Benzo(a)pyrene Dioleopoxide (BPDE)-DNA Adduct Levels in Leukocytes of Smokers in Relation to Polymorphism of CYP1A1, GSTM1, GSTP1, GSTT1, and mEH

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Abstract

Benzo(a)pyrene [B(a)P] dioleopoxide (BPDE)-DNA adducts were measured in the leukocytes of 41 healthy smokers using high-performance liquid chromatography coupled with a fluorimetric detector. The correlation between exposure to B(a)P through smoking and BPDE-DNA adduct levels was poor (r = 0.31), although subjects in the high exposure group [B(a)P > 50 ng/d] had a slightly higher level of adducts compared with the less exposed group (mean ± SE, 1.70 ± 0.3 versus 1.09 ± 0.1; P = 0.057). We studied the effect on BPDE-DNA adducts of individual variations in genes controlling B(a)P metabolism, classifying subjects in “low-risk” and “high-risk” genotypes for smoking-related B(a)P DNA damage. The high-risk group included subjects characterized by a combination of increased B(a)P activation [cytochrome P450 1A1 (CYP1A1) MspI and/or exon 7 Ile105Val allele variants and microsomal epoxide hydrolase (mEH) fast activity] and decreased deactivation ability [presence of glutathione S-transferase M1 (GSTM1) null allele and wild-type glutathione S-transferase P1 (GSTP1)]. The low-risk group included smokers with lower B(a)P activation (wild-type CYP1A1, low or intermediate mEH activity) and higher deactivation capacity (active GSTM1, GSTP1 Ile105Val allele). Subjects in the low-risk group had lower levels of BPDE-DNA adducts compared with subjects in the high-risk genotype group; this difference was significant using two markers (CYP1A1 and GSTM1, median ± SD, 0.77 ± 1.16 versus 1.89 ± 0.39; P = 0.03) or three markers (CYP1A1, GSTM1, and GSTP1, median ± SD, 0.66 ± 0.93 versus 1.43 ± 1.17; P = 0.013). The discrimination between groups was reduced when including mEH as an additional marker (P = 0.085). In conclusion, CYP1A1, GSTM1, and GSTP1 genotyping seems to be a risk predictor of BPDE-DNA adduct formation in leukocytes. (Cancer Epidemiol Biomarkers Prev 2004;13(8):1342–8)

Introduction

Benzo(a)pyrene [B(a)P], a representative of polycyclic aromatic hydrocarbons (PAH), is a classic DNA-damaging carcinogen present in polluted air, food, and cigarette smoke.

Cigarette smokers have relatively high PAH-DNA adduct levels (1, 2) and are often studied with the aim of understanding the mechanisms that correlate exposure, activation, and deactivation of PAH and cancer occurrence.

The best characterized activation pathway of B(a)P consists of covalent binding to DNA of its metabolically active species [i.e., (±)-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE; ref. 3)], resulting in adducts that may lead to mutations relevant for carcinogenesis when occurring on oncogenes or tumor suppressor genes (4).

It has been suggested that individual variations in the susceptibility to the mutagenic and carcinogenic activity of B(a)P might be partially explained by differences in its activation and deactivation pathways. B(a)P is oxidized by a series of well-characterized enzymes such as cytochrome P450 1A1 (CYP1A1), CYP2C9, and CYP3A4 (5, 6) and further metabolized by microsomal epoxide hydrolase (mEH), catalyzing the hydrolysis of B(a)P epoxides into trans-dihydrodiols (7, 8). B(a)P epoxides can also undergo different metabolic fates [e.g., become substrates of glutathione S-transferase enzymes (9-11)]. Other metabolic pathways have been described but are usually considered to be less important in B(a)P activation/deactivation.

Variations in metabolic genotypes in smoking-related cancer have been studied extensively. Four CYP1A1 polymorphisms have been reported; two of these—a thymine/cytosine point mutation in the MspI restriction site and an isoleucine/valine substitution in exon 7 at the heme binding region—result in increased enzyme activity (12).

Different glutathione S-transferase isoenzymes have been identified in human populations, some with tissue-specific expression. The glutathione S-transferase M1
(GSTM1) and glutathione S-transferase T1 (GSTT1) enzymes have been shown to be polymorphic. In both genes, a deletion is responsible for the existence of a null allele associated with the lack of expression of a functional protein (13, 14).

A coding sequence polymorphism in glutathione S-transferase P1 (GSTP1), an A → G transition in nucleotide 303 has also been identified. It results in a change in codon 105 from isoleucine to valine in the hydrophobic binding site and is associated with a variation in catalytic efficiency (15).

The mEH enzyme is encoded by a single gene (EPHX1), and two polymorphic sites have been reported within the coding region: an exon 3 polymorphism results in a tyrosine-to-histidine substitution in residue 113, and an exon 4 polymorphic site results in a 139 histidine-to-arginine substitution. According to in vitro studies, exon 3 and 4 substitutions, respectively, may decrease and increase mEH activity in tissues (8).

Lung, esophagus, and head and neck cancers have been associated with CYP1A1 variants and with GSTM1 deficiency in smokers (12). CYP1A1 and GSTM1 variants also seem to play a role in lung cancer risk in Caucasian nonsmokers (16). The correlation between metabolic polymorphisms and cancer, given a similar exposure, may be explained by varying levels of adducts in target organs, which are correlated with different activation/deactivation genotypes.

Because samples from target organs such as lungs are hard to obtain from healthy subjects, many researchers have tried to study the correlation between metabolic genotypes and DNA adducts by using more accessible blood cells (17-21). CYP1A1 and/or glutathione S-transferase genotypes are often studied in leukocytes or lymphocytes, showing that individuals with high activating CYP1A1/low deactivating GSTM1 genotypes tend to have higher DNA adduct levels (19-28). Similar studies document the influence of mEH, GSTP1, and GSTT1 (29-34).

We studied BPDE-DNA adducts in leukocytes from healthy smokers as a function of exposure to B(a)P and CYP1A1, GSTM1, GSTP1, GSTT1, and mEH polymorphisms, with the goal of finding a panel of simple genetic markers for identifying subjects at increased risk of tobacco-related B(a)P DNA damage. Buffy-coated cells (~50 mL) were centrifuged at 800 × g for 20 minutes at 8°C and frozen at −80°C until analysis, which was completed within 20 days after sampling.

BPDE-DNA Adduct Analysis. Leukocytes and DNA were isolated as described previously in detail (13, 31). DNA (~400 to 500 µg) was used for the analysis of B(a)P tetrols, carried out with a high-performance liquid chromatography system coupled with a fluorimetric detector as reported previously (35).

Genotype Analysis. CYP1A1 MspI and exon 7 Ile<sup>462</sup>Val polymorphisms were studied using a PCR-RFLP assay, as described by Cascorbi et al. (36).

For GSTP1 Ile<sup>105</sup>Val polymorphism, we used a PCR-RFLP method following the protocol from Harries et al. (37). PCR products (20 µL) were incubated with 5 units BsmI (New England Biolabs, Hertfordshire, United Kingdom) for 4 hours at 37°C and loaded on a 3% agarose gel stained with ethidium bromide.

GSTM1 and GSTT1 genotypes were determined using a PCR-based assay, which allows an internal standard–controlled identification of positive or null samples, as described by Arand et al. (38). The PCR reaction was carried out in a 25 µL volume containing 1× PCR buffer, 0.75 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L deoxynucleotide triphosphates, and 1.25 units Taq polymerase (Advanced Biotechnologies, Surrey, United Kingdom). The GSTT1 and albumin primers were at a final concentration of 4 ng/µL, and the GSTM1 primers were at a final concentration of 6 ng/µL. The PCR conditions were 95°C for 5 minutes and 30 cycles at 95°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes and a final extension at 72°C for 5 minutes.

To study mEH Tyr<sup>113</sup>His and His<sup>139</sup>Arg polymorphisms, we used two separate PCR; for the 113 position, the presence of an EcoRV restriction site identified the wild-type alleles, whereas the RsaI restriction enzyme site was cleaved in the variant alleles for position 139 (39). Based on the reported in vitro activities of the different mEH protein variants, some authors have combined the exon 3 and 4 polymorphisms to estimate the mEH activity in vivo. Following the classification of Benhamou et al. (40), we assigned the combined genotypes to three levels of predicted mEH activity: high, intermediate, and low.

PCRs were all done in a Perkin-Elmer 9700 thermal cycler (Perkin-Elmer, Foster City, CA).

We studied the effect of individual variations in genes controlling B(a)P metabolism on BPDE-DNA adduct formation, dividing subjects into “low-risk” and “high-risk” genotypes for smoke-related B(a)P DNA damage.

The high-risk subjects included smokers characterized by a combination of increased B(a)P activation due to CYP1A1 MspI and/or exon 7 Ile<sup>462</sup>Val allele variants (41, 42) and fast mEH enzymatic activity and decreased B(a)P deactivation ability due to GSTM1-null allele and wild-type GSTP1, because the GSTP1 Ile<sup>105</sup>Val allele has greater activity on PAH diol epoxides compared with the wild-type allele (43).

The low-risk group included all smokers carrying no “risk” polymorphism or just one “risk” polymorphism. Figure 1 highlights the critical steps in B(a)P activation and detoxification, whereas the studied polymorphisms...
and their effects on enzymatic function are summarized in Table 1.

**B(a)P Exposure Determination.** Different cigarette brands purchased in Italy were smoked with a homemade smoking machine described by Luceri et al. (44) to sample PAH present in mainstream cigarette smoke. We sampled PAH using a polytetrafluoroethylene filter (type AP10, 2 μm pore size) and ORBO 43 sorbent tube in line with a lit cigarette. PAH were extracted as reported by Lodovici et al. (45).

B(a)P exposure in each subject was calculated by multiplying the number of cigarettes smoked per day with the average B(a)P content in each brand and ranged from 3 to 158 ng/d.

**Statistical Analysis.** The correlation between BPDE-DNA adducts and B(a)P exposure was calculated by regression analysis. Differences between high and low exposed B(a)P groups were analyzed using ANOVA, and data are expressed as means ± SE.

The effects of each polymorphism variation and the combination of low-risk and high-risk genotype on BPDE-DNA adduct levels were analyzed using nonparametric statistics with the Mann-Whitney test. Data are expressed as medians, reporting the interquartile range, SD and, for the smaller sample sizes, min-max ranges. The statistical analysis was carried out using the Stata statistical package (Stata, College Station, TX).

**Results**

BPDE-DNA adducts were influenced by the number of cigarettes smoked (Fig. 2A). When considering B(a)P exposure, obtained by multiplying the average content for each brand by the reported number of cigarettes smoked per day, we found that the group exposed through smoking to low B(a)P (<50 ng/d) had fewer adducts than the high exposed group (>50 ng/d), although this difference was not statistically significant (P = 0.057).

The calculated daily exposure to B(a)P was poorly correlated with BPDE-DNA adducts in different individuals (r = 0.31; Fig. 3). This poor correlation could have been due to the exposure to B(a)P in diet, a variable not controlled in this study. However, in Italy (46), average human exposure to B(a)P through the diet is small (calculated to be ~6 ng/d) compared with smokers’

**Table 1. Polymorphisms studied and their effects on enzymatic functions**

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Effect on function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>T&lt;sub&gt;6235&lt;/sub&gt; → C (MspI restriction site)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>A&lt;sub&gt;4819&lt;/sub&gt; → G (exon 7)</td>
<td>Ile&lt;sup&gt;662&lt;/sup&gt; Val</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Deletion</td>
<td>None (null)</td>
</tr>
<tr>
<td>GSTT1</td>
<td>Deletion</td>
<td>None (null)</td>
</tr>
<tr>
<td>GSTP1</td>
<td>A&lt;sub&gt;303&lt;/sub&gt; → G (exon 5)</td>
<td>Ile&lt;sup&gt;105&lt;/sup&gt; Val</td>
</tr>
<tr>
<td>mEH</td>
<td>Exon 3 T → C</td>
<td>Tyr&lt;sup&gt;113&lt;/sup&gt;His</td>
</tr>
<tr>
<td></td>
<td>Exon 4 A → G</td>
<td>His&lt;sup&gt;138&lt;/sup&gt;Arg</td>
</tr>
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![Figure 1. Critical steps in B(a)P activation and detoxification.](image-url)
intake. Therefore, dietary intake could be a confounding factor only for five subjects enlisted in our study, who smoked two to five cigarettes per day.

We studied the effect of individual variations in metabolic genotypes on BPDE-DNA adduct levels (Table 2). Studying each gene polymorphism by itself, a commonly used approach, no statistically significant variations in DNA adduct levels were observed, with the exception of a borderline significant increase in adducts in the GSTM1-null subjects versus subjects carrying active GSTM1 ($P = 0.063$; Table 2).

Smokers were classified according to a combination of activation/deactivation genotypes. Given the limited sample size, we considered only two combinations of activation and deactivation.

Using just CYP1A1 and GSTM1 as genetic markers, we considered subjects with wild-type CYP1A1 or GSTM1 functional allele as being at low risk for BPDE-DNA formation, whereas those with MspI and/or exon 7 CYP1A1 variants associated with GSTM1-null genotype were at high risk.

In a second classification, the GSTP1 genotype was also considered, and we included subjects with GSTP1 Ile$^{105}$Val allele in the low-risk group, whereas wild-type GSTP1 were classified in the high-risk group.

In a third classification, the predicted mEH enzyme activity was also considered; subjects with low or intermediate mEH enzymatic activity were classified in the low-risk group, whereas subjects with fast mEH activity were included in the high-risk group.

The differences between low-risk and high-risk groups were significant using two markers (CYP1A1 and GSTM1; $P = 0.03$) or three markers (CYP1A1, GSTM1, and GSTP1; $P = 0.013$). The discrimination between groups was reduced ($P = 0.085$) when mEH was included as additional marker (Fig. 4).

**Discussion**

Our data show that BPDE-DNA adducts in smokers, with no occupational exposure to PAH, are poorly correlated with B(a)P exposure through cigarette smoke. Dietary B(a)P exposure was not a confounding factor because the estimated exposure to B(a)P through the diet is low in Italy (46), equivalent to two to five cigarettes; therefore, it is not a significant confounding factor for most smokers in our study (36 of 41).

The classification of smokers into low-risk and high-risk genotypes using CYP1A1 and GSTM1 as genetic markers identified two distinct groups having lower and higher BPDE-DNA adducts. This observation confirms data reported by Brescia et al. (24) on leukocytes of coke-oven workers. Moreover, Rojas et al. (26) found that subjects with active GSTM1 had undetectable BPDE-DNA adducts when the mutation in CYP1A1 was heterozygous, whereas individuals homozygous at MspI CYP1A1 locus and with null GSTM1 genotype had extremely high BPDE-DNA adduct levels.

In the present study, the addition of a third classification factor for polymorphism, GSTP1, increased the discrimination of adduct levels between genotypes. Consequently, GSTP1 should be added as a risk marker of DNA adducts, given the relative ease with which this additional genotyping can be done and also
considering that GSTP1 Ile105Val is a relatively frequent polymorphism (37).

On the contrary, the inclusion of a predicted higher mEH enzymatic activity as a B(a)P activation factor resulted in the muddling of differences between groups. This might be explained by the dual function of mEH, which is involved not only in the production of the final active metabolite of B(a)P but also in its inactivation (47). While, in fact, mEH usually results in detoxification of xenobiotic epoxides, it is also involved in the metabolic activation of PAHs, catalyzing the conversion of B(a)P to 7,8-diol, which is converted by CYP1A1 and CYP3A4 into 7,8-diol-9,10-epoxide, a highly reactive metabolite that can damage DNA, RNA, and proteins (Fig. 1).

We classified the high mEH activity genotype as a risk factor, because the carriers of this genotype have an increased risk of developing PAH-related malignancies (48, 49). Some data in the literature, as well as our own, do not show a significant effect of mEH activity on leukocyte DNA adducts (39), whereas Kuljukka-Rabb et al. (32) found a relationship between mEH activity and adduct levels in coke-oven workers.

The mosaic of different polymorphisms of genes involved in PAH activation/deactivation pathways may improve the validity of leukocyte BPDE adducts as markers of risk for B(a)P carcinogenesis. In this context, it would also be appropriate to study genes involved in the processing of DNA damage such as DNA repair genes.

![Figure 4. BPDE adduct levels in subjects with low-risk and high-risk genotype considering two (CYP1A1 and GSTM1), three (CYP1A1, GSTM1, and GSTP1) or four (CYP1A1, mEH, GSTM1, and GSTP1) polymorphisms. Columns, medians; bars, interquartile ranges. The appropriate significance of the statistical comparisons between groups, as shown above, was calculated with the Mann-Whitney test. Bold numbers, number of individuals in each genotype subgroup.](image-url)
Nevertheless, our data indicate that the levels of leukocyte BPDE adducts in smokers are correlated more with an enhanced capacity to activate and impair deactivation of B(a)P than with the total burden of PAH in tobacco smoke. It is possible to postulate that subjects with enhanced hereditary capacity to activate, and/or low capacity to deactivate, PAH are at higher risk of developing cancer when exposed to cigarette smoke or other sources of PAH environmental pollution. The identification of particularly vulnerable people could allow a more effective preventive action.

References


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