Detection of 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]-Pyridine-DNA Adducts in Normal Breast Tissues and Risk of Breast Cancer

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Abstract
2-Amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP), the most abundant heterocyclic amine (HCA) in cooked food, is a mammary carcinogen in female rats. In humans, consumption of well-done meat and PhIP intake have been associated with an increased risk of breast cancer, but PhIP-DNA adducts have not been analyzed in breast tissues from women having unknown exposure to HCAs. Using an immunohistochemistry (IHC) method, we measured PhIP-DNA adducts in normal breast tissues of 106 women having newly diagnosed breast cancer in comparison with those of 49 women undergoing reduction mammoplasty. The IHC method was first validated in MCF-7 cells treated with different doses of N-hydroxy-PhIP. We detected significant dose-response relationship and correlation (r = 0.94) between the levels of PhIP-DNA adducts detected by IHC and 32P-postlabeling. Using IHC, PhIP-DNA adducts were detected in 82 and 71% of the normal breast tissue sections from the cancer and control patients, respectively. The median (range) absorbance was 0.18 (0 – 0.57) and 0.08 (0 – 0.38) in the cancer and control patients, respectively (P < 0.001). Using the median in the controls as a cutoff point, 71% of the cancer patients and 47% of the controls were distributed in the higher range (χ² = 8.17; P = 0.004). Logistic regression analysis demonstrated an OR of 4.03 (95% CI, 1.41–11.53) after adjusting for age and ethnicity. Stratified analyses did not find any significant effect of age, ethnicity, smoking, well-done meat consumption, dietary intake of PhIP, or polymorphisms of CYP1A1, CYP1B1, NAT2, and GSTM1 genes on the level of PhIP-DNA adducts. However, a potential interactive effect of well-done meat consumption and NAT2 genotype on the level of PhIP-DNA adducts was observed (P = 0.047). This is the first study of detection of PhIP-DNA adducts in breast tissue samples obtained from women having unknown exposure to HCAs. These data strongly support the hypothesis that HCA exposure contributes to human breast cancer among genetically susceptible individuals.

Introduction
Breast cancer is the second leading cause of cancer deaths in American women (1). Environmental factors have long been suspected to contribute to human breast cancers, but no specific agents other than radiation have been definitely implicated (2). Urban residency, dietary fat intake, cigarette smoking, alcohol consumption, and exposure to organochlorine compounds have been suspected to play a role, but the associations are either weak or inconclusive. Several lines of experimental evidence support the hypothesis that exposure to environmental carcinogens is involved in human breast cancer. First, a number of compounds present in the environment are potent mammary carcinogens (3). Second, the anatomical features of the breast make it a susceptible target for chemical carcinogens. For example, lipophilic polycyclic aromatic compounds can be stored and concentrated in the human breast fat pad (4). Human mammary epithelial cells have a high capacity for metabolizing these compounds into DNA binding species and thus can become target cells for carcinogenesis (5). Third, the spectrum of p53 gene mutations in human breast tumors suggests the involvement of exogenous agents in inducing these mutations in a significant portion of the cases (6). Fourth, mutagenicity and genotoxicity have been detected in nipple aspirates (7), breast cyst fluid samples (8), extracts of mammary lipids from women undergoing reduction mammoplasty (9, 10), and human breast milk samples (11). The critical question is: what carcinogens are involved in the etiology of human breast cancer? The recently reported comprehensive Long Island Study failed to find a direct connection between breast cancer and several environmental toxins such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons (12, 13). On the other hand, epidemiological studies have suggested an association between the consumption of well-done meat and an increased risk of breast cancer (14, 15). PhIP, which is present in well-done meat, is the carcinogen that is suspected to be responsible for this increased risk (16).

PhIP is the most abundant HCA in the human diet. HCAs are pyrolys products of amino acids and proteins (17) that are...
formed in a variety of muscle meats when they are cooked. The metabolic activation of HCAs into DNA binding species causing DNA adduct formation is believed to be a key event in HCA-induced carcinogenesis (18). Previous studies have shown that PhIP is metabolized into two major products: N^2-OH-PhIP, which is mutagenic, and 4-hydroxy-amo-ino-PhIP (N^2-OH-PhIP), which is not mutagenic. Both CYP1A1 and CYP1B1, as well as CYP1A2, can catalyze N-hydroxylation of PhIP (19). In fact, CYP1A1 has a greater catalytic efficiency than CYP1A2 in the formation of N^2-OH-PhIP (20). N^2-OH-PhIP and 4-hydroxy-amo-ino-PhIP may undergo acetylation and sulfation to form more highly mutagenic products (21), or they can be conjugated by glucuronidase or sulfotransferase to produce nonmutagenic species that are readily excreted. PhIP has been shown to have the highest carcinogenic potency (22). In addition, animal experiments have shown that long-term exposure to PhIP can induce tumors of the mammary gland in female rats and the colon in male rats (23–25), which are the two most common sites of carcinogenesis in Western countries. PhIP is bioavailable in human populations as demonstrated by its detection in human urine (26–28) and milk (29). Furthermore, PhIP-DNA adducts have been detected in human breast tissue samples obtained from individuals given dietary equivalent levels of ^14C-labeled PhIP using accelerator MS (30). PhIP-DNA adducts have also been detected in exfoliated ductal epithelial cells in human breast milk (31). On the basis of these data, we hypothesize that PhIP exposure plays an important role in human breast carcinogenesis. If this hypothesis were true, we would expect to detect PhIP-DNA adducts in human breast tissues as a marker of exposure and to find an association between the level of PhIP-DNA adducts and risk of cancer. Therefore, in the present study, we examined PhIP-DNA adducts in normal breast tissues of women having or not having breast cancer using an IHC method. We also explored the possible effect of several environmental factors, i.e., smoking and well-done meat consumption, as well as some genetic factors (polymorphisms of four metabolic genes: CYP1A1; CYP1B1; NAT2; and GSTM1) on the level of PhIP-DNA adducts in human breast tissues.

Materials and Methods

Study Subjects and Tissue Samples. A total of 106 normal breast tissue sections was obtained from women with newly diagnosed breast cancer who underwent mastectomy at The University of Texas M. D. Anderson Cancer Center from 1997 to 2000. Forty-nine normal breast tissue sections, which were obtained from noncancer patients undergoing reduction mammoplasty at a different local hospital, were used as controls. The use of human tissue sections was approved by the M. D. Anderson Cancer Center Institutional Review Board. The age range of the study participants was 20–50 years. The median (range) ages of the cancer patients and controls were 43 years (28–50 years) and 30 years (19–50 years), respectively, and the difference was statistically significant (P < 0.001). Seventy-eight percent of the cancer patients and 43% of the control patients were non-Hispanic whites, whereas 11% of the cancer patients and 51% of the controls were African Americans. A questionnaire was administered to collect information on cigarette smoking, alcohol use, reproduction and hormonal history, family history of cancer, dietary habits, and other risk factors. A blood sample was collected from each study participant. Fresh breast tissue samples were fixed in 10% buffered formalin within 5 h after surgery and embedded in paraffin. The paraffin blocks were then cut into 5-μm sections and mounted on aminoalkylsilane-coated slides (Sigma Diagnostics, St. Louis, MO).

In Vitro N-Hydroxy-PhIP Treatment. MCF-7 cells were treated using N-hydroxy-PhIP (NCI Chemical Carcinogen Repository, Kansas City, MO) at a concentration of 0, 0.29, 0.58, 1.17, 2.31, 4.62, 9.37, 18.75, 37.5, 75, 100, 150, or 300 μM for 2 h at 37°C. The cell treatment was performed in triplicates. Upon harvesting, cells were divided into two portions for IHC and ^32P-postlabeling, respectively. For IHC, cells were cytospun onto the slides at a concentration of 1 × 10^5 cells/slide and then fixed in methanol for 10 min at room temperature and stored at −80°C. Also, before being subjected to IHC, the slides were fixed in methanol at −20°C overnight. For ^32P-postlabeling, cells were lysed, and DNA was extracted from them using the phenol/chloroform procedure. PhIP-DNA adducts were analyzed using the intensification procedure of the ^32P-postlabeling assay (32).

IHC. PhIP-DNA adduct detection using IHC was performed as described previously (33). Briefly, the paraffin-embedded sections were baked at 65°C overnight, deparaffinized in xylene, and rehydrated in serial alcohol. Endogenous peroxidase activity was blocked using 1% H_2O_2 in methanol for 20 min. After treatment with RNase and pepsin, the sections were blocked using 3% BSA and normal goat serum. Next, the primary anti-PhIP-DNA adduct polyclonal antibody was incubated with the sections at 4°C overnight in a humid chamber at a dilution of 1:3000. Also, the biotinylated secondary antibody was incubated with the sections at 37°C for 30 min, at a dilution of 1:200. The antibody complex was detected using an avidin-biotin-peroxidase complex solution and visualized using 3,3'-diaminobenzidine (Zymed Laboratories, Inc., San Francisco, CA). A negative control was included in each experiment by omitting the primary antibody. The staining specificity was confirmed using the primary antibody that had been preabsorbed with 2 or 20 μg/ml DNA extract from MCF-7 cells treated with 150 μM N-hydroxy-PhIP.

Image Analysis. Tissue sections that had an epithelial cell content of ≥10% showing nuclear staining were considered to be positive. No staining or very weak staining was defined as negative. Cytoplasmic staining was ignored. The stained nuclei of mammary epithelial cells were captured as grayscale images from three randomly selected low power fields (×100). A threshold was set consistently for all sections analyzed to exclude cells with negative or very low staining intensities. The staining intensity was expressed as absorbance, and the mean absorbance of all evaluated cells in the three fields was used in comparisons of different tissues. A cytopsin sample of MCF-7 cells without PhIP treatment was included in each batch of staining, and the absorbance of this negative control was subtracted as background from each testing sample. To reduce analytical bias, each batch of samples included samples obtained from both cancer and control patients, and the case-control status was blinded to the person who performed the assays.

Genetic Polymorphisms. DNA was isolated from peripheral lymphocytes using the phenol/chloroform procedure. Polymorphisms of the CYP1A1, GSTM1, and NAT2 genes were determined by PCR and RFLP as reported previously (34). The m1 (V432L) and m2 (A453S) polymorphisms of the CYP1B1 gene were detected as previously reported by Bailey et al. (35) with some modifications. The restriction products were separated by electrophoresis with 10% native polyacrylamide gel. An internal control gene was amplified along with the GSTM1 gene. When both GSTM1 and the internal standard were not ampli-
fied, the sample was considered as noninformative. At least 10% of the samples were analyzed in repeats to ensure quality control.

**Statistical Analysis.** The correlation between the absorbance of PhIP-DNA adducts measured by IHC and the relative adduct labeling values measured by 32P-postlabeling was determined by linear regression analysis. The median (range) and mean \( \pm \) SD values of the PhIP-DNA adduct absorbance were compared between cases and controls, and smokers and nonsmokers, as well as between different subgroups using Mann-Whitney test and \( t \) test, respectively. Two-tailed \( P \)s were calculated for the determination of statistical significance; the signification value was \( P < 0.05 \). Logistic regression was applied to calculate the OR and 95% CI for the association between PhIP-DNA adducts and breast cancer risk after adjusting for age and ethnicity.

**Results**

Because the antibody against PhIP-DNA adducts had not been previously tested in human tissue, we first verified the specificity and sensitivity of this antibody in MCF-7 cells exposed to a PhIP derivative, \( N \)-hydroxy-PhIP. A clear increasing nuclear staining was detected in the cells exposed to 1.17–100 \( \mu \)M \( N \)-hydroxy-PhIP, and the staining intensity leveled off at 150 \( \mu \)M (Fig. 1, top panel). The staining intensity of cells exposed to the lower level of \( N \)-hydroxy-PhIP (0.29 and 0.58 \( \mu \)M) was not different from that of unexposed cells (slides not shown). The dose response relationship of increasing staining intensity is also illustrated in Fig. 2, top panel. On average, 458 \( \pm \) 59 cells were analyzed on each slide. When DNA samples obtained from the same cell population were analyzed using 32P-postlabeling, DNA adducts were not detectable in cells treated with 0.29 \( \mu \)M \( N \)-hydroxy-PhIP. At doses from 0.58 to 18.75 \( \mu \)M, two major PhIP-DNA adducts were detected, whereas four additional adducts appeared in cells that received higher doses of \( N \)-hydroxy-PhIP (Fig. 1, bottom panel). A significant dose response relationship was demonstrated within the range of 0.58–100 \( \mu \)M, whereas adducts leveled off at doses \( > 150 \mu \)M (Fig. 2, bottom panel). The intensification factor was 120 for the total adducts and 20–200 for each individual spot (data not shown). There was a significant correlation between the staining intensities and relative adduct labeling values in the 32P-postlabeling analysis (\( r = 0.94 \)).

Because the lowest detectable staining intensity by IHC was seen in cells treated with 1.17 \( \mu \)M \( N \)-hydroxy-PhIP, which had a level of 1.25 adducts/10\(^7\) nucleotides by 32P-postlabeling, the detection limit of the IHC assay is probably \( \sim 1/10^7 \) for PhIP-DNA adducts.
We then measured the level of PhIP-DNA adducts in normal breast tissue sections obtained from the 106 cancer and 49 control patients using IHC and the image analysis method. Nuclear staining of PhIP-DNA adducts was clearly visible in the mammary epithelial cells (Fig. 3). Omitting the primary antibody or using a primary antibody preabsorbed with the antigen resulted in absence or significantly reduced intensity of staining (Fig. 3). All tissue sections were processed in four staining experiments with each negative control of MCF-7 cells showing an average absorbance of 0.14, 0.14, 0.14, and 0.21, respectively (mean ± SD, 0.16 ± 0.035; coefficient of variance, 22%). On average, 801 ± 252 mammary epithelial cells were analyzed in each tissue section. After subtracting the negative control value from sample staining readings, positive staining was recorded in 82% (87 of 106) of the cancer patients and 71% (35 of 49) of the control patients. Image analysis showed that the median (range) absorbance of the positively stained samples was 0.20 (0.02–0.57) for the cancer patients and 0.13 (0.03–0.38) for the controls ($P = 0.001$). Using the median value (0.13) of staining intensity in the control patients as a cutoff point, we found that 70.8% of the cancer patients and 46.9% of the controls were distributed in the higher range ($\chi^2 = 8.17; P = 0.004$, Table 1). Logistic regression analysis demonstrated an OR of 4.03 (95% CI, 1.41–11.53) after adjusting for age and ethnicity ($P = 0.009$).

Using stratified analysis, we have explored the possible effect of several factors on the level of PhIP-DNA adducts in breast tissues. As shown in Table 2, we did not find any significant effect of age, ethnicity, smoking, well-done meat consumption, or polymorphisms of *CYP1A1, CYP1B1, GSTM1, and NAT2* genes on the level of PhIP-DNA adducts in the breast. Moreover, when cancer and control patients were pair-
PhIP-DNA Adducts and Breast Cancer

Table 1  Logistic regression analysis of adjusted ORs and CIs for PhIP-DNA adducts in breast tissues associated risk of cancer

<table>
<thead>
<tr>
<th>Adducts&lt;sup&gt;a,b&lt;/sup&gt; (absorbance)</th>
<th>Cases</th>
<th>Controls</th>
<th>Crude OR (95% CI)</th>
<th>P</th>
<th>Adjusted OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.08</td>
<td>31 (29.2)</td>
<td>26 (53.1)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>&gt;0.08</td>
<td>75 (70.8)</td>
<td>23 (46.9)</td>
<td>2.73 (1.28–5.86)</td>
<td>0.004</td>
<td>4.03 (1.41–11.53)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

<sup>a</sup> The median value of the control group was used as the cutoff point.
<sup>b</sup> Adjusted for age and ethnicity.

Table 2  Stratified analysis of PhIP-DNA adducts in breast tissues (absorbance)<sup>c</sup>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls and cases</th>
<th></th>
<th>Controls</th>
<th></th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&lt;60)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td>n</td>
<td>Median (range)</td>
<td>n</td>
<td>Median (range)</td>
</tr>
<tr>
<td>≤42</td>
<td></td>
<td>80</td>
<td>0.13 (0.05–0.57)</td>
<td>41</td>
<td>0.08 (0.03–0.38)</td>
</tr>
<tr>
<td>&gt;42</td>
<td></td>
<td>75</td>
<td>0.18 (0.05–0.43)</td>
<td>8</td>
<td>0.05 (0.01–0.31)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td>100</td>
<td>0.15 (0.05–0.57)</td>
<td>21</td>
<td>0.06 (0.01–0.31)</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td></td>
<td>55</td>
<td>0.14 (0.05–0.39)</td>
<td>28</td>
<td>0.10 (0.01–0.31)</td>
</tr>
<tr>
<td>Smoking&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>44</td>
<td>0.17 (0.05–0.37)</td>
<td>11</td>
<td>0.05 (0.01–0.31)</td>
</tr>
<tr>
<td>Ever</td>
<td></td>
<td>87</td>
<td>0.15 (0.05–0.43)</td>
<td>24</td>
<td>0.08 (0.02–0.26)</td>
</tr>
<tr>
<td>Never</td>
<td></td>
<td>3</td>
<td>0.16 (0.05–0.43)</td>
<td>6</td>
<td>0.06 (0.01–0.17)</td>
</tr>
<tr>
<td>Well-done meat</td>
<td></td>
<td>106</td>
<td>0.15 (0.05–0.57)</td>
<td>31</td>
<td>0.10 (0.03–0.38)</td>
</tr>
<tr>
<td>CYP1A1</td>
<td></td>
<td>40</td>
<td>0.15 (0.05–0.31)</td>
<td>14</td>
<td>0.09 (0.01–0.18)</td>
</tr>
<tr>
<td>wt/wt</td>
<td></td>
<td>69</td>
<td>0.13 (0.05–0.57)</td>
<td>23</td>
<td>0.06 (0.02–0.24)</td>
</tr>
<tr>
<td>wt/ml</td>
<td></td>
<td>40</td>
<td>0.16 (0.05–0.43)</td>
<td>11</td>
<td>0.09 (0.03–0.31)</td>
</tr>
<tr>
<td>CYP1B1</td>
<td></td>
<td>29</td>
<td>0.17 (0.05–0.38)</td>
<td>4</td>
<td>0.12 (0.03–0.19)</td>
</tr>
<tr>
<td>wt/wt</td>
<td></td>
<td>53</td>
<td>0.15 (0.05–0.57)</td>
<td>9</td>
<td>0.10 (0.06–0.31)</td>
</tr>
<tr>
<td>wt/ml</td>
<td></td>
<td>95</td>
<td>0.17 (0.05–0.40)</td>
<td>37</td>
<td>0.07 (0.02–0.26)</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td>55</td>
<td>0.15 (0.05–0.57)</td>
<td>17</td>
<td>0.07 (0.02–0.26)</td>
</tr>
<tr>
<td>Null</td>
<td></td>
<td>68</td>
<td>0.16 (0.05–0.57)</td>
<td>16</td>
<td>0.08 (0.03–0.31)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All P were >0.05 thus not listed in the table.
<sup>b</sup> Mean age of the study population as the cutoff point.
<sup>c</sup> More than 100 cigarettes lifetime.
<sup>d</sup> CYP1B1 genotype; wt/wt, both m1 and m2 alleles were wild type; wt/ml, one of the two alleles was either heterozygous or homozygous mutant; mt/ml, both alleles were either heterozygous or homozygous mutant.

matched by race and age (17 pairs of samples), the staining intensity was still significantly higher in the cancer patients than in the controls (Table 3). Using linear regression analysis, we found that the case-control status, not age or ethnicity, was the only significant predictor of the level of PhIP-DNA adducts in breast tissue (data not shown).

Finally, we explored the possible gene-environmental interaction on the level of PhIP-DNA adducts in the breast tissues. We did not see any significant interaction between smoking and polymorphisms of the CYP1A1, CYP1B1, NAT2, and GSTM1 genes on the level of PhIP-DNA adducts (Table 4). However, among well-done meat consumers, we found that women with a rapid NAT2 genotype had a higher level of PhIP-DNA adducts than those with a slow NAT2 genotype (P = 0.047, Table 4). No interaction was detected between well-done meat consumption and polymorphisms of CYP1A1, CYP1B1, and GSTM1 genes on the level of PhIP-DNA adducts.

Discussion

We report here the detection of PhIP-DNA adducts in normal breast tissues of women having or not having breast cancer and a significant association between the levels of PhIP-DNA adducts in such tissue and the risk of breast cancer. We observed a borderline significant interaction between consumption of well-done meat and polymorphisms of the carcinogen-metabolizing gene, i.e., NAT2, on the level of PhIP-DNA adducts. To our knowledge, this is the first study to demonstrate the presence of PhIP-DNA adducts in breast tissues from women without preplanned exposure to PhIP. Our data provides evidence supporting the hypothesis that exposure to HCAs in genetically susceptible individuals may contribute to the etiology of human breast cancer.

Several laboratories have attempted to measure HCA-induced DNA adducts in human tissues or lymphocytes. One study (36) reported the detection of PhIP-DNA adducts in only two surgical samples of human colon mucosa, 0 of 12 samples of the pancreas and 0 of 6 samples of bladder epithelium using gas chromatography-MS and 32P-postlabeling. Another study (37) reported the detection of HCA-induced DNA adducts in 3 of 38 human tissue samples using 32P-postlabeling. However, in humans who consume well-done meat, HCA-DNA adducts are undetectable in WBCs using the 32P-postlabeling method.
PhIP-DNA adducts have been detected using accelerator MS in the breast tissues of humans given dietary equivalent levels of PhIP labeled with $^3$H before surgery (30). This observation indicates that at doses derived from the human diet, HCAs can induce DNA-adduct formation in the breast tissues. However, this method is limited to adduct detection in samples previously exposed to $^{14}$C-labeled HCAs. Therefore, it cannot be applied to epidemiological studies in naturally HCA unexposed human populations. The attempts to detect DNA adducts in exfoliated ductal epithelial cells from human breast milk have generated one positive result (finding PhIP-DNA adducts in 30 of 64 tested samples by $^{32}$P-postlabeling-high-performance liquid chromatography; Ref. 31) and one negative result using $^{32}$P-postlabeling-TLC method (38). Both methods require at least 10 μg of native DNA samples.

The availability of the antibody against PhIP-DNA adducts has provided a novel alternate method for the detection of PhIP-DNA adducts in human tissues. Using this antibody, PhIP-DNA adducts have been detected via the IHC method in tissue sections obtained from rats exposed to PhIP (33) and in normal human prostate tissues transplanted into subcutis of athymic nude mice (39). However, as this antibody had not been previously applied in other human tissues, we first examined the specificity and sensitivity of the IHC method in a human mammary epithelial cell system. The specificity of the antibody was examined by using antigen-preabsorbed antibody, which significantly reduced staining intensity in tissue sections (Fig. 3). Furthermore, a clear dose response relationship in nuclear staining was detected in cells exposed to various doses of N-hydroxy PhIP, and the results of IHC were highly correlated to that of the $^{32}$P-postlabeling assay in the exposed cells. The detection limit of the IHC method seemed to be in the range of one adduct/10$^5$–10$^7$ nucleotides. At a dose of 0.58 μM, a level of 0.78 adducts/10$^7$ nucleotides was detected by $^{32}$P-postlabeling, but IHC demonstrated a nuclear staining at a similar intensity to that of the unexposed cells. At doses > 100 μM (adduct level ~1/10$^5$), the staining intensity leveled off. The higher detection limit does not affect the application of this method in human studies because humans are very rarely exposed to such a high dose of PhIP. The lower detection limit of one adduct/10$^5$ nucleotides, on the other hand, is within the range of DNA adducts in biological samples.

When the IHC method was applied in this study, we detected PhIP-DNA adducts in a high percentage of normal breast tissue sections obtained from both cancer patients and controls (82 and 71%, respectively). The high positive rate could be explained by the fact that human exposure to PhIP is quite common. Specifically, PhIP has been detected not only in cooked fish and meat but also in cigarette smoke (40), in beer and wine (41), and in urban air and diesel engine exhaust particulates (42). The high positive rate is also consistent with a previous report that PhIP-DNA adducts were detected in exfoliated ductal epithelial cells from 47% (30 of 64) of lactating women (31). In an effort to cross-reference the level of PhIP-DNA adducts in breast tissues using both $^{32}$P-postlabeling and IHC methods, we failed to positively identify the PhIP adducts in the $^{32}$P-postlabeling TLC assay because of the low level of adducts and limited amount of DNA for multiple rec chromatography analyses. However, the mean staining intensity of 0.10 among controls, which corresponds to 2.6 adducts/10$^7$ nucleotides as detected by $^{32}$P-postlabeling in the cell system, is quite comparable with a previously reported mean level of PhIP adduct (4.7 adducts/10$^7$ nucleotides) in exfoliated ductal epithelial cells from 30 human breast milk samples by the $^{32}$P-postlabeling high-performance liquid chromatography method (31). Detection of PhIP-DNA adducts in the breast provides strong supporting evidence that human breast tissues are susceptible to PhIP-induced DNA damage and carcinogenesis.

Another interesting observation made in this study was the possible interaction of well-done meat intake and NAT2 genotype on the level of PhIP-DNA adducts in breast tissues and risk of breast cancer. Under the same assay conditions, cancer patients displayed a significantly higher level of PhIP-DNA adducts than controls did. This observation supports the hypothesis that PhIP exposure contributes to breast human cancer. Because of difficulties in obtaining normal breast tissue samples from the control patients, our study was limited in that the cancer and control patients were not well matched by age or race. However, using stratified and regression analyses, we did not find a significant effect of age or race on the level of PhIP-DNA adducts. The differences in adduct levels between cancer and control patients remained significant in paired samples matched by age and race. If this finding could be confirmed in a larger scale study using well-matched samples, PhIP could be put at the top of the list of human breast carcinogens.

Another interesting observation made in this study was the possibility of well-done meat intake and NAT2 genotype on the level of PhIP-DNA adducts in breast tissues. Individuals with a history of well-done meat consumption and a rapid NAT2 genotype showed relatively higher levels of PhIP-DNA adducts in the breast tissues than those with a slow NAT2 genotype ($P = 0.047$, Table 4). This finding is consistent with the previous studies that showed a significant relationship between breast cancer risk and consumption of well-done meat among women with a rapid/intermediate NAT2 genotype (43, 44). We are well aware that our sample size in this study was very limited, and the genetic polymorphisms we examined are far from comprehensive. We are currently conducting a large-scale study with detailed assessment of PhIP exposure and more genetic polymorphisms to confirm these observations and to further explore factors that contribute to PhIP-related breast carcinogenesis.

Reporting this method for PhIP-DNA adduct detection in human tissues and the preliminary data in a population study may further stimulate research efforts to test the

<p>| Table 3  PhIP-DNA adducts (absorbance) in samples matched by age and race |
| --- | --- | --- | --- |</p>
<table>
<thead>
<tr>
<th>Pair</th>
<th>Race*</th>
<th>Case</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>28</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>29</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td>30</td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>30</td>
<td>0.29</td>
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<tr>
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</table>

Median (range) 0.17 (0.03–0.33) 0.06 (0.0–0.31)

* A, African American; H, Hispanic; W, white.

$^b P = 0.019$ by Mann-Whitney test when zero values were included; $P = 0.062$ when zero values were excluded.
The hypothesis that well-done meat consumption could increase the risk of breast cancer by inducing DNA damage in the target tissues, and women who are genetically susceptible to such exposures may have a higher risk of breast cancer.

Acknowledgments
We thank Yingqiu Du for technical support in DNA adducts analysis and genotyping. We also thank Dr. Maureen E. Goode and Judy King for editorial assistance.

References
6. Biggs, P. J., Warren, W., Venitt, S., and Stratton, M. R. Does a genotoxic hypothesis that well-done meat consumption could increase the risk of breast cancer by inducing DNA damage in the target tissues, and women who are genetically susceptible to such exposures may have a higher risk of breast cancer.

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References


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Jijiang Zhu, Ping Chang, Melissa L. Bondy, et al.


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