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# Effect of aflatoxin metabolism and DNA adduct formation on hepatocellular carcinoma among chronic hepatitis B carriers in Taiwan

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***Background/Aims:*** **Aflatoxins (AFs) are established hepatic carcinogens in several animal species. This study was performed to establish whether aflatoxin exposure may affect the risk of developing hepatocellular carcinoma in chronic hepatitis B virus carriers.**
***Methods:*** **Urinary AF metabolites were measured for 43 HCC cases and 86 matched controls nested in a cohort of 7342 men in Taiwan. Thirty hepatocellular carcinoma cases and 63 controls were also tested for $AFB_1$-albumin adducts.**
***Results:*** **There was a dose-response relationship between urinary $AFM_1$ levels and risk of hepatocellular carcinoma in chronic hepatitis B virus carriers. Comparing the highest with the lowest tertile of urinary $AFM_1$ levels, the multivariate-adjusted odds ratio (OR) was 6.0 (95% confidence interval (CI)=1.2–29.0). The hepatocellular carcinoma risk associated with $AFB_1$ exposure was more striking among the hepatitis B virus carriers with detectable $AFB_1$-$N^7$-guanine adducts in urine. Compared with chronic hepatitis B virus carriers who were negative for $AFB_1$-albumin adducts and urinary $AFB_1$-$N^7$-guanine, no elevated risk was observed for those who were positive for either marker. But an extremely high risk of hepatocellular carcinoma among those having both markers was found (OR=10.0, 95% CI=1.6–60.9). The proportion of $AFB_1$ converted to $AFM_1$ decreased with the progress of liver disease, whereas the formation of $AFP_1$ increased. The difference in patterns of $AFB_1$ metabolite formation was an independent risk factor for hepatocellular carcinoma after adjustment for total $AFB_1$ excretion. There was a synergistic interaction between glutathione S-transferase M1 genotype and $AFB_1$ exposure in hepatocellular carcinoma risk.**
***Conclusions:*** **$AFB_1$ intake and expression of enzymes involved in $AFB_1$ activation/detoxification may play an important role in hepatitis B virus-related hepatocarcinogenesis.**

***Key words:*** **$AFB_1$-albumin adducts; Chronic hepatitis B virus carriers; Glutathione S-transferase M1; Hepatocellular carcinoma; Urinary aflatoxin metabolites.**

LIVER CANCER, largely hepatocellular carcinoma (HCC), is one of the most common cancers in the world with an estimated half to one million new cases per year (1). Considerable evidence indicates that HCC is multifactorial in origin. Our previous studies have linked many risk factors to the development of HCC (2–8). One of the most striking epidemiologic characteristics of HCC is its remarkable geographic variation. High incidence areas cluster in the Far East and tropical Africa (1). Although chronic hepatitis B virus (HBV) infection has been well documented as the most important risk factor of HCC in these areas (1,9), ingestion of aflatoxins, especially aflatoxin $B_1$ ($AFB_1$), has long been implicated as another dominant cause. The current contention is that aflatoxin exposure may interact with chronic HBV infection to produce HCC.

Aflatoxins are carcinogenic in several animal species, but their carcinogenic potency differs with species (10). In humans, ecological studies conducted in Africa and Southeast Asia have revealed a strong correlation be-

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tween the contamination of foodstuffs by aflatoxins and incidence of HCC (11,12). The hypothesis that $AFB_1$ has a causative role in the etiology of human HCC is also supported by the finding that a large fraction of the tumor tissues and adjacent nonmalignant liver tissues from HCC patients in certain geographic areas of high aflatoxin ingestion contained an AGG to AGT mutation at codon 249 of the p53 tumor suppressor gene (13,14), a mutation that is preferentially induced in cultured HepG2 human hepatocytes exposed to $AFB_1$ (15). However, case-control studies of HCC risk and aflatoxin intake, as assessed by questionnaire, have yielded inconsistent results (16,17). Dietary questionnaire is inadequate to measure aflatoxin intake because the content of aflatoxins in individual foods can vary widely due to the geographic and seasonal variations. This method may also suffer from severe recall bias.

Molecular dosimetry methods have been developed to quantitate aflatoxin exposure on an individual basis. It is now possible to measure levels of the covalent adducts of $AFB_1$ on DNA and protein and urinary excretion of aflatoxin metabolites (18–25). In a recent case-control study involving a total of 50 HCC cases which was nested in a cohort study in Shanghai, the presence of aflatoxin metabolites in urine was associated with a significantly increased risk of HCC. This excess risk of HCC was particularly high among chronic HBV carriers (20). Our previous studies using an indirect immunofluorescence assay have demonstrated that 50–70% of the liver tissues from HCC patients in Taiwan had a detectable level of $AFB_1$-DNA adducts (18). Two recent nested case-control studies in Taiwan using biomarkers of aflatoxin exposure have provided additional evidence for a role of aflatoxin intake in development of HCC (23,24). There is a 8-fold variation in liver cancer mortality by place of residence in Taiwan (1). Data of the two previous nested case-control studies in Taiwan were based on a cancer-screening project (CSP) carried out in the Penghu Islets and seven rural townships, respectively (23,24). The liver cancer mortality in the Penghu Islets was reported to be the highest in Taiwan (1). We have carried out a large-scale prospective study on the multifactorial etiology of HCC in Taipei metropolitan area since 1988. More than 70% of the subjects in this study were government employees who had higher educational backgrounds and better health care, while approximately 20% of the subjects of CSP study were illiterate. Preliminary results from this prospective study demonstrated a strong association between elevated serum levels of $AFB_1$-albumin adducts and HCC risk (25). In the present work, we examined the effect of aflatoxin exposure, as assessed by various urinary aflatoxin metabolites and $AFB_1$-albumin adducts, on the risk of developing HCC among chronic HBV carriers. We paid a special attention to the role of metabolism in aflatoxin-related hepatocarcinogenesis.

## Subjects and Methods

*Study subjects*

The cohort characteristics and method of cancer follow-up has been described in detail previously (8). Briefly, between August 1988 and June 1992, a cohort of 4841 male asymptomatic hepatitis B virus surface antigen (HBsAg) carriers and 2501 male noncarriers aged 30–65 years was recruited from the Government Employee Central Clinics and the Liver Unit of Chang-Gung Memorial Hospital in Taipei, Taiwan. At entry into this cohort study, each study participant was personally interviewed to obtain information on demographic characteristics, habits of cigarette smoking and alcohol drinking, dietary consumption frequency (including the frequency of consuming peanuts and fermented bean products, which are thought to be the major aflatoxin-contaminated foodstuffs in Taiwan), as well as personal and family history of major chronic diseases. Urine and blood samples from study subjects were also drawn and frozen at $-30°C$ or $-70°C$ until subsequent analysis. This study was approved by the Department of Health and is in compliance with regulations for the protection of human research subjects.

All HBsAg carriers in this study undergo both ultrasonography and $\alpha$-fetoprotein measurement every 6–12 months. Individuals with ultrasonographic images compatible with HCC and/or with an elevated $\alpha$-fetoprotein level more than 20 ng/ml are referred to hospitals for further confirmatory examinations, including fine-needle aspiration cytology and image diagnosis. Follow-up of HBsAg noncarriers is carried out by annual physical examination including serum $\alpha$-fetoprotein test. The response rate to the periodic follow-up examinations was approximate 72% for HBsAg carriers and 80% for HBsAg noncarriers. Information on HCC occurrence and vital status of study subjects who did not participate in the follow-up examinations was obtained from both computerized data files of the national death certification and the cancer registry system in Taiwan.

By December 31, 1994, we had carried out approximately 34578.8 person-years of follow-up, an average of 4.7 years per person. Fifty HCC cases were identified during the follow-up period. All HCC cases were diagnosed on the basis of either pathological/cytological examinations or an elevated $\alpha$-fetoprotein level ($\geq$400 ng/ml) combined with at least one positive im-

age on angiography, sonography, and/or computerized tomography. Because most HCC patients in Taiwan are chronic HBV carriers and long-term infection with HBV might alter aflatoxin metabolism, it is essential to examine whether there is difference in the profile of various aflatoxin metabolites excreted in the urine between HBsAg-positive and HBsAg-negative subjects. One HBsAg-positive and one HBsAg-negative control was chosen for each case from cohort members without HCC on the date the disorder was diagnosed in the case. The controls were matched to the index case on age (±5 years) and date of questionnaire interview and urine collection (±3 months). All HBsAg-positive controls remained asymptomatic throughout the follow-up period, except two who were subsequently affected with liver cirrhosis. Analysis of urinary aflatoxins was carried out on a total of 43 matched case-control sets based upon the availability of urine samples.

*Laboratory analyses*

Serum HBsAg was assayed using a radioimmunoassay (Abbott Laboratories, North Chicago, IL). All but 13 study subjects (six HCC cases and seven controls) were also tested for antibodies against hepatitis C virus (anti-HCV). Anti-HCV was examined in duplicate by a second generation enzyme immunoassay (Abbott Laboratories, North Chicago, IL) according to the manufacturer's instructions. Positive samples from the first test were retested. Only the repeatedly positive samples were considered anti-HCV positive.

For extraction of aflatoxin metabolites in urine, a 5 ml aliquot urine was incubated with 5 ml of 0.1 M $Na_2SO_4$ and 5 ml of 0.1 M acetic acid containing 710 units of $\beta$-glucuronidase for 18 h at 37°C. Precipitated material was removed by centrifugation of the sample at 3000 rpm for 10 min. The supernatant was then treated with 5 ml chloroform, and the mixture vortexed for 1 min. The sample was centrifuged for 5 min at 3000 rpm to aid in the solvent separation. Following centrifugation, the chloroform layer was removed and treated with 5 ml deionized water. The mixture was vortexed and centrifuged at 3000 rpm for 5 min. The chloroform layer was removed, evaporated to dryness under a stream of $N_2$ gas, and then n-hexane (2 ml) and trifluoroacetic acid (200 $\mu$l) were added. After 1 h at 40°C, 0.8 ml of deionized water:acetonitrile (9:1, v/v) was added, the solution was vortexed for 1 min, and the lower aqueous layer was transferred to a vial for high performance liquid chromatography (HPLC) analysis.

Levels of aflatoxin metabolites in urine were analyzed by reverse-phase HPLC as previously described (22). HPLC was performed with a Waters system incorporating two M510 pumps, an M680 system controller, an M717 autoinjector and an M470 scanning fluorescence detector. Quantitation of various aflatoxin metabolites was accomplished with Millennium 2010 chromatography manager (Waters Assoc., Milford, MA). The HPLC column used was a $C_{18}$ 10-$\mu$m (300×3.9 mm) $\mu$Bondapak column (Waters Assoc., Milford, MA). Gradient elution was used to improve the resolution of very similar structures of various aflatoxin metabolites. An aliquot of 25 $\mu$l extracted samples was injected into the HPLC system. For analysis of $AFM_1$, $AFB_1$-$N^7$-guanine, $AFG_1$, and $AFB_1$, chromatographic separation was obtained by elution for 12 min with 15% acetonitrile:water and then the system was automatically switched to a 22% acetonitrile:water solvent mix. The flow rates were 1.5, 0.8, 0.3, and 1.0 min/ml at 0–12, 13–21, 22–35, and 36–41 min, respectively. Elutes were measured by fluorescence detection with 365 nm excitation and 430 nm emission wavelength for $AFM_1$ and with 500 nm emission for $AFB_1$-$N^7$-guanine, $AFG_1$ and $AFB_1$. The HPLC analysis for $AFP_1$ was done with a 30-min elution with 12% acetonitrile:water followed by a 12–22% acetonitrile:water linear gradient generated over 12 min, and then at 22% acetonitrile. The flow rates were 1.5, 0.3, 1.5, 0.3, 1.0, and 0.3 ml/min at 0–17, 18–19, 20–29, 30–41, 42–44, and 45–52 min, respectively. $AFP_1$ was measured by monitoring the fluorescence emission at 500 nm with the excitation wavelength at 365 nm. Authentic standards were used to determine retention times for the derivatives of interest and to generate calibration curves for individual aflatoxins. All aqueous mobile phases before use were adjusted by orthophosphoric acid and triethylammonium formate buffer to pH 3.0. According to this HPLC procedure, the minimum detectable concentration of aflatoxins in the urine samples analyzed was about 0.05 ng/ml. Serum levels of $AFB_1$-albumin adducts were tested by a competitive enzyme-linked immunosorbent assay as published (23–25). The glutathione S-transferase M1 (GSTM1) genotyping was carried out by a polymerase chain reaction assay (26).

*Statistical methods*

Correlation between quantitative data was assessed by the Spearman rank correlation coefficient. Conditional logistic models were used to derive the matched crude and multivariate-adjusted odds ratios (ORs) associated with urinary levels of aflatoxin metabolites. The test for trend of matched ORs across tertiles was based on Wald's tests with consecutive scores 1, 2, 3 assigned to the first (low), second (medium), and third (high) tertile

of urinary aflatoxin levels. Statistical adjustment of the percentage of the amount of individual $AFB_1$ metabolite in total $AFB_1$ metabolites in urine was performed by the residual method (27). The significance of the difference in the median percentage of individual $AFB_1$ metabolite in total $AFB_1$ metabolites in urine between two groups was examined by the Wilcoxon rank-sum test. In the stratified data analyses, relative risks were estimated by unmatched ORs. Mantel's chi-square test for a trend was used to examine the dose- response relationship. The Mantel-Haenszel test for the homogeneity of ORs across strata was calculated as a test for interaction. All *p*-values were calculated from two-sided tests of statistical significance.

## Results

The mean ages (standard deviation) of HCC cases and matched controls were 51.0 (10.0) and 51.3 (9.9) years, respectively. Almost all HCC cases were HBsAg carriers; the one HBsAg-negative case was anti-HCV positive. Anti-HCV was detected in 10.8% of HCC cases (4/37) and in 5.1% of controls (4/79).

We measured urinary concentrations of $AFB_1$, two of its main metabolite, $AFM_1$ and $AFP_1$, $AFB_1$-$N^7$-guanine adducts, as well as $AFG_1$. All study subjects were positive for $AFM_1$, 81.4% for $AFP_1$, 42.6% for $AFB_1$-$N^7$-guanine adducts, 27.9% for $AFB_1$ and only 12.4% for $AFG_1$. Urine samples collected between July and September had a higher level of total aflatoxins than the samples collected in other months. However, the monthly difference in the levels of total aflatoxins was not statistically significant.

The correlations between urinary aflatoxin metabolites and monthly consumption frequency of peanuts and fermented bean products within the 6 months before urine collection among HBsAg-positive controls were examined using Spearman rank correlation coefficient. Urinary $AFM_1$ levels were positively correlated with the consumption frequency of peanuts and fermented bean products (correlation coefficient [r]=0.35, $p$=0.026). Although $AFP_1$ was found to greatly contribute to the overall level of urinary aflatoxin metabolites, there was no significant correlation between the dietary consumption frequency of aflatoxins and $AFP_1$ excretion. No significant correlation with the consumption frequency of aflatoxins was observed for $AFB_1$-$N^7$-guanine, $AFB_1$, and $AFG_1$. Correlations among the various aflatoxin metabolites in urine were also analyzed. There was a significant correlation between $AFM_1$ and $AFB_1$-$N^7$-guanine adducts ($r$=0.32, $p$=0.046). Correlations among other urinary aflatoxin metabolites were not significant.

The associations of HCC risk with various aflatoxin metabolites in urine among HBsAg carriers are shown in Table 1. The single HBsAg-negative HCC case was not included in the analysis. There was a significant dose-response relationship between urinary $AFM_1$ levels and HCC risk (trend test, $p$=0.04). After adjustment for educational level, ethnicity, habitual alcohol drinking, and status of cigarette smoking, the matched

TABLE 1

Conditional logistic regression analysis of the associations between various aflatoxin metabolites in urine and risk of HCC among HBsAg carriers

| Individual urinary aflatoxin (ng/ml) | Group | Controls | HCC | Odds ratio (95% CI) | |
|---|---|---|---|---|---|
| | | *n* (%) | *n* (%) | Crude | Adjusted[a] |
| $AFM_1$[b] | <1.61 | 18 (41.9) | 9 (21.4) | 1.0[+] | 1.0[+] |
| | 1.61–2.85 | 10 (23.2) | 10 (23.8) | 1.9 (0.6–6.2) | 1.9 (0.5–7.2) |
| | >2.85 | 15 (34.9) | 23 (54.8) | 3.5 (1.0–11.8) | 6.0 (1.2–29.0) |
| $AFP_1$[b] | <0.68 | 13 (30.2) | 10 (23.8) | 1.0 | 1.0 |
| | 0.68–2.90 | 14 (32.6) | 14 (33.3) | 1.4 (0.5–4.3) | 1.6 (0.5–5.3) |
| | >2.90 | 16 (37.2) | 18 (42.9) | 1.7 (0.5–6.0) | 2.0 (0.5–8.0) |
| $AFB_1$-$N^7$-guanine[c] | <0.21 | 30 (69.8) | 24 (57.1) | 1.0 | 1.0 |
| | 0.21–0.36 | 6 (13.9) | 12 (28.6) | 3.6 (0.9–13.7) | 5.3 (1.1–25.2) |
| | >0.36 | 7 (16.3) | 6 (14.3) | 1.7 (0.4–6.4) | 2.8 (0.6–12.9) |
| $AFB_1$ | Undetectable | 34 (79.1) | 25 (59.5) | 1.0 | 1.0 |
| | Detectable | 9 (20.9) | 17 (40.5) | 2.3 (0.9–6.1) | 2.0 (0.7–5.8) |
| $AFG_1$ | Undetectable | 38 (88.4) | 38 (90.5) | 1.0 | 1.0 |
| | Detectable | 5 (11.6) | 4 ( 9.5) | 0.8 (0.2–3.4) | 0.4 (0.1–2.6) |

CI, confidence interval.

[+] Test for trend was statistically significant.

[a] Educational level, ethnicity, habitual alcohol drinking, and cigarette smoking status (categorized as never, ≤16.3 and >16.3 pack-years) were also included in the conditional logistic model.

[b] Categorized according to the tertile distribution of all controls.

[c] Categorized according to the tertile distribution of all controls with detectable $AFB_1$-$N^7$-guanine adducts in urine.

TABLE 2

Odds ratios (ORs) of HCC by urinary levels of $AFM_1$ and $AFB_1$-$N^7$-guanine adducts among HBsAg carriers

| Tertile of urinary $AFM_1$ (ng/ml) | $AFB_1$-$N^7$-guanine | | | | | |
|---|---|---|---|---|---|---|
| | Undetectable | | | Detectable | | |
| | Controls | HCC | OR (95% CI) | Controls | HCC | OR (95% CI) |
| <1.61 | 12 | 8 | 1.0 | 6 | 1 | 1.0+ |
| 1.61–2.85 | 5 | 6 | 1.8 (0.4–8.0) | 5 | 4 | 4.8 (0.4–58.0) |
| >2.85 | 7 | 7 | 1.5 (0.4–5.9) | 8 | 16 | 12.0 (1.2–117.4) |

CI, confidence interval.
+$p$=0.014, test for trend.

OR comparing the highest with the lowest tertile of urinary $AFM_1$ levels was 6.0 (95% confidence interval [CI]=1.2–29.0, $p$=0.026). Higher urinary levels of $AFB_1$-$N^7$-guanine adducts were also positively associated with the risk of HCC; however, a monotonic dose-response relation was not observed. There was some elevation in HCC risk associated with urinary levels of $AFP_1$, but this association did not reach statistical significance. More HCC cases than controls had detectable levels of $AFB_1$ in urine. HBsAg carriers with detectable urinary $AFG_1$ had no increase in risk of HCC. The relationships between various aflatoxin metabolites and HCC were not materially changed when adjustment was also made for anti-HCV status.

Since $AFM_1$ was positively correlated with $AFB_1$-$N^7$-guanine, the odds ratio of developing HCC in relation to $AFM_1$ was further analyzed by levels of the urinary $AFB_1$-DNA adducts (Table 2). Among HBsAg carriers with detectable $AFB_1$-$N^7$-guanine in urine, the dose-response relationship between urinary $AFM_1$ levels and HCC risk remained significant with the increase in the magnitude of the odds ratios (trend test, $p$=0.014). Among HBsAg carriers who had no detectable $AFB_1$-$N^7$-guanine in urine, the odds ratios of HCC associated with elevated urinary $AFM_1$ levels were only approximate 1.5 and none were statistically significant.

Among the 42 HBsAg-positive patients with HCC included in this study, 11 (26.2%) were diagnosed within 1 year, 12 (28.6%) between 1 and 2 years, and 19 (45.2%) more than 2 years after urine collection. The odds ratios of HCC associated with urinary levels of $AFM_1$ and $AFB_1$-$N^7$-guanine increased after exclusion of the 23 matched case-control sets in which the HCC cases were diagnosed within 2 years after urine collection. In a multiple logistic regression analysis adjusted for educational level, status of cigarette smoking, and habitual alcohol drinking, the OR of HCC was 4.7 (95% CI=0.6–35.5, $p$=0.13) and 10.2 (95% CI=1.6–65.6, $p$=0.01), respectively, for HBsAg carriers in medium and high tertile of $AFM_1$. The multivariate-adjusted OR of HCC for urinary $AFB_1$-DNA adducts was 6.8 (95% CI=1.0–46.9, $p$=0.05) in the medium tertile and 5.3 (95% CI=0.4–73.7, $p$=0.2) in the high tertile, respectively.

For a total of 93 study subjects (29 HBsAg-positive HCC cases, one HBsAg-negative HCC case, 31 HBsAg-positive controls, and 32 HBsAg-negative controls) there was data available for both $AFB_1$-albumin adducts and urinary aflatoxin metabolites. The correlation coefficient between $AFB_1$-albumin adducts and total urinary $AFB_1$ metabolites was −0.11 ($p$=0.28). We further analyzed the relationship of the presence of $AFB_1$-albumin adducts and/or urinary $AFB_1$-$N^7$-guanine with the risk of HCC among chronic HBV carriers. Compared with those who were negative for both markers, no elevated risk was observed for individuals who were positive for either $AFB_1$-albumin adducts or urinary $AFB_1$-$N^7$-guanine. But an extremely high risk of HCC among those having both markers was found (OR=10.0, 95% CI=1.6–60.9, $p$=0.007) (Table 3).

Ingested $AFB_1$ is converted by microsomal cytochrome P450 to various derivatives, including $AFM_1$, $AFP_1$, and an unstable 8,9-epoxide metabolite which can covalently bind to DNA (28). To address the issue

TABLE 3

Odds ratios (ORs) of HCC by status of $AFB_1$-albumin adducts and urinary $AFB_1$-$N^7$-guanine adducts among HBsAg carriers[a]

| $AFB_1$-albumin adduct | $AFB_1$-$N^7$-guanine adduct | Controls | HCC | OR (95% CI) |
|---|---|---|---|---|
| Undetectable | Undetectable | 10 | 6 | 1.0+ |
| Undetectable | Detectable | 11 | 6 | 0.9 (0.2–3.8) |
| Detectable | Undetectable | 8 | 5 | 1.0 (0.2–4.7) |
| Detectable | Detectable | 2 | 12 | 10.0 (1.6–60.9) |

CI, confidence interval.
[a] 13 HCC cases and 12 controls had no sufficient serum samples for analysis of $AFB_1$-albumin adducts.
+ $p$=0.012, test for trend.

TABLE 4

Adjusted percentage[a] of the amount of individual $AFB_1$ metabolite in total $AFB_1$ metabolites excreted in urine among HBsAg-negative controls, HBsAg-positive controls, and HCC cases

| Individual $AFB_1$ metabolite in total $AFB_1$ (%) | HBsAg-negative controls | HBsAg-positive controls | Late-onset[b] HCC | Early-onset[c] HCC |
|---|---|---|---|---|
| | Median (Range) | Median (Range) | Median (Range) | Median (Range) |
| $AFM_1$ | 71.1 (9.3–100) | 51.4 (2.0–100) | 55.4 (21.7–100) | 44.8 (2.2–100) |
| $AFP_1$ | 25.4 (0.0–89.0) | 42.8 (0.0–97.1) | 40.5 (0.0–75.4) | 48.6 (0.0–94.3) |
| $AFB_1$-$N^7$-guanine | 0.7 (0.0–14.7) | 0.7 (0.0–20.3) | 1.8 (0.0–47.6) | 0.7 (0.0–8.6) |
| $AFB_1$ | 0.2 (0.0–11.2) | 0.4 (0.0–7.2) | 0.2 (0.0–4.8) | 0.4 (0.0–2.7) |

[a] Adjusted for the effects of educational level, ethnicity, habits of cigarette smoking and alcohol drinking.
[b] HCC cases diagnosed more than 2 years after urine collection.
[c] HCC cases diagnosed within 2 years after urine collection.

of whether metabolism of $AFB_1$ is influenced by long-term infection with HBV, thus affecting the association between urinary aflatoxin biomarkers and HCC risk, the percentages of the various $AFB_1$ metabolites in the total $AFB_1$ excretion (including $AFM_1$, $AFP_1$, $AFB_1$-$N^7$-guanine, and $AFB_1$) were compared between HBsAg-negative and positive controls. The proportion of $AFM_1$ in total $AFB_1$ in urine was higher among HBsAg-negative than positive controls, whereas the proportion of $AFP_1$ was lower among HBsAg-negative controls. However, the differences in the proportions of $AFB_1$ conversion to $AFM_1$ and $AFP_1$ were not statistically significant between the two groups. The proportion of $AFM_1$ in total $AFB_1$ was also lower in the early-onset HCC cases diagnosed within 2 years after urine collection than in the late-onset cases who were diagnosed more than 2 years after urine collection. The contribution of the amount of $AFB_1$-$N^7$-guanine adducts and $AFB_1$ to total $AFB_1$ excreted in urine was low. The difference in either the proportion of $AFB_1$-$N^7$-guanine adducts or the proportion of the unmetabolized $AFB_1$ in total $AFB_1$ metabolites between any two groups was not significant (Table 4).

TABLE 5

Odds ratios (ORs) of HCC associated with the production of $AFP_1$ and $AFB_1$ relative to the production of $AFM_1$ and $AFB_1$-$N^7$-guanine adducts among HBsAg carriers

| $\Sigma(AFP_1+AFB_1)/\Sigma(AFM_1+AFB_1$-$N^7$-guanine) | Controls | HCC | Adjusted[a] OR (95% CI) | $p$ |
|---|---|---|---|---|
| All subjects | | | | |
| ≥1.0 | 19 | 18 | 1.0 | |
| <1.0 | 24 | 24 | 1.0 (0.4–2.5) | 0.97 |
| Subjects with <2 years between urine collection and HCC diagnosis | | | | |
| ≥1.0 | 11 | 11 | 1.0 | |
| <1.0 | 13 | 12 | 0.6 (0.2–2.2) | 0.42 |
| Subjects with ≥2 years between urine collection and HCC diagnosis | | | | |
| ≥1.0 | 8 | 7 | 1.0 | |
| <1.0 | 11 | 12 | 5.4 (0.8–34.7) | 0.07 |

[a] Adjusted for the effects of total urinary $AFB_1$ metabolite level, educational level, and habits of cigarette smoking and alcohol drinking.

The risk of HCC in relation to the capacity to produce $AFP_1$ and $AFB_1$ relative to $AFM_1$ and $AFB_1$-$N^7$-guanine adducts is shown in Table 5. Because the conversion rate of $AFB_1$ to $AFM_1$ appeared to decrease with the progression of HCC, the association with HCC of the ratio $AFP_1+AFB_1/AFM_1+AFB_1$-$N^7$-guanine was examined separately for two distinct follow-up periods. A higher activity for production of $AFM_1$ and $AFB_1$-$N^7$-guanine was associated with the development of HCC when the analysis was restricted to the HCC cases who were diagnosed more than 2 years after urine collection. After adjustment for total urinary $AFB_1$ levels and other HCC risk factors, HBsAg carriers with a ratio <1.0 exhibited a 5-fold increase in risk of HCC compared with the carriers with a ratio ≥1.0.

Table 6 shows the relationship of HCC with urinary levels of $AFM_1$ and $AFB_1$-$N^7$-guanine adducts stratified by GSTM1 genotype. The association of HCC with urinary $AFM_1$ levels was more striking among HBsAg carriers with the GSTM1-null genotype. A test for statistical interaction between elevated $AFM_1$ levels and the GSTM1-null genotype was significant based on a multiplicative model (test for homogeneity, $p=0.007$). The difference in the odds ratios of HCC for the positivity of urinary $AFB_1$-DNA adducts between GSTM1-null and GSTM1-nonnull HBsAg carriers was not significant.

## Discussion

HCC is the most common cause of cancer mortality in Taiwan (1,9). Although HBV has been well documented as the main causative agent for HCC in this

TABLE 6

Odds ratios of HCC associated with urinary $AFM_1$ and $AFB_1$-$N^7$-guanine by GSTM1 genotype among HBsAg carriers[a]

| Urinary aflatoxin level | GSTM1 +/+ and +/0 | | | | GSTM1 0/0 | | | |
|---|---|---|---|---|---|---|---|---|
| | Odds Ratio | | | | Odds Ratio | | | |
| | Controls | HCC | Crude | Adjusted[b] (95% CI) | Controls | HCC | Crude | Adjusted (95% CI) |
| $AFM_1$ | | | | | | | | |
| Low[c] | 3 | 7 | 1.0 | 1.0 | 11 | 1 | 1.0 | 1.0 |
| High | 7 | 7 | 0.4 | 0.5 (0.1–3.7) | 11 | 15 | 15.0[d] | 11.1 (1.1–115.2) |
| $AFB_1$-$N^7$-guanine | | | | | | | | |
| Undetectable | 6 | 8 | 1.0 | 1.0 | 12 | 4 | 1.0 | 1.0 |
| Detectable | 4 | 6 | 1.1 | 2.0 (0.3–16.0) | 10 | 12 | 3.6 | 5.8 (1.1–30.8) |

[a] GSTM1 genotype was unavailable for 12 HCC cases and 11 controls because of insufficient DNA samples.
[b] Adjusted for educational level and habits of cigarette smoking and alcohol drinking.
[c] Categorized according to the median urinary $AFM_1$ level among all controls.
[d] Test for homogeneity of the odds ratios across GSTM1 genotypes was significant.

area, considerable evidence suggests that HCC is not an inevitable consequence of chronic infection with HBV (1–9). The weather of Taiwan is warm and humid, which is good for fungal growth. The importance of aflatoxin exposure in the etiology of HCC has long been suspected. The aflatoxins that produce the most severe pre- and postharvest contamination of foodstuffs include aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$. $AFB_1$ has been extensively studied *in vitro* and *in vivo* because it is the most prevalent and carcinogenic of the four. This prospective study demonstrated that $AFB_1$ exposure was positively associated with HCC risk among chronic hepatitis B carriers. This association cannot be explained on the basis of confounding, since it was statistically significant even when adjustment was made for known HCC risk factors. The urine samples were frozen at −30°C until the analysis of aflatoxins. The duration of urine storage prior to testing was the same for cases and controls. Even though the levels of aflatoxin metabolites may change with time, this may only bias the relative risk estimates associated with aflatoxins toward the null. The association between $AFB_1$ exposure and risk of HCC was stronger after exclusion of patients diagnosed within 2 years of urine collection, suggesting that our results are unlikely to be explained by bias due to an effect of preclinical cancer. The data of this study support a role of $AFB_1$ exposure in the HBV-related hepatocarcinogenesis.

A number of molecular dosimetry markers of aflatoxin exposure have been investigated (18–25). In monkeys, approximately 30–40% of the administered $AFB_1$ dose was excreted in urine (29). Unmetabolized aflatoxins ($AFB_1$, $AFG_1$) and the hydroxylated ($AFM_1$, $AFQ_1$) and demethylated metabolites ($AFP_1$) of $AFB_1$ have been measured in human urine. $AFM_1$, $AFP_1$, and $AFB_1$-$N^7$-guanine adducts are the three major aflatoxin metabolites identified in human urine (19,20,22). Available evidence suggests that both urinary levels of $AFM_1$ and $AFB_1$-$N^7$-guanine correlate well with dietary exposure to $AFB_1$ in areas with high aflatoxin exposure. But the excretion of $AFP_1$ in urine was not dose-related to the $AFB_1$ intake, despite $AFP_1$ being quantitatively a major aflatoxin metabolite in human urine (19). Peanuts and peanut products have been demonstrated to be one of the major aflatoxin-contaminated foodstuffs in Taiwan. Although there was no comprehensive survey regarding distribution, frequency, and concentration of aflatoxin contamination in Taiwan, a moderate but significant correlation between urinary $AFM_1$ level and consumption frequency of peanuts and fermented bean products was observed in this study. Since the exposure to $AFB_1$ may be through sources other than peanuts and fermented bean products and dietary questionnaires may suffer from recall bias, the correlation coefficient between urinary $AFM_1$ levels and the consumption frequency of peanuts and fermented bean products may not be high. Our data are consistent with previous reports suggesting that $AFM_1$ is the predominant aflatoxin in urine and a good internal dosimeter reflecting exposure. However, we did not find a significant correlation of dietary consumption frequency of aflatoxins with excretion of $AFB_1$-$N^7$-guanine in urine. Less than one half of the urine samples had detectable levels of $AFB_1$-$N^7$-guanine in this study. The relatively low level of $AFB_1$-$N^7$-guanine in urine might explain the observation that the correlation between aflatoxin intake and urinary $AFB_1$-DNA adducts was not as good as that found for $AFM_1$ in this study.

In animal models, covalent binding of $AFB_1$ with

hepatic DNA is a critical step in $AFB_1$-induced hepatocarcinogenesis (30). Our previous studies showed that a significant proportion of liver tissues from HCC patients in Taiwan had detectable levels of $AFB_1$-DNA adducts (18). However, tissue levels of $AFB_1$-DNA adducts may be affected by cancer through disease-induced changes in diet and/or alterations in the $AFB_1$ metabolism. The major product formed by the interaction of $AFB_1$ with hepatic DNA *in vivo* is $AFB_1$-$N^7$-guanine (31). In animals, the excretion of $AFB_1$-guanine adduct into urine was dose-related. There was a good correlation between the adduct excretion and the adduct formation in the liver DNA (32). In this study, high urinary $AFB_1$-$N^7$-guanine adduct levels were associated with increased risk of HCC. The HCC risk associated with $AFB_1$ exposure was more striking among chronic HBV carriers with detectable concentrations of urinary $AFB_1$-DNA adducts. However, no evidence of a dose-related effect on the development of HCC was found for increasing levels of the $AFB_1$-DNA adducts. The process between $AFB_1$ exposure and hepatic DNA damage is complex. Adducts initially formed in hepatocytes may be lost from DNA through spontaneous reactions and/or active DNA repair (33,34). For a given level of exposure to $AFB_1$, there may be considerable interindividual variation in the persistence of $AFB_1$-DNA adducts formed in hepatocytes due to discrepancy in the DNA repair activity. Since the repair activity was not assessed in this study, the HCC risk of some persons who had a higher level of urinary $AFB_1$-$N^7$-guanine adducts caused by a higher DNA repair activity may thus be misclassified.

The previous report from a CSP study in Taiwan (24) used $AFB_1$-albumin adducts and total urinary aflatoxin metabolites analyzed by competitive ELISA to investigate the relationship between aflatoxin exposure and the development of HCC. The antibody used to measure total urinary aflatoxin metabolites had a high level of recognition for $AFB_1$. It also cross-reacted with $AFB_2$, $AFM_1$, $AFG_1$, and $AFP_1$, but not with $AFB_1$-$N^7$-guanine adducts. However, HPLC analysis of the urine samples in this study showed that the presence of the $AFB_1$-$N^7$-guanine adducts in urine was a significant predictor for HCC risk. No significant association was observed between urinary $AFB_1$ levels and HCC. This finding illustrates the limitation of the measurement of only total quantity of multiple compounds recognized by a given antibody.

Urinary aflatoxin metabolites and DNA adducts reflect only the dietary exposure to aflatoxins on the previous few days. It is unlikely that a single measurement of urinary aflatoxins accurately indicates long-term exposure of an individual. Thus, misclassification of long-term exposure resulting from single urinary measurements may underestimate the true association between aflatoxin exposure and HCC risk in this study. Our data show no significant seasonal variation in urinary aflatoxin levels. It is possible that aflatoxin exposure is fairly constant among residents in Taiwan over time. Several traditional Chinese foods are frequently contaminated with aflatoxins in Shanghai (20). The source of exposure to aflatoxins in Taiwan merits further studies.

To date there are no biomarkers reflecting long-term exposure to aflatoxins. Although serum levels of $AFB_1$-albumin adducts have been suggested to be a marker revealing $AFB_1$ exposure integrated over a longer time period, this marker can only reflect intake over a period of weeks/months because the half-life of albumin in humans is about 20 days (21). Since there are many competing biotransformation pathways for metabolism of $AFB_1$ (28), it is not surprising to find no significant correlation between $AFB_1$-albumin adducts and urinary levels of total $AFB_1$ metabolites in this study. However, it is interesting to observe that the odds ratio of developing HCC is extremely high for chronic HBV carriers who are positive for both $AFB_1$-albumin adducts and $AFB_1$-$N^7$-guanine adducts. This result suggests that the HBV carriers with high aflatoxin exposure and/or high enzyme activity for metabolism of $AFB_1$ to its epoxide may be at the highest risk of HCC.

Changes in the expression of various forms of cytochrome P450 during the development of hepatitis have been shown in a recent animal study (35). It was also reported that microsomal fractions from HCC patients produced higher levels of $AFP_1$ than observed in microsomes prepared from normal liver tissue (36). In this study, the proportion of $AFB_1$ converted to $AFM_1$ was higher among HBsAg-negative controls compared with HBsAg-positive controls, while the conversion of $AFB_1$ to $AFP_1$ was lower among HBsAg-negative controls. The early-onset HCC cases diagnosed within 2 years after urine collection also appear to have a lower conversion rate of $AFB_1$ to $AFM_1$ and a higher rate of $AFB_1$ converted to $AFP_1$ than the late-onset cases who were diagnosed more than 2 years after urine collection. These findings suggest that the biotransformation of $AFB_1$ to $AFM_1$ might decrease with the progress of the liver disease. The relative risk of HCC associated with urinary $AFM_1$ in this study might thus be underestimated.

The acute toxicity of $AFM_1$ was found to be nearly equivalent to that of $AFB_1$ in rats (37). $AFM_1$ is also genotoxic in *in vitro* studies and a relatively potent hepatic carcinogen in animal studies, although it is con-

siderably less potent than $AFB_1$ (38,39). In contrast, $AFP_1$ is much less toxic than $AFB_1$ and shows little mutagenic activity (38). The difference in patterns of $AFB_1$ metabolite formation by microsomal oxidation has been suggested to be a critical factor for the susceptibility of a given species to the carcinogenic effect of $AFB_1$ (40). In this study, the relative activity for biotransformation of $AFB_1$ to less carcinogenic products and to more toxic forms by microsomal oxidations was defined as the ratio of the sum of the amounts of $AFP_1$ and unmetabolized $AFB_1$ to the sum of the amounts of $AFM_1$ and $AFB_1$-$N^7$-guanine adducts excreted in urine. Our results suggest that a decreased ratio was an independent risk factor for HCC among chronic HBV carriers after adjustment for total $AFB_1$ excretion in urine and other HCC risk factors. Biotransformation of $AFB_1$ is a complex, multi-pathway process (28). An *in vitro* study with human liver microsomes suggested cytochrome P450 1A2 (CYP1A2) is the principal P450 responsible for oxidation of $AFB_1$ to $AFM_1$. CYP1A2 is also the dominant P450 enzyme responsible for the activation of $AFB_1$ to $AFB_1$-8,9-epoxide, which can form adducts with DNA, at the low $AFB_1$ concentrations relevant to dietary exposure in humans (41). Our result showing a significant correlation between $AFM_1$ and $AFB_1$-$N^7$-guanine in urine would support the hypothesis that the same P450 is responsible for the biotransformation of $AFB_1$ to these metabolites. Although the metabolism of $AFB_1$ to $AFM_1$ and the activation of $AFB_1$ to $AFB_1$-8,9- epoxide may be catalyzed by the same enzyme, they are two competing pathways of $AFB_1$ biotransformation. This may explain why the magnitude of the correlation between $AFM_1$ and $AFB_1$-$N^7$-guanine adducts is not high in this study. A 100-fold interindividual variation in enzyme levels of CYP1A2 has been observed in human liver tissues (42). This study suggests that individuals with relatively high hepatic CYP1A2 activities may be at greater risk for hepatocarcinogenesis from $AFB_1$ exposure than those with low CYP1A2 activities.

Phase II detoxification enzymes may also play a critical role in susceptibility to $AFB_1$-induced hepatocarcinogenesis. The primary pathway for $AFB_1$ detoxification in mammals is through glutathione S-transferase (GST)-mediated conjugation of $AFB_1$-8,9-epoxide with glutathione. The difference in cytosolic GST activity toward $AFB_1$-8,9-epoxide has been linked with species susceptibility to $AFB_1$ (43). The GSTs are a family of multifunctional isoenzymes in which the $\mu$ form (GSTM1) is present in about 50% of individuals (44). GSTM1 is rich in liver and has been demonstrated to play an important role in detoxification of the DNA reactive metabolites of $AFB_1$ in human lymphocytes (45). In this study, chronic HBV carriers with the GSTM1-null genotype were at much higher risk of $AFB_1$-related HCC than carriers with the GSTM1-nonnull genotype. This result is consistent with those observed in previous experimental studies and supports the importance of GSTM1 genotype in $AFB_1$-related hepatocarcinogenesis in humans. Although our data suggest that a synergistic interaction of chronic HBV infection, $AFB_1$ intake, and genetic susceptibility may exist in the development of HCC, the carcinogenicity of $AFB_1$ in humans may be ultimately determined by a complex set of biological processes. In addition to the expression of enzymes involved in the activation/detoxification of $AFB_1$, nutritional factors have also been suggested to be important in modulating HCC risk in relation to $AFB_1$ exposure. We have demonstrated that vitamins A and C can inhibit the formation of $AFB_1$-DNA adducts in woodchuck hepatocytes (46). The combined effect of nutritional factors and GSTM1 genotype on the risk of $AFB_1$-related HCC among chronic hepatitis B carriers is being investigated in our ongoing study.

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