It is not possible to analyze the data, because one column contains α single data

[A, B] p<0.05 (Mann-Whitney test)

Table 4. Mean number of CA (including gap) in uncultured lymphocytes related to *GSTM1* and *CYP2A6* genotypes

	CA frequency (%) (mean ± SD)	P value
GSTM1 Genotype		
Positive (n=15)	1.40±1.06	
Null (n=9)	1.11±1.36	P>0.05
CYP2A6 Genotype		
Wild (n=16)	1.00±0.89	
Mutant* (n=7)	1.88±1.46	P>0.05

The frequencies of CA were analyzed in peripheral blood lymphocytes by CA assay. The mean frequencies of CA were 1.86±1.17 in smoker subjects (n=14) and 0.50±0.53 in non-smoker subjects (n=10). We found a statistically significant increase in CA frequency in smoker subjects compared to non-smoker subjects ($p<0.05$) (Table 3).

We also compared female subjects to male subjects to determine whether there are any effects of sex on CA, and found no significant differences ($p>0.05$); thus sex was not a confounding factor in our study (Table 3).

Of 24 genomic DNA samples analyzed, 16 subjects were carrying the *CYP2A6*1* (wild) allele. No heterozygous or homozygous subjects were identified for the *CYP2A6*2* allelic variant. Seven heterozygous and one homozygous subjects were identified for *CYP2A6*3* allelic variant. Allelic frequencies were detected as 0.83 for *CYP2A6*1* allele and 0.17 for *CYP2A6*3* alleles in 24 subjects (Table 2). The allele frequencies were stratified by smoking habits, and in 14 smokers were 0.79, 0.21 for *CYP2A6*1* and *CYP2A6*3*, respectively, versus 0.85 and 0.15 in 10 non-smokers. CA frequency was statistically higher in smoker subjects with *CYP2A6*1* alleles than in non-smoker subjects with *CYP2A6*1* alleles ($p=0.018$) (not shown). CA frequency was slightly increased in subjects with *CYP2A6* mutant alleles compared to those with *CYP2A6* wild alleles ($p>0.05$) (Table 3). Nicotine and cotinine excretion in smokers was analyzed from urine samples by GC. In genotyped subjects, we chose six subjects carrying three different *CYP2A6* alleles (W, n=3; H, n=2 and M, n=1) (Table 2). *CYP2A6* wild allele was used as a control for *CYP2A6* mutant allele. Our results showed that cotinine level is lower in subjects carrying the homozygous *CYP2A6*3* allele (subject 13) than in subjects with *CYP2A6*1* alleles. There was a slight decrease in the levels of cotinine in subjects with *CYP2A6*3* heterozygous alleles (subjects 6 and 22) (Table 2). In *CYP2A6*3* variant allele subjects, the levels of cotinine were decreased compared to *CYP2A6*1* subjects, but this decrease was not statistically significant due to small sample size (not shown).

In *GSTM1* cases, nine subjects were identified with null and 15 with *GSTM1* positive allele. The description of the studied subjects is given in Table 2. The frequency of *GSTM1* null genotype was 43% in smoker subjects and 30% in non-smoker subjects. CA frequency was not significantly different in *GSTM1*-positive and -null subjects ($p>0.05$). When the frequency of CA in smoker subjects was compared to that in non-smoker *GSTM1*-positive subjects, we observed that CA frequency in smoker subjects was higher than in non-smoker *GSTM1*- positive subjects ($p=0.012$) (not shown). On the other hand, we found a slight increase in smoker *GSTM1*-positive subjects compared to *GSTM*-null subjects, but the difference was not statistically significant ($p>0.05$) (Table 3). However, a significant association was observed between *GSTM1*-null and cigarette smoking in subjects who smoked more than 15 cigarettes/day. We did not observe similar results in the subjects who smoked less than 15 cigarettes/day.

DISCUSSION

Susceptibility to genotoxic exposure varies among individuals due to acquired or inherited characteristics. During the last few years, increasing attention has been focused on genetic polymorphisms that could modulate human response to genotoxic insult. In principle, any polymorphisms that affect xenobiotic metabolism or cellular response to DNA damage could alter individual sensitivity to genotoxins.

It is generally accepted that chromosomal mutations are causal events in the development of neoplasia, and it has earlier been postulated, but not proven,

that increased chromosomal damage may reflect an enhanced risk in healthy subjects. Such associations could not be seen for SCE or MN.

In the present study, the influence of *GSTM1* and *CYP2A6* genotypes on smoking-induced genotoxicity in the same subjects of a Turkish population was studied, and the *CYP2A6* genotype and phenotype were evaluated. We found a statistically significant increase in frequency of CA in smokers compared to non-smoker subjects ($p<0.05$) (Table 2). This increase suggests that smoker subjects appear to be more susceptible to smoking-related cancers (such as lung and bladder cancers) than non-smoker subjects. We detected allele frequency 0.17 for *CYP2A6*3* and 0.83 for *CYP2A6*1* in our study group, and these frequencies are concordant with the results of Oscarson et al.[9]. In our previous study, we noticed a poor correlation between the *CYP2A6* genotype and phenotype determined in vivo using the probe drug coumarin by using the original genotyping method described to detect the *CYP2A6*1* and *CYP2A6*3* alleles. The majority of Turkish subjects (68%) excreted less than 60% of the 2 mg dose as coumarin metabolism[10].

CYP2A6 genotype–smoking association and the levels of cotinine were decreased in subjects with *CYP2A6*3* variant allele compared to those with *CYP2A6*1* allele, but the difference was not statistically significant. According to the data, there was a relationship between *CYP2A6* activity measured by urinary nicotine metabolite ratio in smoker subjects and *CYP2A6* genotype. The lack of a statistically significant association may be due to the small sample size. The findings can be supported in the future with a large scale study group.

The frequency of the *GSTM1*-null genotype was 37.5% in our study population. The result concerning GSTM1 is consistent with Aktafl et al.[17] The frequency of CA was not significantly different in *GSTM1*-positive and -null subjects ($p>0.05$). However, a significant association between *GSTM1*-null and cigarette smoking in subjects who smoked more than 15 cigarettes/day was observed. We did not observe similar results with cigarette smoking in subjects who smoked less than 15 cigarettes/day. Our results indicate that the genotoxic effect of smoking on chromosomes appears to be influenced by smoking intensity in relation to the *GSTM1* genotype. The *GSTM1* genotype might serve as a protective gene in the individuals who smoked more than 15 cigarettes/day. Because the number of study subjects is too low for the combination of relatively small allele frequencies, we omitted all data on the combination of polymorphisms.

In our previous study, we observed a slight increase in the frequency of CA in *GSTM1*-null subjects compared with positive subjects, but it was not statistically significant18 ($p>0.05$). Scarpato et al.19 carried out a study about the influence of *GSTM1* and *GSTT1* polymorphisms on the frequency of CA in smokers and pesticide-exposed greenhouse workers. They found that a statistically significant increase ($p=0.026$) in baseline CA frequency was observed in *GSTM1*-null in comparison with *GSTM1*–positive subjects. Our earlier results15 were similar to theirs. Salama et al.[20] also reported similar results and found that *GSTM1* and *GSTT1* polymorphisms played a role in modifying the genotoxicity of tobacco-specific nitrosamines. Lei et al.[21] investigated the association between metabolic polymorphisms (*GSTM1*, *XRCC1*) and smoking-related DNA damage by sister chromatid exchange (SCE). They found significant increases in the frequency of SCE in smokers, and could not find any significant interaction between cigarettes smoked per day with *GSTM1* on SCE frequency. There are conflicting studies on the association between polymorphisms (GSTs and NAT2) and smoking behavior[5,15,18]. Smits et al.[22] found no association between polymorphisms in *CYP1A1*, *GSTM1*, *GSTT1*, *NAT2* and *GSTP1* genes and tobacco consumption. Many studies indicate that subjects lacking full functional *CYP2A6* may have a decreased risk of developing tobacco-related cancers[23-25].

A pharmacogenetic hypothesis formulated for lung cancer mentions that people who inherit high *CYP2A6* metabolic capacity will have the highest capacity to metabolize nicotine. These subjects will also metabolically activate nitrosamines in smoker subjects. Without smoking, subjects will also develop lung tumors by activating nitrosamines from the diet[26]. Although phenotyped subjects were small among the

number of smoker subjects, *CYP2A6*1* smoker subjects (subjects 11, 21 and 23) had increased mean cotinine excretion levels compared to their mean nicotine levels. On the contrary, smokers with *CYP2A6*3* variant allele (subjects 6, 9 and 22) had decreased mean cotinine excretion levels compared to their mean nicotine levels as measured by GC. Smoker subject 13, who was mutant for *CYP2A6*3*, had higher nicotine than cotinine excretion. The observed data for nicotine/cotinine metabolic ratio in genotyped smoker individuals supports with the important role of the *CYP2A6* gene in nicotine metabolism. An in vivo study further stressed the importance of *CYP2A6* in nicotine metabolism, as individuals homozygous for a *CYP2A6* gene deletion displayed only 15% of urinary cotinine levels compared with individuals carrying at least one active *CYP2A6* gene after smoking the same number of cigarettes[9]. Earlier reports suggest *CYP2D6* (debrisoquine hydroxylase) may contribute to the in vivo metabolism of nicotine; however, the contribution is still in question since it has not been reproduced[27]. In addition, *CYP2A6* inhibitors can be used as a new approach to treat tobacco dependence.

We have demonstrated in this study *CYP2A6* genetic polymorphism using a combination of two sets of the PCR system as described by Fernandez-Salguero et al.[13]. Recently, different laboratories were unable to show a single genuine *CYP2A6*3* allele by applied genotyping method. They developed one-step PCR-based genotyping protocol for the *CYP2A6*3* allele, which highly specifies *CYP2A6* gene polymorphism[28, 29]. It would be interesting to re-evaluate our samples by the recent robust genotype method to confirm the *CYP2A6* allele frequency. However, the distribution of *CYP2A6* genotypes is in agreement with the study performed by oligonucleotide ligation assay, which was carried out to detect the major variant *CYP2A6* allele in the Turkish population. New methods which can enable the rapid screening of novel polymorphisms are now becoming available[9]. Thanks to the *CYP2A6* polymorphism studies, several novel alleles were found and reliable genotyping methods are now available for the prediction of *CYP2A6* polymorphisms. Although several new *CYP2A6* polymorphisms might be described in the future, *CYP2A6* phenotyping studies suggest that these alleles are the most common inactive alleles, especially in Caucasian populations.

We have already carried out *CYP2A6-GSTM1* genotyping to clarify the impacts of genotypes on cigarette–smoking-related genotoxicity. It is also possible that combinations of other polymorphic enzymes involved with nicotine metabolism, such as *CYP1A1*, *CYP1B1*, *CYP1A2*, and *CYP2D6*, may be better predictors of cancer susceptibility than the polymorphisms examined. The study of the relationship among human genetic polymorphisms and cancer susceptibility will undoubtedly have increasingly important implications for risk assessment and the prevention, early diagnosis, and intervention of clinical disease and cancer. The role of the *CYP2A6* polymorphism in smoking behavior and risk of cancer is very unclear. Thus, well-designed studies are needed. Furthermore, the question of how the *CYP2A6* polymorphism plays a role in determining an individual's disposition of drugs that are primarily metabolized by this enzyme remains to be answered. There is still a growing need for expanding genotype-phenotype studies with respect to the *CYP2A6* gene due to its importance in various chemical- and tobacco-related cancers for carriers of *CYP2A6* variant and *GSTM1* null alleles, cancer risk; CA frequency predicts the overall cancer.

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