Urinary 8-oxodeoxyguanosine, aflatoxin B1 exposure and hepatitis B virus infection and hepatocellular carcinoma in Taiwan

Hui-Chen Wu¹, Qiao Wang¹, Lian-Wen Wang¹, Hwai-I Yang⁴, Habibul Ahsan², Wei-Yann Tsai³, Li-Yu Wang¹,⁵, Shu-Yuan Chen¹,⁶, Chien-Jen Chen⁴,⁷ and Regina M.Santella¹,²

¹Departments of Environmental Health Sciences, ²Epidemiology and ³Biostatistics, Mailman School of Public Health of Columbia University, New York, NY 10032, USA ⁴Graduate Institute of Epidemiology, College of Public Health, National Taiwan University, Taipei, Taiwan ⁵Present address: Graduate Institute of Aboriginal Health, Tzu Chi University, Hualien, Taiwan ⁶Present address: Graduate Institute of Public Health, College of Medicine, Tzu Chi University, Hualien, Taiwan ⁷Present address: Genomics Research Center, Academia Sinica, Taipei, Taiwan

To whom correspondence should be addressed. Tel: +1 212 305 1996; Fax:+1 212 305 5328; Email: rps1@columbia.edu

Introduction

In Taiwan, primary hepatocellular carcinoma (HCC) is the leading cause of cancer death for males and the second for females. Epidemiological evidence suggests that dietary exposure to aflatoxin B1 (AFB1), a mold contaminant, and chronic infection with hepatitis B virus are major risk factors for HCC (1). In a previous prospective study in Taiwan, using biomarkers to assess exposure to AFB1, we demonstrated that the presence of AFB1-albumin adducts as well as urinary AFB1 metabolites were associated with increased risk of HCC (2). A viral–chemical interaction was also observed (2). Similar results were observed in a study carried out in China (3).

Although, a number of studies have demonstrated that increasing AFB1 exposure results in increasing HCC risk, the underlying mechanisms leading to development of HCC are not fully understood. One possible mechanism of AFB1-related hepatocarcinogenesis is the induction of oxidative DNA damage in liver tissue (4,5). Reactive oxygen species (ROS) have also been suggested to be involved in the progression of chronic liver disease and the occurrence of HCC (6). In addition, oxidative DNA damage is generally regarded as a significant contributory cause of cancer from environmental exposures (7). 8-Oxodeoxyguanosine (8-oxodG) is a sensitive marker of the DNA damage due to hydroxyl radical attack at the C8 of guanine (8). In vitro treatment of hepatocytes with AFB1 resulted in a dose-dependent increase in ROS formation (4). Exposure of rats to AFB1 produced a time- and dose-dependent increase in 8-oxodG, the most abundant DNA lesion caused by ROS, in hepatic DNA (5). These data suggest that AFB1-induced oxidative DNA damage may constitute an important pathway in AFB1 hepatocarcinogenesis.

The urinary excretion of products of damaged nucleotides in cellular pools or in DNA may be important biomarkers of exposure to relevant carcinogens and may predict cancer risk. For urinary 8-oxodG, although the nucleotide pool may be a significant source (9), recent data suggest that it is the result of DNA repair and does not result from diet or cell death (10). Urinary excretion of 8-oxodG has been used as a biomarker for the assessment of oxidative DNA damage in both clinical and occupational settings (11–13). A clinical study also found that the determination of 8-oxodG is useful in assessing malignancy in HCC (14).

There are limited data on the effect of AFB1 exposure and the combined effect of chronic hepatitis B virus infection and AFB1 exposure on the level of urinary 8-oxodG. The significance of AFB1-induced oxidative DNA damage in the carcinogenicity of AFB1 has not been well investigated or has any long-term follow-up study investigated the effect of oxidative damage on the development of HCC. The specific aims of this study were to investigate, among subjects without HCC, whether oxidative DNA damage, as assessed by urinary excretion 8-oxodG, is associated with AFB1 exposure, as assessed by urinary AFB1 metabolites and to examine the role of urinary 8-oxodG levels in HCC risk using data and biospecimens collected in a community-based Cancer Screening Program cohort. We performed a case–control study of 364 subjects enrolled in a previous nested case–control study of susceptibility to AFB1-related HCC (2,15).

Materials and methods

Study cohort

Subjects are from the community-based Cancer Screening Program cohort recruited in Taiwan. This study was approved by Columbia University’s Institutional Review Board as well as the Research Ethics Committee of the College of Public Health, National Taiwan University, Taipei, Taiwan. Written informed consent was obtained from all subjects and strict quality controls and safeguards were used to protect confidentiality. The cohort characteristics and methods of screening and follow-up have been described in detail previously (2,15). Briefly, individuals who were between 30 and 64 years old and lived in seven towns in Taiwan, three located on Penghu Islets with the highest HCC incidence in Taiwan and the other four from Taiwan Island were recruited between July 1990 and June 1992 with a total of 12 020 males and 11 923 females. Participants were personally interviewed based on a structured questionnaire regarding epidemiological information and donated a 20 ml fasting blood sample as well as a spot urine aliquot at recruitment. Biospecimens were transported on dry ice to a central laboratory at the National Taiwan University and were kept at −70°C until transport on dry ice to Columbia University for analysis. Epidemiological information included sociodemographic characteristics, habits of alcohol intake and cigarette smoking, health history and family history of cancers. Habitual cigarette smoking was defined as having smoked >4 days/week for at least 6 months. Information about duration and intensity of smoking was collected. The cohort characteristics of this study versus those of the original Cancer Screening Program cohort are given in Table 1.

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was also obtained. Habitual alcohol intake was defined as drinking alcohol-containing products >4 days/week for at least 6 months. Blood samples were tested in Taiwan for serological markers, including alanine transaminase (ALT), aspartate transaminase (AST), α-fetoprotein (AFP). Hepatitis B virus surface antigen (HBsAg), antibody against hepatitis C virus (anti-HCV) and AFP were assayed using standard kits (Abbott Laboratories, North Chicago, IL). Both ALT and AST levels were determined with a serum chemistry autoanalyzer (Hitachi Model 736; Hitachi, Co., Tokyo, Japan) using commercial reagents (Biomerieux, Mercy l’Etoile, France). Anti-HCV and AFP were assayed in all males and females who resided in Hu-Hsi and Pai-Hsa on the Penghu Islets. The other assays were carried out on samples from all participants. Any participant who had an elevated level of ALT (≥45 IU/ml), AST (≥40 IU/ml) or AFP (≥20 ng/ml) who had a family history of HCC or liver cirrhosis, whereas others were examined annually. Any suspected HCC cases identified during follow-up were referred for confirmatory diagnosis as described above. 

### Study subjects

A total of 241 cases were newly diagnosed with HCC between February 1991 and June 2004. A total of 1246 controls were randomly selected from cohort subjects who were not affected with HCC through the follow-up period by matching to each case by age (±5 years), gender, residential township and date of recruitment (±3 months). The number of matched controls per case varied depending on the number of eligible controls with available specimens and ranged from two to six. Baseline urine samples were available from 74 cases and 290 controls and shipped to Columbia University on dry ice for determination of urinary 8-oxodG as well as AFB1 metabolites. In their distributions by gender, age, residential area and smoking status, subjects for whom specimens were available were similar to those for whom specimens were unavailable. 8-oxodG and AFB1 metabolites in urine

Before examination, urine samples were centrifuged at 2000g for 10 min to remove any suspended cell debris. The supernatants were used for the determination of 8-oxodG as well as AFB1 metabolites. Urinary AFB1 metabolites were determined by competitive enzyme-linked immunosorbent assay (ELISA) using an AFB1 specific antibody. Urinary 8-oxodG was determined using a standard curve of serially diluted AFB1. The color was developed by adding 100 μl of p-nitrophenyl phosphate substrate (1 mg/ml of 1M diethanolamine) per well and incubating the plates for 30 min at 37°C. The absorbance was measured with a microplate reader at 405 nm. Urinary 8-oxodG was expressed as pmol 8-oxodG per ml. Samples were assayed by duplicate analysis in duplicate wells. Statistical methods

The e2 test was used to examine differences in the distribution of variables between cases and controls. To characterize the levels of urinary excretion of 8-oxodG and the potential factors modulating these levels in a Taiwanese population, a total of 290 control subjects were used. A multivariate-adjusted linear regression model was constructed to compute regression coefficients. Levels of urinary 8-oxodG and urinary AFB1 metabolites were natural log transformed to normalize the distribution. Pearson partial correlation coefficient was used to determine the correlation between urinary 8-oxodG and AFB1 metabolites adjusted by age and gender. To evaluate the dose–response relationship between levels of urinary 8-oxodG and AFB1 metabolites, subjects were divided into quartiles based on the distribution of urinary AFB1 metabolites for the total control subjects. Then, a multivariate-adjusted logistic regression model was constructed to determine whether there was a trend. To examine the independent and combined effects of the level of urinary 8-oxodG on HCC risk, both case and control subjects were analyzed. Urinary 8-oxodG was used to divide subjects into two groups: those with levels above the median value for all control samples versus those below the median. To evaluate the dose–response relationship between levels of urinary 8-oxodG and HCC risk, subjects were divided into quartiles based on control values. HBsAg, smoking, alcohol consumption, body mass index (BMI) and urinary AFB1 metabolites-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were derived from conditional logistic regression models stratified on the matching factors to estimate the OR and 95% CI for the association between levels of urinary 8-oxodG and HCC risk. The test for trend of adjusted ORs across tertiles was based on Wald’s test with consecutive scores 1, 2, 3 and 4 assigned to the first, second, third, and fourth quartile of urinary 8-oxodG. All analyses were performed with SAS software 9.0 (SAS Institute, Cary, NC). All statistical tests were based on two-tailed probability.

Results

The sociodemographic characteristics of HCC cases with and without baseline urines were similar except that the frequency of positive anti-HCV was significantly higher in cases with baseline urine samples compared with cases without baseline urine samples (Table I). Controls with baseline urine samples were significantly younger than controls without baseline urine samples. There was a significant difference in frequency of HBsAg positive between controls with and without urine samples (15.9 and 30.3%, respectively). There were a total of 74 (57 males and 17 females) HCC cases and 290 (229 males and 61 females) controls with baseline urine available. The mean ages were 52 ± 8 and 52 ± 9 years for cases and controls, respectively. The highest percentage of positive HBsAg status was significantly higher in cases compared with controls (56.8 versus 15.9%, P < 0.0001) as was that of positive anti-HCV antibody (31.9 versus 6.1%, P < 0.0001). The distributions of habitual smoking were similar for cases and controls (48.7 and 45.5%, respectively). There was no significant difference in frequency of alcohol consumption between cases and controls (21.6 and 17.0%, respectively). Mean levels of urinary 8-oxodG were 247 ± 157 and 250 ± 169 pmol/ml for cases and controls, respectively. Mean levels of urinary 8-oxodG were highest in female controls (318 ± 187 pmol/ml), followed by male HCC cases (254 ± 166 pmol/ml), and female HCC cases (224 ± 125 pmol/ml). Urinary AFB1 metabolites were detectable in 97.3% (72 of 74) and 98.3% (285 of 290) HCC cases, and controls, respectively. The mean levels of urinary AFB1 metabolites were 67.1 ± 47.9 and 58.0 ± 41.9 fmol/ml for cases and controls, respectively. Results of the linear regression analysis of multiple factors associated with levels of urinary 8-oxodG are shown in Table II. Among controls, after multivariate adjustment, females had a statistically higher level of urinary 8-oxodG compared with males (P = 0.0002). Age, BMI, HBsAg, smoking and alcohol consumption were not associated with urinary 8-oxodG level. The levels of urinary excretion of AFB1 metabolites were positively associated with urinary 8-oxodG level in both HCC cases and controls (P = 0.02 and P < 0.0001, respectively). Each 1 fmol/ml of urinary AFB1 metabolites was associated with a 1.28–1.38 pmol/ml increase in urinary 8-oxo-dG concentration (P < 0.0001). The level of urinary 8-oxo-dG was correlated with urinary AFB1 metabolites, with Pearson partial correlation coefficient of 0.41 (P < 0.0001) for controls and 0.37 (P = 0.0013) for...
HCC cases (data not shown). Figure 1 displays the strong positive relationship among controls between the levels of ln urinary 8-oxodG and the levels of ln urinary AFB1 metabolites. Subjects were divided into quartiles based on the distribution of urinary AFB1 metabolites in controls to evaluate the dose–response relationship with urinary 8-oxodG (Table III). When compared with control subjects in the lowest quartile of urinary AFB1 metabolites, there was an increase in detection of high level of urinary 8-oxodG, with adjusted ORs of 1.4 (95% CI 0.7–2.8), 3.4 (95% CI 1.6–7.0) and 4.8 (95% CI 2.3–10.3) for subjects in the second, third and fourth quartile, respectively (\( P_{\text{trend}} \), 0.0001).

The association of urinary 8-oxodG with HCC risk is given in Table IV. After adjustment for HBsAg status, smoking, alcohol drinking, BMI and urinary AFB1 metabolites, the OR for those with urinary 8-oxodG levels above the median compared with those with levels below the median was 0.8 (95% CI 0.4–1.6). When urinary 8-oxodG levels were stratified into quartiles based on control values, HCC risk decreased with adjusted ORs of 0.8 (95% CI 0.3–2.0), 0.7 (95% CI 0.3–2.0) and 0.7 (95% CI 1.0–4.2) for subjects with adducts in the second, third and fourth quartile, respectively, compared with those in the lowest quartile.

The combined effect of urinary 8-oxodG and HBsAg is given in Table V. Subjects positive for HBsAg and with urinary 8-oxodG below the median had a significantly increased HCC risk (OR 11.4, 95% CI 3.9–33.3) compared with subjects negative for HBsAg and with urinary 8-oxodG below the median (\( P_{\text{for linear trend}} \), 0.0001).

Discussion

We investigated the relative contributions of environmental determinants to the concentration of urinary excretion of 8-oxodG in a well-characterized Chinese adult population living in an area with high AFB1 exposure. We found that the major factors determining urinary excretion of 8-oxodG in this population were gender and level of urinary AFB1 metabolites. Our results provide data on humans supporting the hypothesis that exposure to AFB1 contributes to increased oxidative stress and that AFB1 may play a role in HCC by enhancing ROS formation and causing oxidative DNA damage as well as bulky adducts.

Females had significantly higher urinary 8-oxodG levels than males among controls, consistent with another study (18). This gender difference was consistent with another study (18).

Table I. Sociodemographic characteristics of subjects with and without baseline urines

<table>
<thead>
<tr>
<th>Variable</th>
<th>HCC cases (( n = 74 ))</th>
<th>Controls (( n = 290 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects (( n = 74 ))</td>
<td>No urine cases (( n = 167 ))</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Age (years) (Mean, SD)</td>
<td>53.2 (8.7)</td>
<td>53.9 (7.9)</td>
</tr>
<tr>
<td>BMI (Mean, SD)</td>
<td>24.8 (3.6)</td>
<td>24.3 (3.6)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>23.0</td>
</tr>
<tr>
<td>Male</td>
<td>57</td>
<td>77.0</td>
</tr>
<tr>
<td>HBsAg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td>43.2</td>
</tr>
<tr>
<td>Positive</td>
<td>42</td>
<td>56.8</td>
</tr>
<tr>
<td>Anti-HCV(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>47</td>
<td>68.1</td>
</tr>
<tr>
<td>Positive</td>
<td>22</td>
<td>31.9</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>38</td>
<td>51.4</td>
</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>48.7</td>
</tr>
<tr>
<td>Alcohol(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>58</td>
<td>78.4</td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>21.6</td>
</tr>
</tbody>
</table>

\(^a\) Anti-HCV data missing for 33 and 79 subjects with and without baseline urine.  
\(^b\) Alcohol data missing for one and three subjects with and without baseline urine.  
P values for the chi-square test for categorical variables except for + indicating P value for t-test for continuous variable.

Table II. Multivariable linear regression analysis on determinants for ln urinary concentration 8-oxodG (pmol/ml)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>HCC cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>( P )</td>
</tr>
<tr>
<td>Gender (F versus M)</td>
<td>0.42</td>
<td>0.0002</td>
</tr>
<tr>
<td>Age (years) (( \geq 55 ) versus (&lt; 55 ))</td>
<td>-0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>BMI (( \geq 25 ) versus (&lt; 25 ))</td>
<td>0.09</td>
<td>0.27</td>
</tr>
<tr>
<td>HBsAg (+ versus -)</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>Smoking (Yes versus No)</td>
<td>-0.02</td>
<td>0.87</td>
</tr>
<tr>
<td>Alcohol (Yes versus No)</td>
<td>-0.08</td>
<td>0.49</td>
</tr>
<tr>
<td>ln urinary AFB1 (fmol/ml)</td>
<td>0.32</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

CI 3.9–33.3) compared with subjects negative for HBsAg and with urinary 8-oxodG below the median (\( P \) for linear trend <0.0001).

Fig. 1. Correlation of ln urinary AFB1 metabolites (fmol/ml) with ln urinary 8-oxodG (pmol/ml) (\( n = 290 \), \( R = 0.41 \), \( P < 0.001 \)).

Discussion

We investigated the relative contributions of environmental determinants to the concentration of urinary excretion of 8-oxodG in a well-characterized Chinese adult population living in an area with high AFB1 exposure. We found that the major factors determining urinary excretion of 8-oxodG in this population were gender and level of urinary AFB1 metabolites. Our results provide data on humans supporting the hypothesis that exposure to AFB1 contributes to increased oxidative stress and that AFB1 may play a role in HCC by enhancing ROS formation and causing oxidative DNA damage as well as bulky adducts. Females had significantly higher urinary 8-oxodG levels than males among controls, consistent with another study (18). This gender difference was consistent with another study (18).
difference might be due to differences in metabolism or DNA repair capacity (19). It has been noted that HCC is two to three times more frequent in males than in females in Taiwan, despite their similarity in HBsAg carrier status (20). The gender difference in the excretion of urinary 8-oxodG might contribute to the increased susceptibility to hepatocarcinogenesis in males.

Habitual cigarette smoking and alcohol intake have been reported to increase levels of urinary 8-oxodG (13,21,22). Other studies, however, failed to find an effect of smoking or alcohol intake on the urinary excretion of urinary 8-oxogG (23–25). In the present study, we did not find statistically higher urinary 8-oxogG among subjects having habitual cigarette smoking or alcohol intake compared with subjects without these exposures. This may be partly due to the fact that a higher percentage of non-smokers were female. Data on the correlation between age of subjects and urinary 8-oxogG are conflicting. Whereas a positive association with age was observed among prostate cancer patients older than 70 years (26), no effect of age was observed in our and another longitudinal study (22). Although obesity is associated with oxidative stress, consistent with our results, the levels of oxidized bases in DNA lymphocytes or other accessible cells will reflect the steady-state levels, i.e., the balance between damage and repair, albeit only in a surrogate for target tissues. The assessment of damage in various biological matrices, such as DNA, serum and urine, is vital to understanding the role of oxidative damage in hepatocarcinogenesis.

ROS generated in inflamed tissues can cause injury to target cells and also damage DNA, and may be involved in the progression of viral hepatitis as well as hepatocarcinogenesis (6). 8-oxoG in liver tissues and circulating leukocytes of those with chronic liver disease and HCC are increased in comparison with normal individuals (28). An occupational study conducted in Taiwan found that workers positive for HBsAg or anti-HCV had higher levels of urinary 8-oxoG (13). We were unable to reproduce the association between viral infection and urinary 8-oxoG level.

There was a non-significant inverse association of HCC risk with increased levels of urinary 8-oxoG. Our study of breast cancer and a study by others of lung cancer both found lower risk at higher levels of urinary 8-oxoG (18,27). Potential sources of urinary level of 8-oxoG include diet (19,29) and cell death (10). Thus, decreased risk with higher levels of urinary 8-oxoG may be related to higher DNA repair capacity. However, other data are not consistent with this hypothesis. Increased urinary 8-oxoG levels were observed among subjects with various cancers in a small study (31) and a second prospective study of lung cancer found no difference (18). Although, some case–control studies have suggested that the urinary excretion of 8-oxoG is increased among cancer patients (31,32), this could be a consequence of the disease with ongoing oxidative stress, inflammation and tissue turnover (33).

To the best of our knowledge, no published studies have measured urinary 8-oxoG to investigate the effect of AFB1 exposure on oxidative stress in humans. The use of urinary 8-oxoG as a biomarker of oxidative damage of AFB1 exposure has several advantages: (i) easy sample collection, (ii) urinary 8-oxoG is regarded as a suitable biomarker of oxidative stress (13), (iii) measuring urinary 8-oxoG by enzyme-linked immunosorbent assay requires small amounts of material allowing the analysis of stored biospecimens, (iv) it avoids the artifactual formation of 8-oxoG during DNA isolation (34) and (v) urinary 8-oxoG level is stable in storage for years (35).

Although the use of urinary 8-oxoG to monitor DNA oxidation remains attractive, our results must be interpreted with caution. First, for the use of urinary excretion of 8-oxoG as a biomarker reflecting the general average risk of a promutagenic oxidative adduct in DNA of all tissues and organs, extensive repair is assumed. In contrast, the levels of oxidized bases in DNA lymphocytes or other accessible cells will reflect the steady-state levels, i.e., the balance between damage and repair, albeit only in a surrogate for target tissues. The assessment of damage in various biological matrices, such as DNA, serum and urine, is vital to understanding the role of oxidative damage in hepatocarcinogenesis. Second, the putative causal role of AFB1 exposure remains attractive, our results must be interpreted with caution. First, for the use of urinary excretion of 8-oxoG as a biomarker reflecting the general average risk of a promutagenic oxidative adduct in DNA of all tissues and organs, extensive repair is assumed. In contrast, the levels of oxidized bases in DNA lymphocytes or other accessible cells will reflect the steady-state levels, i.e., the balance between damage and repair, albeit only in a surrogate for target tissues. The assessment of damage in various biological matrices, such as DNA, serum and urine, is vital to understanding the role of oxidative damage in hepatocarcinogenesis.

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There was a non-significant inverse association of HCC risk with increased levels of urinary 8-oxoG. Our study of breast cancer and a study by others of lung cancer both found lower risk at higher levels of urinary 8-oxoG.
cytotoxic effects of ROS in large amounts. Differences in the activity of repair enzymes could alter dramatically the relative amount of the nucleoside detected in urine. Third, there is concern that the small sample size in our case–control study limits statistical power to detect a small decrease in HCC risk. Moreover, the controls with available urine may not be representative of the general reference population due to the higher frequencies of younger as well as HbsAg-negative subjects. However, the cases and controls were comparable with regard to sociodemographic characteristics such as age or gender that may affect the HCC risk or levels of urinary 8-oxoG. In this case, selection bias is unlikely to occur. Fourth, levels of 8-oxoG are not a quantitative marker of damage to DNA by all reactive species, because >10 different oxidative modifications have been observed in DNA (36,37).

In summary, we found, among controls, a statistically positive association between urinary 8-oxoG, a biomarker of oxidative stress, and urinary AFB1 metabolites, a biomarker of AFB1 exposure. With each 1 fmol/ml increase in urinary AFB1 concentration, urinary 8-oxoG concentration increased by 1.28–1.38 pmol/ml. These results strongly suggest that AFB1 exposure may result in an increased risk of oxidative DNA damage. In terms of HCC risk, a non-significant inverse relationship between urinary 8-oxoG was observed, consistent with the hypothesis that the increased levels of excretion may also be a measure of increased DNA repair. Our results provide information on the application of biomarkers in human populations at high risk for cancer and that AFB1-induced oxidative DNA damage may, in addition to the formation of AFB1-DNA adducts, have an important role in AFB1 carcinogenicity.

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Conflict of Interest Statement: None declared.

References


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