

Comparison of Polymorphisms in Genes Involved in Polycyclic Aromatic Hydrocarbon Metabolism with Urinary Phenanthrene Metabolite Ratios in Smokers

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Abstract

The hypothesis that interindividual differences among smokers in the metabolism of polycyclic aromatic hydrocarbons (PAH) are related to lung cancer risk has been extensively investigated in the literature. These studies have compared lung cancer risk in groups of smokers with or without polymorphisms in genes involved in PAH metabolism. We believe that carcinogen metabolite phenotyping, involving the actual measurement of PAH metabolites, would be a better way to investigate differences in lung cancer risk. With this goal in mind, we have developed methods for quantifying phenanthrene metabolites in urine. Phenanthrene is the simplest PAH with a bay region, a feature closely associated with carcinogenicity. The urinary metabolite *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydropheanthrene (PheT) is a measure of metabolic activation, whereas phenanthrols (HOPhe) are a measure of detoxification. In this study, we quantified urinary PheT/HOPhe ratios in 346 smokers who were also genotyped for 11 polymorphisms in genes involved in PAH metabolism: *CYP1A1MspI*, *CYP1A1I462V*, *CYP1B1R48G*, *CYP1B1A119S*, *CYP1B1L432V*, *CYP1B1N453S*, *EPHX1Y113H*, *EPHX1H139R*,

GSTP1I105V, *GSTP1A114V*, and *GSTM1* null. The geometric mean molar PheT/3-HOPhe ratio was 4.08 (95% confidence interval, 3.79-4.39). Ten percent of the smokers had PheT/3-HOPhe ratios of ≥ 9.90 . We found a significant association between the presence of the *CYP1A1I462V* polymorphism and high PheT/3-HOPhe ratios ($P = 0.02$). This effect was particularly strong in females and in combination with the *GSTM1* null polymorphism. In contrast, the *CYP1B1R48G* and *CYP1B1A119S* polymorphisms were associated with significantly lower PheT/3-HOPhe ratios, particularly in Blacks. There were no consistent significant effects of any of the other polymorphisms on PheT/3-HOPhe ratios. The highest 10% of PheT/3-HOPhe ratios could not be predicted by the presence of any of the 11 polymorphisms individually or by certain combinations. The effects of the *CYP1A1I462V* polymorphism observed here, particularly in combination with *GSTM1* null, are quite consistent with reports in the literature. However, the results of this study indicate that genotyping is not an effective way to predict PAH metabolism at least as represented by PheT/HOPhe ratios. (Cancer Epidemiol Biomarkers Prev 2006;15(10):1805-11)

Introduction

Lung cancer is the leading cause of cancer death in the world (1). Approximately 90% of lung cancer is caused by cigarette smoking (2). Polycyclic aromatic hydrocarbons (PAH) in cigarette smoke are widely considered to be among the most important causative agents for lung cancer in smokers (3-5). One of these compounds, benzo(*a*)pyrene (BaP), has recently been evaluated by the IARC as carcinogenic to humans (6).

PAHs require metabolic activation to exert their carcinogenic effects, and there are competing detoxification pathways (7, 8). The metabolic activation process produces DNA adducts, which are critical in the carcinogenic process because they cause mutations in important growth control genes, such as *KRAS* and *p53* (4, 9, 10). The metabolic activation of PAH to DNA adducts varies widely among individuals (11-17). These well-established facts lead to the major hypothesis that

smokers who efficiently metabolically activate PAH will be at a higher risk for lung cancer. This hypothesis has been extensively tested in molecular epidemiology studies, which have investigated polymorphisms in genes involved in the metabolic activation and detoxification of PAH. The results of these studies have been mixed, but there are indications that certain polymorphisms in *CYP1A1* and *GST* genes may lead to enhanced lung cancer risk (18-27).

PAH metabolism is extremely complex. It is unlikely that interindividual differences in metabolism can be assessed simply by measurement of variants in one or a few genes involved in the process. We believe that carcinogen metabolite phenotyping, which is the actual measurement of metabolites resulting from PAH activation and detoxification, is a more reliable way to identify those smokers who efficiently metabolically activate PAH and therefore may be at higher risk for lung cancer. We are pursuing this line of research by quantifying phenanthrene metabolites in human urine (28-30). Phenanthrene is the simplest PAH molecule with a bay region, a feature closely associated with carcinogenicity (8, 31). Phenanthrene metabolism is similar in many ways to that of BaP (Fig. 1), but the products are far more abundant in human urine, and therefore, phenanthrene metabolites are practical biomarkers (28). A major metabolic activation pathway of BaP proceeds through formation of *anti*-BaP-7,8-diol-9,10-epoxide (BPDE), which forms adducts with DNA (7, 32). Most of the BPDE that is produced reacts with H₂O, yielding *trans*, *anti*-BaP-tetraol. An analogous pathway of phenanthrene

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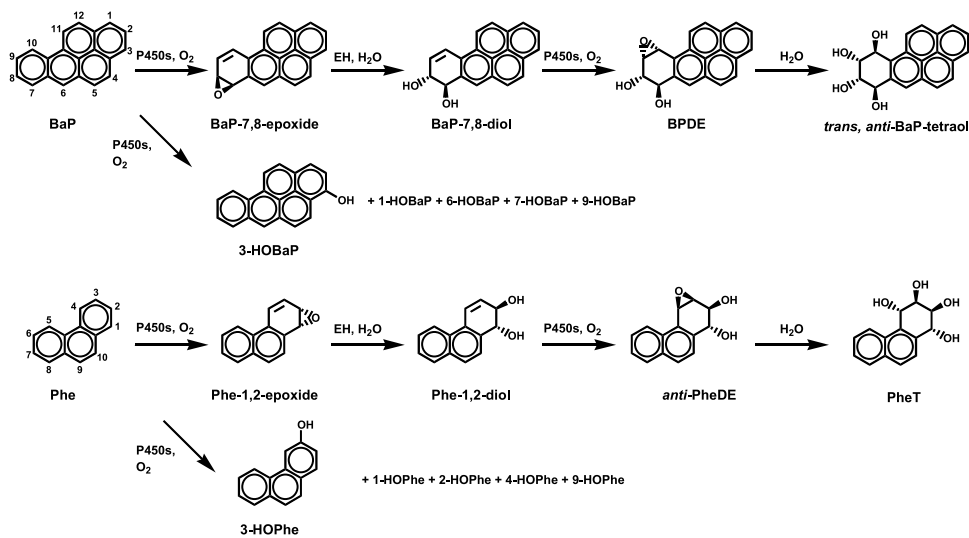


Figure 1. Metabolism of BaP to *trans, anti*-BaP-tetraol via BPDE (metabolic activation pathway) and to phenols, such as 3-HOBaP (metabolic detoxification pathway), and corresponding metabolism of phenanthrene (*Phe*) to PheT and phenanthrols, such as 3-HOPhe.

metabolism yields *r-1,t-2,3,c-4*-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT), which we consider to be a measure of PAH metabolic activation by the diol epoxide pathway (33). Phenanthrene and BaP are both metabolized to phenols (HOBaP and HOPhe), which are considered to be detoxification products (7, 33). In previous studies, we have developed methods for quantifying PheT and HOPhe in human urine and investigated the longitudinal stability of these measures in smokers and nonsmokers (28-30). We propose that the ratio PheT/HOPhe will be characteristic of an individual and that smokers with higher PheT/HOPhe ratios should be at higher risk for lung cancer.

In the present study, we have determined urinary PheT/HOPhe ratios in 346 smokers who were also genotyped for 11 polymorphisms in PAH metabolizing genes. The polymorphisms are *CYP1A1*MspI, *CYP1A1*I462V, *CYP1B1*R48G, *CYP1B1*A119S, *CYP1B1*L432V, *CYP1B1*N453S, *EPHX1*Y113H, *EPHX1*H139R, *GSTP1*I105V, *GSTP1*A114V, and *GSTM1* null. *CYP1A1* and *CYP1B1* code for cytochromes P450 1A1 and 1B1. These cytochrome P450 enzymes catalyze both the metabolic activation of BaP and phenanthrene by the diol epoxide pathway and their detoxification by phenol formation (Fig. 1; refs. 33-38). *EPHX1* codes for microsomal epoxide hydrolase, which is involved in the metabolic activation of BaP and phenanthrene through the diol epoxide pathway (7, 39). *GSTM1* and *GSTP1* code for glutathione *S*-transferases, which catalyze the detoxification of BPDE and other PAH diol epoxides (40-43). Our goal was to investigate the association of urinary PheT/HOPhe ratios with particular polymorphisms in these genes and to determine whether these polymorphisms, individually or in combination, could predict high PheT/HOPhe ratios.

Materials and Methods

Study Design. The study was approved by the University of Minnesota Research Subjects' Protection Program Institutional Review Board Human Subjects Committee (Minneapolis, MN). Smokers were recruited through flyers posted on the University of Minnesota campus and near campus and from advertisements in newspapers and on radio and television. Interested participants called the Tobacco Use Research Center and were told that the study would involve submission of urine and blood samples. All subjects signed a consent form. Two hundred and forty-three subjects gave first morning urine samples and 30 mL blood (collected in tubes containing EDTA) specifically for this study. The remaining subjects were drawn

from other ongoing studies in smokers, and in these cases, baseline samples were used before any intervention. All of these samples were also first morning urines, and 30 mL blood were collected as above. The other studies were clinical trials involving smoking reduction (32 subjects; ref. 44), high-dose nicotine patch (15 subjects), or switch to smokeless products (13 subjects). These other studies also involved a cross-sectional analysis of smoking behavior and biomarkers in adults (42 subjects; ref. 45) and adolescents (1 subject). The diets of the subjects and their medications were not considered in this study.

Analysis of Urine. Urinary PheT and 1-HOPhe, 2-HOPhe, 3-HOPhe, and 4-HOPhe were determined by gas chromatography-mass spectrometry as described previously, except that [D_{10}]PheT was used as internal standard in the PheT analysis (28, 29). PheT, 3-HOPhe, and total HOPhe were expressed as pmol/mL urine, and PheT/HOPhe ratios are molar. Total HOPhe is the sum of 1-HOPhe, 2-HOPhe, 3-HOPhe, and 4-HOPhe.

Genotyping. DNA was isolated from leukocytes using a GFX genomic blood DNA purification kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Genotyping was done by BioServe Biotechnologies (Laurel, MD) using Masscode, a high-throughput method that can simultaneously do multiple genotyping analyses (46). Briefly, for polymorphism site amplification (PCR1), DNA (3.5 ng) was added to 384-well PCR plates and dried at 70°C. Two external primers used to amplify the polymorphism site were normalized to 0.15 μ mol/L and mixed, and 3 μ L of the mix were added to the wells containing the dried DNAs. The plates were dried, and 2 μ L of a PCR master mix containing 1.73 μ L H₂O, 0.2 μ L of 10 \times buffer (Qiagen, Valencia, CA), 0.04 μ L of 10 mmol/L deoxynucleotide triphosphate (Roche, Indianapolis, IN), 0.01 μ L of 100% formamide, and 0.02 μ L of 5 units/ μ L HotStarTaq (Qiagen) were added. Touchdown PCR protocol was used, with an initial 20 minutes of incubation at 95°C and then 15 cycles of 95°C for 15 seconds, 65°C for 15 seconds ($n - 1$ °C per cycle), and 72°C for 30 seconds. An additional 20 cycles with annealing at 50°C were done.

For allelic discrimination PCR (PCR2), two allele-specific primers, differing at their 5' ends by a tag-specific sequence and at their 3' ends with the complementary base of the two possible alleles, were used. These primers create a template for the two universally tagged primers with photochemically cleavable mass spectrometry tags. Their 5' end sequences are identical to those of the allele-specific primers. The higher concentration of the universal tag primers in PCR2 favors

their extension over the allele-specific primers. PCR2 mix consisted of 0.25 μ L of a primer mix (0.01 μ L of a 50 μ mol/L stock of each allele-specific primer and 0.23 μ L of a 44 \times stock of universally tagged primer mix) and 7.75 μ L of master mix. The master mix was composed of 6.48 μ L H₂O, 1.0 μ L of 10 \times salt mix [300 mmol/L Tris-HCl (pH 8.7), 17 mmol/L MgCl₂, 360 mmol/L KCl, 40 mmol/L (NH₄)₂SO₄], 0.16 μ L of 10 mmol/L each of deoxynucleotide triphosphate, 0.04 μ L of 100% formamide, and 0.072 μ L of 5 units/ μ L HotStarTaq. After addition of 8 μ L of PCR2 mix, a touchdown PCR protocol identical to PCR1 was done.

After PCR2 was completed, the PCR products were pooled from 384-well plates into a 96-well QIAquick (Qiagen) plate using a Beckman (Fullerton, CA) Multimek 96. The samples in the QIAquick plate were then subjected to four washes: an initial wash with 500 μ L of binding solution [3.0 mol/L guanidine hydrochloride, 0.9 mol/L tetramethylammonium chloride in 50 mmol/L 2-[N-morpholino]ethanesulfonic acid monohydrate buffer (pH 5.5)] followed by two washes with 750 μ L of 2.5 mol/L guanidine hydrochloride in 3.57 mmol/L 2-[N-morpholino]ethanesulfonic acid monohydrate buffer (pH 5.5) and a final wash with 750 μ L, 100 mmol/L ammonium formate in 90% ethanol. The plates were then dried by centrifugation. To each well of the QIAquick plate, 75 μ L of high-performance liquid chromatography grade H₂O were added to elute the DNA. After a 2-minute incubation, the DNA samples were eluted into a 96-well plate by centrifuging at 1,000 \times g for 1 minute. The plate was centrifuged at 3,000 \times g for 5 minutes to pellet column material that eluted with the DNAs.

An autoinjector HTS PAL (CTC Analytics, Zwingen, Switzerland) was used to load the samples into the mass spectrometer. Before the samples reached the mass spectrometer, they were passed through a photolysis unit containing a mercury lamp emitting UV light at 254 nm to cleave the cleavable mass spectrometry tags from the purified PCR products. An Agilent (Palo Alto, CA) 1100 series LC/MSD with an atmospheric pressure chemical ionization chamber was used to analyze the samples.

Statistical Analyses. All statistical analyses were implemented using Statistical Analysis System statistical software version 9.1 (SAS Institute, Inc., Cary, NC). Socio-demographic characteristics were summarized by standard descriptive statistical methods. PheT/3-HOPhe and PheT/total HOPhe ratios were investigated on the logarithmic scale because histograms showed that their distributions were heavily skewed with rather long right tails. Statistical comparisons were made using the two-sample *t* test or one-way ANOVA depending on whether the factor had two or more than two categories. Adjustments for multiple comparisons were done with the Tukey method. Data were transformed back and expressed as geometric means and their 95% confidence intervals (95% CI).

Potential covariates predicting the natural log of PheT/3-HOPhe included the 11 polymorphisms and certain logical combinations of these. A multivariate linear regression model with a stepwise method was used to identify which covariates remained by specifying 0.1 as both a significance level for including variables and for eliminating variables. Then, those covariates remaining were used to predict the natural log of PheT/3-HOPhe and list the top 10% of predicted values. The κ coefficient was used to assess the agreement between predicted and actual top 10% of values.

Results

The mean age of the subjects was 39.7 \pm 12.2 (SD) years (range, 18-73; data were missing for seven subjects). There were 127 (36.7%) males and 219 females. The racial distribution was

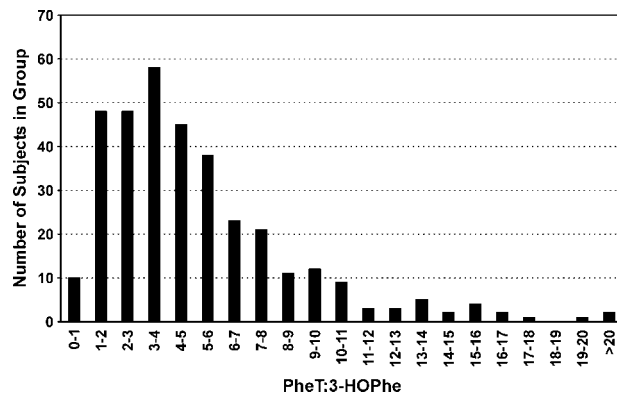


Figure 2. Frequency plot of PheT/3-HOPhe ratios in 346 smokers.

68.6% White (236 subjects), 23.3% Black (80), and 8.1% other (28). Data were missing for two subjects. The mean number of cigarettes smoked per day was 19.4 \pm 9.1 (range, 1-60), with 39.8% of the subjects smoking 1 to 15 cigarettes per day, 42.2% of the subjects smoking 16 to 25 cigarettes per day, and 18.0% of the subjects smoking 26 to 60 cigarettes per day.

The distribution of molar PheT/3-HOPhe ratios is illustrated in Fig. 2. The geometric mean PheT/3-HOPhe ratio was 4.08 (95% CI, 3.79-4.39), and the mean PheT/total HOPhe ratio was 1.18 (95% CI, 1.09-1.27). Ten percent of the smokers had PheT/3-HOPhe ratios of \geq 9.90, whereas the corresponding value for PheT/total HOPhe was 2.70.

There were no significant differences in PheT/3-HOPhe ratios, PheT, 3-HOPhe, or total HOPhe between males and females or among the three categories of cigarettes per day. There was a weak positive trend for increasing PheT/3-HOPhe ratio with age in the three categories of \leq 30, 31 to 50, and $>$ 50 ($P = 0.05$), which became somewhat stronger when adjusted by race ($P < 0.03$). There were significant differences in PheT/3-HOPhe ratios, PheT/total HOPhe ratios, and PheT among racial groups ($P < 0.01$). PheT/3-HOPhe and PheT/total HOPhe ratios were significantly higher in Whites and other racial groups than in Blacks ($P < 0.01$). PheT was significantly higher in Whites than in Blacks ($P < 0.01$).

The relationship of PheT/3-HOPhe ratios to polymorphisms is summarized in Table 1. There was no significant effect of the *CYP1A1*MspI polymorphism (also known as *CYP1A1**2A). The PheT/3-HOPhe ratio was significantly higher in individuals heterozygous or homozygous for *CYP1A1*H462V [also known as *CYP1A1**2C; 5.68 (95% CI, 4.20-7.68)] than in normals [3.99 (95% CI, 3.70-4.31); $P = 0.02$]. A gene dosage effect was not observed, as the ratio for heterozygotes was greater than for homozygotes. However, there were only four (1.2%) homozygotes. Individuals heterozygous in *CYP1A1*H462V were significantly overrepresented in the top 10% and underrepresented in the bottom 10% of PheT/3-HOPhe ratios ($P = 0.03$). With one exception, all subjects heterozygous or homozygous for *CYP1A1*H462V were also either heterozygous or homozygous for *CYP1A1*MspI. Therefore, the effects on PheT/3-HOPhe ratios in individuals with both polymorphisms (also known as *CYP1A1**2B) were virtually identical to those with only a *CYP1A1*H462V polymorphism.

The PheT/3-HOPhe ratio was significantly lower in individuals heterozygous or homozygous for *CYP1B1*R48G [3.59 (95% CI, 3.23-3.98)] than in normals [4.88 (95% CI, 4.39-5.41); $P < 0.0001$]. It was also significantly lower in individuals heterozygous or homozygous for *CYP1B1*A119S [3.48 (95% CI, 3.15-3.85)] than in normals [4.98 (95% CI, 4.49-5.52); $P < 0.0001$]. A gene dosage effect was observed for both of these *CYP1B1* variants. Haplotypes of the *CYP1B1* gene are summarized in Table 2. In all cases, PheT/3-HOPhe ratios

Table 1. Effects of polymorphisms on PheT/3-HOPhe ratios in 346 smokers

Gene polymorphism	rs number	% Occurrence			Geometric mean PheT/3-HOPhe (95% CI)				P*
		Heterozygotes	Homozygotes	Combined	Normal	Heterozygotes	Homozygotes	Combined	
<i>CYP1A1</i> MspI	4646903	26.1	1.55	27.6	4.26 (3.91-4.65)	3.93 (3.38-4.57)	4.81 (2.11-11.0)	3.98 (3.44-4.60)	0.42
<i>CYP1A1</i> I462V	1048943	5.59	1.18	6.77	3.99 (3.70-4.31)	6.02 (4.22-8.59)	4.31 (2.16-8.60)	5.68 (4.20-7.68)	0.02
<i>CYP1B1</i> R48G	10012	39.4	12.7	52.1	4.88 (4.39-5.41)	3.76 (3.33-4.23)	3.11 (2.50-3.88)	3.59 (3.23-3.98)	<0.0001
<i>CYP1B1</i> A119S	1056827	40.9	12.8	53.7	4.98 (4.49-5.52)	3.61 (3.23-4.05)	3.09 (2.50-3.82)	3.48 (3.15-3.85)	<0.0001
<i>CYP1B1</i> L432V	1056836	43.7	25.2	68.9	4.18 (3.61-4.85)	3.83 (3.45-4.24)	4.59 (3.91-5.38)	4.09 (3.75-4.47)	0.79
<i>CYP1B1</i> N453S	1800440	21.5	2.65	24.2	4.04 (3.71-4.40)	4.26 (3.66-4.96)	8.49 (5.34-13.5)	4.59 (3.96-5.34)	0.14
<i>EPHX1</i> Y113H	1051740	36.5	10.5	47.0	4.12 (3.70-4.58)	4.14 (3.68-4.66)	4.46 (3.68-5.40)	4.21 (3.81-4.66)	0.78
<i>EPHX1</i> H139R	2234922	34.4	5.21	39.6	4.14 (3.78-4.55)	4.03 (3.48-4.67)	4.25 (3.10-5.84)	4.06 (3.56-4.64)	0.80
<i>GSTP1</i> I105V	947894	45.8	14.0	59.8	4.31 (3.84-4.84)	4.00 (3.57-4.49)	4.23 (3.40-5.25)	4.05 (3.67-4.48)	0.43
<i>GSTP1</i> A114V	1799811	12.7	0	12.7	4.11 (3.80-4.45)	3.99 (3.19-5.00)	—	3.99 (3.19-5.00)	0.80
<i>GSTM1</i> null	None	—	64.6	64.6	3.80 (3.37-4.29)	—	4.42 (4.00-4.90)	4.42 (4.00-4.90)	0.07

*Normal versus combined.

†Not done.

were significantly lower in the haplotype groups than in normals ($P < 0.01$).

PheT/3-HOPhe and PheT/total HOPhe ratios in subjects heterozygous or homozygous for both *CYP1A1*I462V and *CYP1B1*R48G or *CYP1A1*I462V and *CYP1B1*A119S were not significantly different from normals. Subjects homozygous for *CYP1B1*N453S had significantly higher PheT/3-HOPhe ratios [8.49 (95% CI, 5.34-13.5)] than normals [4.04 (95% CI, 3.71-4.40); $P = 0.004$; Table 1].

There were no significant effects of *EPHX1* or *GSTP1* polymorphisms on the PheT/3-HOPhe ratio (Table 1). The PheT/3-HOPhe ratio was higher [4.42 (95% CI, 4.00-4.90)] in *GSTM1* null individuals than in those with *GSTM1* present [3.80 (95% CI, 3.37-4.29)], but this difference was not significant ($P = 0.07$).

The relationship of molar PheT/total HOPhe ratios to polymorphisms was similar to that seen for the PheT/3-HOPhe ratios.

We also examined the relationship between polymorphisms and PheT/3-HOPhe ratios (Table 1) in the 243 subjects who were specifically recruited for this study. The results were essentially the same as those shown in Table 1, except that, for *CYP1B1*N453S, the combined heterozygote and homozygote ratio [4.73 (95% CI, 3.92-5.71)] was now significantly higher ($P = 0.03$) than that in normals [3.77 (95% CI, 3.42-4.15)].

When the data were stratified by gender, a strong relationship was observed between the *CYP1A1*I462V polymorphism and PheT/3-HOPhe ratio in females. The ratio was significantly higher in females heterozygous or homozygous for this polymorphism [6.49 (95% CI, 4.28-9.85)] than normals [3.80 (95% CI, 3.43-4.21); $P < 0.01$]. A gene dosage effect was not observed, but there were only four homozygotes. No significant effect of *CYP1A1*I462V on PheT/3-HOPhe ratio was observed in males. Similar results were seen for PheT/total HOPhe ratio.

The data were further stratified, forming groups of ≤ 15 , 16 to 25, and ≥ 26 cigarettes per day. The strongest relationships between PheT/3-HOPhe or PheT/total HOPhe ratios and *CYP1A1*I462V polymorphism were seen in females in the 16 to 25 cigarettes per day group. The PheT/3-HOPhe ratio was 7.74

(95% CI, 4.38-13.7) in females heterozygous or homozygous for this polymorphism compared with normals [3.74 (95% CI, 3.12-4.47); $P = 0.04$]. PheT itself was also significantly higher in this group in heterozygous or homozygous females [9.73 (95% CI, 4.42-21.5)] than in normals [3.30 (95% CI, 2.76-3.93); $P = 0.002$], but no effects were observed on 3-HOPhe or total HOPhe.

The combination of *CYP1A1*I462V and *GSTM1* null was investigated. In individuals who were either homozygous or heterozygous for *CYP1A1*I462V and also had the *GSTM1* null genotype ($n = 15$), the PheT/3-HOPhe ratio [6.85 (95% CI, 4.50-10.4)] was significantly higher than in normals [$n = 102$; 3.78 (95% CI, 3.33-4.29); $P < 0.01$]. Similar results were seen for PheT/total HOPhe ratio. Stratifying by gender, this combined effect was restricted to females and was significant for PheT/3-HOPhe ($P = 0.002$), PheT/total HOPhe ($P < 0.001$), and PheT ($P = 0.019$). Smokers with the combination of *CYP1A1*I462V homozygous or heterozygous and *GSTM1* null were significantly overrepresented in the top 10% and underrepresented in the bottom 10% of PheT/3-HOPhe ratios ($P = 0.02$). There were no consistent significant effects on PheT/3-HOPhe ratios of the combination of *CYP1A1*I462V and *GSTP1* polymorphisms or of the combination of *CYP1A1*MspI and *GSTM1* null or *GSTP1* polymorphisms.

The significantly lower PheT/3-HOPhe and PheT/total HOPhe ratios seen in the individuals with *CYP1B1*R48G and *CYP1B1*A119S polymorphisms were further investigated. This seems to be due mainly to an increase in 3-HOPhe and total HOPhe, respectively, in these individuals and is also related to gender and race. Whereas PheT levels were not significantly different among individuals with polymorphisms in these genes compared with normals, 3-HOPhe and total HOPhe levels were higher in heterozygotes of both genotypes than in normals. Stratifying by gender, there was a significant decrease in PheT/3-HOPhe ratios in females who were heterozygous or homozygous in *CYP1B1*R48G and *CYP1B1*A119S, with a gene dosage effect. For *CYP1B1*R48G, levels of PheT/3-HOPhe were 4.83 (95% CI, 4.18-5.57) in normals, 3.79 (95% CI, 3.25-4.41) in heterozygotes, and 2.51 (95% CI, 1.79-3.50) in homozygotes ($P < 0.01$ for normals versus heterozygotes or homozygotes),

Table 2. Effects of *CYP1B1* haplotypes on PheT/3-HOPhe ratios

Haplotype	% Occurrence*	Geometric mean PheT/3-HOPhe (95% CI)		P
		Normal	Haplotype	
R48G + A119S + L432V	36.7	5.05 (4.25-5.99)	3.77 (3.34-4.25)	<0.01
R48G + A119S	48.3	4.93 (4.43-5.48)	3.53 (3.18-3.93)	<0.0001
R48G + L432V	37.6	5.03 (4.25-5.96)	3.80 (3.38-4.28)	<0.01

*Either homozygous or heterozygous in each.

whereas the corresponding values for *CYP1B1*A119S were 4.95 (95% CI, 4.30-5.71), 3.63 (95% CI, 3.12-4.22), and 2.46 (95% CI, 1.83-3.30; $P < 0.0001$). There was also a significant decrease in PheT/3-HOPhe levels ($P < 0.02$) in heterozygous males of both genotypes, but the effect was weaker and there was no gene dosage relationship. Stratifying by cigarettes per day, the strongest significant effects of the *CYP1B1*R48G and *CYP1B1*A119S polymorphisms in decreasing PheT/3-HOPhe ratio were seen in the 16 to 25 cigarettes per day group. Smokers heterozygous or homozygous for either *CYP1B1*R48G or *CYP1B1*A119S were significantly overrepresented in the bottom 10% and underrepresented in the top 10% of PheT/3-HOPhe ratios ($P < 0.001$).

The relationship between race, *CYP1B1* polymorphisms, and PheT/3-HOPhe ratios was further investigated. The occurrence of *CYP1B1*R48G and *CYP1B1*A119S polymorphisms was significantly greater in Blacks than Whites. For *CYP1B1*R48G, 68.8% of Blacks and 46.2% of Whites were either homozygotes or heterozygotes ($P = 0.0006$). For *CYP1B1*A119S, 71.4% of Blacks and 47.8% of Whites were either homozygotes or heterozygotes ($P = 0.0003$). PheT/3-HOPhe ratios were lower in Blacks than in Whites. After adjustment for race, the relationship between the presence of *CYP1B1*R48G and lower PheT/3-HOPhe ratio or PheT/total HOPhe ratio was no longer significant. With respect to *CYP1B1*A119S, the relationship between the presence of the polymorphism and lower ratios remained after adjustment for race. Thus, the higher prevalence of these polymorphisms in Blacks seems to be a significant factor in determining the lower ratios in Blacks than in Whites.

The strongest relationship between a combination of polymorphisms and PheT/3-HOPhe ratios in the top 10% of our subjects was found for *CYP1A1*I462V and *GSTM1* null. Nevertheless, only four of these subjects had both polymorphisms and 29% did not have either one. We investigated whether the highest 10% of PheT/3-HOPhe ratios could be predicted by any of the 11 polymorphisms individually or by certain combinations. The combinations were as follows: *CYP1A1*I462V and *GSTM1* null, *CYP1A1*MspI and *GSTM1* null, and each of the four *CYP1B1* polymorphisms individually with *GSTM1* null. The model could not accurately predict high PheT/3-HOPhe ratios.

Discussion

Some interesting effects of polymorphisms in *CYP* genes were observed in this study. The *CYP1A1*I462V polymorphism was associated with a significant increase in phenanthrene metabolic activation as determined by PheT/HOPhe ratios, and this effect was particularly strong in female smokers and in combination with *GSTM1* null. We also observed a consistent modifying effect of the *CYP1B1*R48G and *CYP1B1*A119S polymorphisms, in which the metabolic activation of phenanthrene was decreased. An important result of this study was that the polymorphisms alone or in logical combinations could not predict elevated PheT/HOPhe ratios, indicating that genotyping alone is not sufficient for evaluating individual differences in PAH metabolism.

The effects of the *CYP1A1*I462V polymorphism on metabolism have been examined in several studies, with mixed results (reviewed in refs. 13, 47). Several studies showed higher metabolism of ethoxyresorufin or BaP in mitogen-stimulated human lymphocytes treated with 3-methylcholanthrene or noninduced (17, 48-50). Increased levels of BaP-DNA adducts in lung tissue, particularly in combination with *GSTM1* null (51), or higher PAH-DNA adducts in leukocytes (52) have also been observed. Other studies showed little or no effect of the polymorphism on ethoxyresorufin or PAH metabolism in lung microsomes (47) or in systems using

expressed enzymes (53, 54). Conversion of BaP-7,8-diol to BPDE proceeded with lower K_m values but also lower rates with expressed enzymes from the *CYP1A1*I462V variant (55). There was no effect of this polymorphism on excretion of 1-hydroxypyrene in nonoccupationally exposed individuals, but after adjusting for age, ethnicity, and number of cigarettes per day, smokers with at least one variant allele excreted ~2-fold more 1-hydroxypyrene than those with wild-type genotype (56). There is no pattern in these data that would be entirely consistent with our observation of higher PheT/HOPhe ratios, although some studies, similar to ours, reported increased metabolic activation.

The *CYP1A1*I462V polymorphism was not associated overall with lung cancer risk in a cohort of men in Shanghai, China, although there was some suggestion that having at least one valine allele might be related to increased risk of lung cancer among smokers of <20 cigarettes per day, particularly among *GSTM1* null individuals (57). Quite similar results were obtained in two other studies in China (58, 59). A study among Chinese women showed that the *CYP1A1*I462V genotype was associated with a significantly elevated risk of lung cancer in both smokers and nonsmokers, with the risk being greater in the latter (60). Another study showed a higher risk of lung cancer in nonsmoking Chinese women with the *CYP1A1*I462V genotype than in those with the normal genotype (61). A pooled analysis of the Genetic Susceptibility to Environmental Carcinogens database found that there was a significant gene dosage effect for the *CYP1A1*I462V polymorphism with respect to lung cancer in the United States and that the effect seemed to be stronger in Caucasians than Asians. The effect of the polymorphism was strongest in never-smoking females (22). Collectively, the results of these studies are quite consistent with our data, if one assumes that higher PheT/HOPhe ratios are associated with higher risk for lung cancer, as theory would indicate.

Several studies have examined the effects of *CYP1B1* variants on PAH metabolism. Variant enzymes expressed from *CYP1B1*L432V and *CYP1B1*A119S had similar activity to wild-type in the conversion of BaP to mutagens, whereas *CYP1B1*A119S but not *CYP1B1*L432V had slightly higher activity than wild-type for conversion of BaP-7,8-diol to mutagens (62). Expressed enzymes from all 16 haplotypes of the common *CYP1B1* variants studied here had no major effects on the conversion of BaP-7,8-diol to BPDE (63). Conversion of BaP to BaP-7,8-diol was significantly decreased in expressed enzyme from *CYP1B1*N453S compared with wild-type, but no other differences were observed for the variants studied here (64). Cellular levels of the protein from the *CYP1B1*N453S genotype were also found to be lower than those of the other variants (65). Collectively, the results of these studies would suggest little effect of the *CYP1B1* polymorphisms studied here on phenanthrene metabolism, yet our results clearly showed a decrease in PheT/HOPhe ratios in individuals with the *CYP1B1*R48G and *CYP1B1*A119S variants, as well as the three haplotypes summarized in Table 2, compared with those with the wild-type gene. The decrease in ratios observed here may have been due to an increase in HOPhe levels, which could result from effects of variant *CYP1B1* enzymes not accounted for in these studies. The low ratios were also associated with race, as the prevalence of the polymorphisms was greater in Blacks than Whites.

There is relatively little information available on *CYP1B1* polymorphisms and lung cancer. One study found an association of the *CYP1B1*A119S polymorphism with lung cancer in a Japanese population (66). A second found no relationship between the *CYP1B1*L432V polymorphism and lung cancer in a Danish cohort (67). However, this polymorphism was associated with an increased risk for lung cancer in never smokers and was more strongly associated with lung cancer when there was some household exposure to environmental

tobacco smoke (68). Our results would suggest a protective effect of the *CYP1B1*R48G and *CYP1B1*A119S polymorphisms in smokers.

GSTM1 and *GSTP1* code for proteins that are good catalysts of PAH diol epoxide detoxification (40, 42, 43). Therefore, it is logical that this pathway of PAH metabolism would decrease in individuals with *GSTM1* deleted. The *GSTP1*I105V polymorphism codes for a protein with higher activity toward PAH diol epoxides than the normal form (41). Many studies have examined the relationship between *GSTM1* null genotype, alone or in combination with other polymorphisms, and lung cancer in smokers (reviewed in refs. 19, 47). These studies may indicate a slight elevation in risk for lung cancer in people with *GSTM1* null genotype. Recent meta-analyses and pooled analyses indicate no effect of *GSTM1* null genotype on lung cancer risk but do show combined effects of *CYP1A1*MspI and *CYP1A1*I462V polymorphisms with *GSTM1* null in increasing lung cancer risk (20, 21, 27). Mixed results have been obtained in studies of *GSTP1* polymorphisms and lung cancer (69-76). Our data do not show consistent significant effects of *GSTM1* null or *GSTP1* polymorphisms on phenanthrene metabolism, but as discussed above, a significant increase in PheT/HOPhe ratios was observed in individuals with combined *CYP1A1*I462V polymorphism and *GSTM1* null genotype, consistent with theory and with previous studies.

A limitation of this study was that only one measurement of urinary phenanthrene metabolites was conducted for each subject. We do not know if the PheT/HOPhe ratios were stable over time in our 346 subjects. In a previous study, we measured urinary phenanthrene metabolites in 12 smokers who provided samples daily for 7 days and then weekly for 6 weeks (30). PheT/HOPhe ratios were relatively stable in some subjects but quite variable in others. We concluded that multiple sampling would be advisable when comparing individuals, as in this study. However, because of the size of this study, it was not feasible to do multiple sampling and analyses.

A recent study by Lodovici et al. (77) used an approach similar to ours. They compared levels of BPDE-DNA adducts in leukocytes of 41 smokers with combinations of genotypes. They found that the combination of *CYP1A1*MspI or *CYP1A1*I462V with *GSTM1* null was associated with significantly higher BPDE-DNA adducts similar to our finding with PheT/3-HOPhe ratios. Including *GSTP1* wild-type in this "high-risk" group was also associated with significantly higher BPDE-DNA adducts, but the relationship was weakened by inclusion of *EPHX1* genotypes. Overall, their results in a smaller group of smokers were quite consistent with ours.

The results of this study show that polymorphisms in PAH metabolizing genes cannot accurately predict phenanthrene metabolism in smokers. As phenanthrene is structurally similar to and is metabolized by the same enzymes as other PAH, it is also very likely that genetic polymorphisms could not accurately predict the metabolism of other PAH. This calls into question the strategy of genotyping for identification of individuals who might be particularly susceptible or resistant to PAH carcinogenesis. This conclusion is logical because enzyme activities are not dictated by genotype alone. Differences in enzyme expression, induction, inhibition, etc. will affect PAH metabolic activation and detoxification. Although metabolic phenotyping is presently more technically demanding than genotyping, current advances in analytic chemistry will lead to rapid phenotyping methods suitable for incorporation into large epidemiologic studies.

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