Analyses of bronchial bulky DNA adduct levels and CYP2C9, GSTP1 and NQO1 genotypes in a Hungarian study population with pulmonary diseases

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Carcinogen–DNA adducts may represent an intermediate end-point in the carcinogenic cascade and may reflect exposure to chemical carcinogens, as well as susceptibility and, ultimately, cancer risk. Interindividual variability in activity of enzymes involved in the metabolism of polycyclic aromatic hydrocarbons to mutagenic diol epoxides may predict adduct levels and, indirectly, lung cancer risk. Using 32P-postlabeling methods, the levels of bulky DNA adducts were determined in macroscopically normal bronchial tissues obtained from resected lobes of 143 Hungarian patients with lung malignancy and other pulmonary conditions. DNA from normal tissue was also evaluated for polymorphisms in cytochrome P450 2C9 (CYP2C9) at two sites, codons 144 (Arg/Cys) and 359 (Ile/Leu), for glutathione S-transferase P1 (GSTP1) at codon 105 and for NAD(P)H:quinone oxidoreductase (NQO1) at codon 187 (Pro/Ser). Using the Mann–Whitney U-test and analysis of variance, levels of adducts were evaluated in relation to variant genotypes, separately for smokers and non-smokers. As previously reported, bulky DNA adduct levels in smokers (n = 104) were estimated to be 54% higher than in non-smokers (n = 39) (8.6 ± 4.2 versus 5.6 ± 3.3 per 10^6 nucleotides, respectively, P < 0.01). Adduct levels were 16–29% higher in individuals with the homozygous Ile359/Ile359 CYP2C9 allele than in those heterozygous for the variant allele (Ile359/Leu359) [8.8 ± 4.3 (n = 84) versus 7.6 ± 3.5 (n = 20) for smokers and 5.8 ± 3.5 (n = 32) versus 4.5 ± 1.3 (n = 7) for non-smokers], although differences were not statistically significant. There were no clear differences in adduct levels in relation to genotypes of NQO1 or GSTP1. Although numbers of patients in this study are large in relation to many studies of carcinogen–DNA adducts, it is still possible that significant differences were not noted for polymorphisms in xenobiotic metabolizing enzymes due to relatively small numbers in stratified data.

Introduction

Carcinogens in cigarette smoke are clearly the major risk factors for human lung cancer, as documented in epidemiological studies (1–3). Exposure to tobacco smoke carcinogens, including polycyclic aromatic hydrocarbons (PAHs) and aromatic amines, may be monitored by measurement of PAH–serum albumin adducts (4) and 4-aminobiphenyl–hemoglobin adducts (5), respectively. Bulky DNA adducts have been measured in human lung and bronchial tissues by 32P-postlabeling in samples from cancer patients and have provided evidence of DNA damage caused by smoking (6,7).

Benzo[a]pyrene (B[a]P), a major carcinogenic component of tobacco smoke, is known to undergo metabolic activation to exert its carcinogenic effects. Moreover, formation of anti-B[a]P 7,8-diol 9,10-epoxide (anti-BPDE), a metabolically activated form of B[a]P, has been evidenced by the detection of anti-BPDE–DNA adducts in human lung and colon mucosa by sensitive HPLC/fluorometric assays (8).

Different forms of cytochromes P450 (CYPs) (e.g. CYP1A1, CYP1A2, CYP2C9 and CYP3A4) have been shown to catalyze the formation of an ultimate mutagenic and genotoxic diol epoxide of B[a]P (9–11). In a study using human laryngeal tissues, total DNA adduct levels correlated strongly with expressed levels of CYP1A1, CYP3A4 and CYP2C forms, indicating that metabolic activation of tobacco smoke carcinogens by these CYPs is an important determinant of genotoxicity (12). Forms of human glutathione S-transferase (GST) are known to catalyze the detoxification of B[a]P by conjugation of epoxide metabolites of B[a]P with glutathione (13). Among forms of GST expressed in human tissues, GSTP1 has been shown to be expressed in the highest abundance in lung tissue (13). NAD(P)H:quinone oxidoreductase (encoded by NQO1 or DIA4) is known to catalyze the reduction of the quinone moiety to a hydroquinone to reduce the production of oxygen radicals (14) and has been shown to prevent the formation of B[a]P quinone–DNA adducts (15). Genetic polymorphisms have been identified in the CYP1A1 (16,17), CYP2C9 (18), GSTP1 (19) and NQO1 (20) genes. These genetic polymorphisms are associated with amino acid substitutions in open reading frames or in flanking regions that may affect catalytic properties and/or expressed levels of those enzymes. The amino acid substitutions in the observed genetic polymorphisms of CYP2C9, GSTP1 and NQO1 have been reported respectively as follows: Arg144Cys and Ile359Leu (18), Ile105Val (19) and Pro187Ser (20). A polymorphism at codon 105 in the GSTP1 gene was recently found to be associated with susceptibility to bladder, testicular and lung cancer (19,24). Though not statistically significant, slightly increased and decreased lung cancer risk was observed with the CYP2C9*2 and CYP2C9*3 alleles, respectively, in a population in Los Angeles County, California (25,26). Little is known of the association of genetic polymorphisms of NQO1 with carcinogen–DNA adducts and related susceptibility to malignant diseases, except

Abbreviations: anti-BPDE, anti-benzo[a]pyrene 7,8-diol 9,10-epoxide; B[a]P, benzo[a]pyrene; CYP, cytochrome P450; GST, glutathione S-transferase; NQO1, NAD(P)H:quinone oxidoreductase; PAH, polycyclic aromatic hydrocarbon.
Table I. Hungarian lung disease patients for which levels of bronchial bulky DNA adducts and CYP2C9, GSTP1 and NQO1 genotypes have been determined

<table>
<thead>
<tr>
<th></th>
<th>Smokers (Sm, Ex-Sm)</th>
<th>Non-smokers (Ex-Sm, N-Sm)</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>n, total</td>
<td>104 (79, 25)</td>
<td>39 (19, 20)</td>
<td>143</td>
</tr>
<tr>
<td>Age range</td>
<td>22–69</td>
<td>34–74</td>
<td>22–74</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>52.7 ± 9.2</td>
<td>54.5 ± 9.7</td>
<td>53.2 ± 9.4</td>
</tr>
<tr>
<td>n, male/female</td>
<td>83/21 (63/16, 20/5)</td>
<td>26/13 (16/3, 10/10)</td>
<td>109/34</td>
</tr>
</tbody>
</table>

a Sm, current smokers who smoked up to the day of the pulmonary surgery; Ex-Sm, short-term ex-smokers who gave up smoking for a maximum of 1 year before surgery (range 1 week–1 year).

b Ex-Sm, long-term ex-smokers who gave up smoking for >1 year before surgery (range 2–20 years); N-Sm, life-time non-smokers.

2 SQ, squamous cell carcinoma; A, adenocarcinoma; SC, small cell carcinoma; LC, large cell carcinoma; OT, other type of lung malignancy; non-malign., non-malignant lung disease.

3 One patient had both squamous cell and small cell carcinomas.

Materials and methods

Study population

Demographic characteristics of the study samples in which CYP2C9, GSTP1 and NQO1 genotypes were evaluated in relation to genetic polymorphisms in CYP1A1 and GSTM1. In that analysis, it was found that, although GSTM1 and CYP1A1 MspI genotypes did not affect adduct levels, the GSTM1 genotype appeared to be a risk factor for squamous cell carcinoma. To our knowledge, associations between levels of BPDE adducts in the lung and genetic polymorphisms in CYP2C9, GSTP1 and NQO1 have not been evaluated.

In the present study using additional samples, the relationship between DNA adduct levels in bronchial tissues from patients with pulmonary diseases and genetic polymorphisms in CYP2C9, GSTP1 and NQO1 was examined.

DNA adduct measurements

Bulky DNA adducts were determined by the 32P-postlabeling procedure as described previously (28) using nuclease P1 digestion of the normal nucleotides for determining the 3’-end of the PCR products was performed using fI (New England Biolabs). The PCR reaction was performed in a mixture which consisted of 10 mM Tris–HCl buffer (pH 8.3), 2% DMSO, 1.5 mM MgCl2, 0.1% (v/v) Triton X-100, 50 mM KCl, 250 µM dNTPs, 1 µM forward (5’-ACGCA-CATCCTCTTCTCCCTC-3’) and reverse (5’-TACTTGGCTGGTTGA TGTCC-3’) primers, 2.5 U Tau polymerase and 50 ng of genomic DNA. PCR reactions were performed for 35 cycles at a heat-denaturing temperature of 94°C for 30 s, an annealing temperature of 58°C for 30 s and an elongation temperature of 72°C for 2 min after a Perkin Elmer DNA thermal cycler 9600. The PCR products were purified on a MicroSpin S-300 column (Pharmacia, Uppsala, Sweden) and digested with AvsI (New England Biolabs, Beverly, MA) and analyzed by 3% Metaphor agarose (FMC, Rockland, ME) gel electrophoresis. PCR products derived from CYP2C9 Arg144 and CYP2C9 Cys144 alleles were detected as DNA fragments of 259 and 57 bp or as a single DNA fragment of 316 bp, respectively. A PCR-based genotyping method was also developed for CYP2C9 ile359Leu using a forward (5’-TGCACGAGTTCCAGAGATGTC-3’) and a reverse primer (5’-AAA-CATGGAATTCTCAGTTAG-3’) as described by Wang et al. (30). Reaction mixtures for PCR amplification consisted of the same components as for the CYP2C9Arg144Cys polymorphism, except that 0.5 µM of the forward and reverse primers were used. PCR amplification was carried out as in the method for determining the CYP2C9Arg144Cys polymorphism, except for an annealing temperature of 66°C for 30 s. Restriction enzyme digestion was performed using NsiI (New England Biolabs) after purification of the PCR products on MicroSpin S-300 columns. PCR products derived from the CYP2C9 ile359 negative allele gave DNA fragments of 110 and 21 bp, whereas those derived from the CYP2C9 Leu359 allele were not digested by NsiI and gave a single fragment of 131 bp.

Determination of genotype at the GSTP1 locus

Since two variant cDNAs encoding Ile and Val at codon 105 were isolated for GSTP1, the Ile105Val polymorphism was detected by a PCR-based genotyping method. The PCR reaction was performed in a mixture which consisted of 10 mM Tris–HCl buffer (pH 8.3), 2% DMSO, 1.5 mM MgCl2, 0.1% (v/v) Triton X-100, 50 mM KCl, 250 µM dNTPs, 1 µM forward (5’-ACGCA-CATCCTCTTCTCCCTC-3’) and reverse (5’-TACTTGGCTGGTTGA TGTCC-3’) primers, 2.5 U Tau polymerase and 50 ng of genomic DNA. PCR reactions were performed for 35 cycles at a heat-denaturing temperature of 94°C for 30 s, an annealing temperature of 66°C for 30 s and an elongation temperature of 72°C for 2 min. Subsequently, PCR products, purified on a Sepharose S-200 Spin column (Pharmacia), were treated with AvsI (New England Biolabs). Using this protocol, PCR products (440 bp) produced from a GSTP1 Ile105 allele were not digested by AvsI, whereas those from the GSTP1 Val105 allele gave DNA fragments of 213 and 227 bp after digestion with AvsI. Although Harries et al. recently reported a similar method for GSTP1 genotyping (19), different primer sets were used in order to yield larger DNA fragments with higher sensitivity against agar gel electrophoresis. Both methods were used in a subset of samples (n = 20), yielding identical GSTP1 genotypes.

Determination of genotypes at the NQO1 locus

The Pro187Ser polymorphism was also detected by PCR-based genotyping. The primers used were 5’-ATTCTCTAAGTGGCCTGAG-3’ and 5’-AATCTGTGTCGTGTTTCTC-3’. The PCR reaction was performed in a mixture (50 µl) which consisted of 10 mM Tris–HCl buffer (pH 8.3), 2% DMSO, 2.5 mM MgCl2, 0.1% (v/v) Triton X-100, 50 mM KCl, 200 µM dNTPs, 1 µM forward and reverse primers, 2.5 U Taq DNA polymerase (Promega) and 50 ng of genomic DNA. PCR reactions were performed for 35 cycles at a heat-denaturing temperature of 94°C for 30 s, an annealing temperature of 66°C for 30 s and an elongation temperature of 72°C for 2 min. Restriction enzyme digestion was performed using HindIII (New England Biolabs). The frequent allele (NQO1 Pro187) was detected as one band with a length of 318 bp, whereas the infrequent allele was detected as 154 and 164 bp bands.

Statistical analysis

Comparison of DNA adduct levels between smokers and non-smokers and within genotypes was made using the Mann–Whitney U-test and analysis of variance.

Results

Bulky DNA adduct levels in human bronchial samples

Bulky DNA adducts were measured in relation to CYP2C9, GSTP1 and NQO1 genotypes from 143 individual bronchial...
The function of CYP2C9 in the metabolism of tobacco smoke PAH carcinogens such as B[a]P is complex. CYP2C9 has been shown to catalyze both activation and inactivation reactions using cDNA expression systems in bacteria, yeast and mammalian cells (31–33). This bioactivation involves pathways leading to 9-hydroxy-B[a]P-4,5-oxide and also 7,8-diol-9,10-epoxide formation from B[a]P-7,8-diol. In human lung, which is the target tissue of tobacco smoke carcinogens, protein expression of CYP2C9 has been verified along with CYP1A1, CYP2E1, CYP3A4/5 and CYP2D6, although CYP1A1 was the most abundant pulmonary CYP (34,35). In spite of low content of CYP2C9 in lung tissues, a relatively high capacity of metabolic activation of B[a]P by cDNA-expressed CYP2C9 has been reported (31). Furthermore, higher affinity of CYP2C9 for B[a]P was suggested as compared with CYP1A1 and CYP2E in a kinetic study on B[a]P metabolism (31). Tissue DNA adducts with BPDE, an ultimate carcinogen formed through the metabolism of B[a]P, were detected in human white blood cells and lungs by HPLC/synchronous fluorescent spectrometry (36,37). Administration of B[a]P-trans-7,8-dihydrodiol and 9-hydroxy-B[a]P to rats resulted in BPDE–DNA adducts and possibly an adduct of B[a]P-4,5-oxide and also 9-hydroxy-B[a]P-4,5-oxide in lung, liver and peripheral blood (38). This indicates that proximate carcinogens formed through the metabolism of B[a]P were transported to target tissues by circulation and that CYP2C9 is likely to influence the metabolism of tobacco smoke PAHs in lungs as well as in liver, where CYP2C9 is most abundantly expressed.

Discussion

In the present study, the influence of CYP2C9, GSTP1 and NQO1 genotypes on the levels of smoking-related DNA adducts was examined in a Hungarian study population with pulmonary diseases. A significant difference was observed in the bulky DNA adduct levels in bronchial tissues between smokers and non-smokers, as analyzed in more detail previously (28). In this study, the CYP2C9*Ile359 allele was associated with higher adduct levels. CYP2C9 genetic polymorphisms were recently evaluated in relation to lung cancer risk among Caucasians in Los Angeles County, California (25,26). Slightly increased risk of lung cancer was observed with the CYP2C9*2 allele (Cys144, Ile359) (25,26), whereas the CYP2C9*3 allele (Arg144, Leu359) was associated with slightly decreased lung cancer risk (26). Our present results on the CYP2C9*Ile359 allele are consistent with the study in Los Angeles County. On the other hand, no association was found among smoking-related adducts and genotypes of CYP2C9*Arg144Cys, GSTP1*Ile105Val or NQO1*Pro187Ser.

Table II. Levels of bronchial bulky DNA adducts in smokers and non-smokers with genotypes for CYP2C9, GSTP1 and NQO1 genes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Smokers (n)</th>
<th>Non-smokers (n)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9*Arg144Cys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a/a</td>
<td>8.6 ± 4.4 (71)</td>
<td>5.7 ± 3.3 (26)</td>
<td>0.015</td>
</tr>
<tr>
<td>a/b</td>
<td>8.4 ± 3.7 (32)</td>
<td>5.2 ± 3.4 (13)</td>
<td>0.001</td>
</tr>
<tr>
<td>b/b</td>
<td>9.4 (1)</td>
<td>6.0 (2)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

| CYP2C9*Ile359Leu    |             |                 |         |
| a/a                 | 8.8 ± 4.3 (84) | 5.8 ± 3.5 (32) | 0.001   |
| a/b                 | 7.6 ± 3.5 (20) | 4.5 ± 1.3 (7)  | 0.001   |
| b/b                 | - (0)        | - (0)           | 0.05    |

| GSTP1*Ile105Val     |             |                 |         |
| a/a                 | 9.2 ± 3.7 (50) | 5.4 ± 3.1 (19) | 0.001   |
| a/b                 | 8.0 ± 4.6 (41) | 6.0 ± 3.6 (17) | 0.001   |
| b/b                 | 7.8 ± 4.6 (13) | 4.0 ± 2.5 (3)  | 0.001   |

| NQO1*Pro187Ser      |             |                 |         |
| a/a                 | 8.9 ± 4.3 (77) | 5.7 ± 3.3 (35) | 0.001   |
| a/b                 | 7.5 ± 3.3 (20) | 4.9 ± 1.6 (3)  | 0.001   |
| b/b                 | 8.4 ± 5.4 (7)  | 4.1 (1)         | 0.001   |

| All                 | 8.6 ± 4.2 (104) | 5.6 ± 3.3 (39) | 0.001   |

4Bulky DNA adduct levels were determined by 32P-post-post-labeling methods as described in Materials and methods and are expressed as the mean ± SD per 10^8 nucleotides. Genotypes for CYP2C9, GSTP1 and NQO1 were analyzed as described in Materials and methods. Numbers in parentheses represent the number of subjects. a and b are frequent and less frequent alleles, respectively. a/a and b/b represent homozygotes and a/b represents heterozygotes. Frequent alleles in this study population: Arg144 and Ile359 for CYP2C9; Ile105 for GSTP1; Pro187 for NQO1.

4Smokers: current smokers, who smoked up to the time of surgery and short-term ex-smokers who stopped smoking <1 year before surgery. Non-smokers: lifetime non-smokers and long-term ex-smokers who stopped smoking ≥1 year before surgery.

4Level of significance for comparison of mean adduct levels between genotypes using one way analysis of variance.

4Level of significance for comparison of mean adduct levels between smokers and non-smokers (Mann-Whitney U-test).
Fig. 1. Effect of smoking and different alleles of xenobiotic metabolizing enzymes on bulky adduct levels in bronchial tissue DNA. The levels of bronchial DNA adducts (per 10^8 nucleotides) were compared according to the different alleles of CYP2C9 Arg144Cys, CYP2C9 Ile359Leu, GSTP1 Ile105Val and NQO1 Pro187Ser. Genotypes a and b are defined as described in the legend to Table I. Adduct levels in smokers with the frequent allele for each polymorphic site are set as 100%. The 100% values for each genotype are as follows: 8.6 for CYP2C9 Arg144 homozygotes; 8.8 for CYP2C9 Ile359 homozygotes; 9.2 for GSTP1 Ile105 homozygotes; 8.9 for NQO1 Pro187 homozygotes. The data are expressed as means ± SD. Numbers in parentheses represent the numbers of subjects. ‘Non-smokers’ includes life-time non-smokers and ex-smokers who stopped smoking more than a year after surgery. ‘Smokers’ includes current smokers and ex-smokers who stopped smoking <1 year before surgery.

the different alleles of CYP2C9 in alteration of enzyme capacities in the bioactivation or detoxification of tobacco smoke carcinogens such as B[a]P, although the amino acid substitutions have been reported to cause impaired (s)-warfarin, tolbutamide and phenytoin metabolism (39–42). The two different forms encoded by GSTP1 showed different catalytic properties depending on the substrate used in cDNA expression systems (43). The amino acid substitution (Pro→Ser) at codon 187 in NQO1 was suggested to result in deficient DT-diaphorase activity toward dichlorophenolindophenol using a human colon cancer cell line (44).

In the present study, levels of bulky DNA adducts were higher in bronchial tissues of those patients who were homozygous for the frequent Ile359 allele of CYP2C9, for which a role in the bioactivation of tobacco smoke carcinogens has been proposed. No clear relationship was observed between adduct levels and the polymorphisms of CYP2C9(Arg144), GSTP1 and NQO1. Although additional studies are needed to verify the function of different alleles of the CYP2C9 gene, our preliminary results and those of others (39–43) indicate that low CYP2C9 activity is associated with increased genotoxicity.

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References


