

Aflatoxin B₁ Exposure, Hepatitis B Virus Infection, and Hepatocellular Carcinoma in Taiwan

Hui-Chen Wu,¹ Qiao Wang,¹ Hwai-I Yang,⁴ Habibul Ahsan,^{2,6} Wei-Yann Tsai,^{3,5} Li-Yu Wang,¹ Shu-Yuan Chen,¹ Chien-Jen Chen,⁴ and Regina M. Santella¹

Departments of ¹Environmental Health Sciences, ²Epidemiology, and ³Biostatistics, Mailman School of Public Health of Columbia University, New York, New York; ⁴Graduate Institute of Epidemiology, College of Public Health, National Taiwan University, Taipei, Taiwan; ⁵Department of Statistics, National Cheng Kung University, Tainan, Taiwan; and ⁶Departments of Health Studies, Medicine, and Human Genetics and Cancer Research Center, The University of Chicago, Chicago, Illinois

Abstract

To evaluate the role of aflatoxin B₁ (AFB₁) exposure on risk of hepatocellular carcinoma (HCC), a case-control study nested within a community-based cohort was conducted. Baseline blood and urine samples were used to determine the level of AFB₁-albumin adducts and urinary AFB₁ metabolites. Conditional logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) to assess the effect of AFB₁ exposure on risk of HCC. The adjusted ORs (95% CIs) were 1.54 (1.01-2.36) and 1.76 (1.18-2.58), respectively, for those with AFB₁-albumin adducts and urinary AFB₁ metabolite levels above the mean compared with those with levels below the mean. When compared with subjects in the lowest quartile of urinary AFB₁ metabolites, there was an increase in risk of HCC, with adjusted ORs (95% CIs) of 0.57 (0.14-2.43), 1.43 (0.32-6.42), and 4.91 (1.18-20.48;

$P_{\text{trend}} = 0.02$), respectively, among noncarriers of hepatitis B virus (HBV) infection. The adjusted OR (95% CI) was 7.49 (5.13-10.93) for carriers of hepatitis B surface antigen compared with noncarriers, regardless of AFB₁ status. The ORs (95% CI) were 10.38 (5.73-18.82) and 15.13 (7.83-29.25) for carriers of hepatitis B surface antigens with levels of AFB₁-albumin adducts and urinary AFB₁ metabolites above the mean, respectively. The combined effect of aflatoxin exposure and HBV infection did not differ by duration of follow-up. Consistent with our previous study with fewer subjects, these data show that AFB₁ exposure is a risk factor for HCC risk. However, in this larger study, the effect of combined AFB₁ exposure and HBV infection is more consistent with an additive than a multiplicative model. (Cancer Epidemiol Biomarkers Prev 2009;18(3):846-53)

Introduction

In Taiwan, primary hepatocellular carcinoma (HCC) is the leading cause of cancer death for males and the second for females. Epidemiologic evidence suggests that dietary exposure to aflatoxin B₁ (AFB₁) and chronic infection with hepatitis B virus (HBV) are major risk factors for HCC (1-3).

The biotransformation of AFB₁, primarily in the liver, is intimately linked with its toxic and carcinogenic effects (4). AFB₁ undergoes an initial two-electron oxidation, yielding an active intermediate, AFB₁-exo-8,9-epoxide, which is later detoxified through a variety of metabolic processes (5). Epoxidation of AFB₁ to the exo-8,9-epoxide is a critical step in the genotoxic pathway of this carcinogen. The exo-epoxide is highly unstable and binds with DNA to form the predominant *trans*-8,9-dihydro-8-(*N*⁷-guanyl)-9-hydroxy-AFB₁ (AFB₁-*N*⁷-Gua)

adduct, a promutagenic DNA lesion (5, 6). In addition to formation of the 8,9-oxide, AFB₁ also undergoes metabolism to yield stable urinary metabolites, which are poorer substrates for epoxidation, such as AFM₁, AFQ₁, and AFP₁ (5). The reactive epoxides are detoxified by glutathione *S*-transferase-mediated conjugation (7).

Biomarkers of AFB₁ exposure include AFB₁-DNA, serum AFB₁-albumin and urinary AFB₁ metabolites such as AFM₁, and AFB₁-mercapturic acid (5). In general, AFB₁-albumin adducts have been recognized as long-term markers of AFB₁ exposure. Because albumin adducts are as long lived as albumin, which has a half-life of 21 days in humans, they provide information on accumulated exposure over a period of 2 to 3 months (8, 9). Measurement of the adduct levels in urine, however, provides a noninvasive means of estimating the levels of AFB₁ exposure.

We have developed antibody-based methods for measurement of AFB₁ exposure, including AFB₁-albumin and urinary AFB₁ metabolites, and we have shown that dietary exposure to AFB₁ increased HCC risk among the Taiwanese population using a case-control study nested within a community-based Cancer Screening Program cohort (10, 11). Our previous study with a total of 3 to 4 years of follow-up found that in HBV-infected males, there was an adjusted odds ratio (OR) of 2.8 [95% confidence intervals (95% CI), 0.9-9.0] for detectable compared with nondetectable AFB₁-albumin adducts

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Note: Current address for L.-Y. Wang: Graduate Institute of Aboriginal Health, Tzu Chi University, Hualien, Taiwan. Current address for S.-Y. Chen: Graduate Institute of Public Health, College of Medicine, Tzu Chi University, Hualien, Taiwan. Current address for H.-I. Yang and C.-J. Chen: Genomics Research Center, Academia Sinica, Taipei, Taiwan.

Requests for reprints: Regina M. Santella, Department of Environmental Health Sciences, Mailman School of Public Health of Columbia University, 630 West 168th Street, RM19-418, New York, NY 10032. Phone: 212-305-1996; Fax: 212-305-5328. E-mail: rps1@columbia.edu

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and 5.5 (95% CI, 1.3-23.4) for high compared with low levels of AFB₁ metabolites in urine (11). There are limited data on the effect of AFB₁ exposure among noncarriers of hepatitis B surface antigen (HBsAg) due to the small number of HCC cases without chronic HBV infection. The specific aim of this study, which encompasses larger numbers of subjects as a result of the continued follow-up of the cohort, was to investigate the role of AFB₁ exposure, as assessed by AFB₁-albumin adducts as well as urinary excretion AFB₁ metabolites, in HCC risk using data and biospecimens collected in the Cancer Screening Program cohort (10, 11).

Materials and Methods

Study Cohort. Subjects were from a 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 (10, 11). Briefly, individuals who were between 30 and 64 y old and lived in seven townships in Taiwan, three located on Penghu Islets, which has the highest HCC incidence rate in Taiwan, and the other four from Taiwan Island, were recruited between July 1990 and June 1992. A total of 12,020 males and 11,923 females were enrolled. Participants were personally interviewed based on a structured questionnaire regarding epidemiologic information and donated a 20 mL fasting blood sample as well as a spot urine at recruitment. Biospecimens were transported on dry ice to a central laboratory at the National Taiwan University and stored at -70°C . Epidemiologic information collected at baseline 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 more than 4 d a week for at least 6 mo. Information about duration and intensity was also obtained. Habitual alcohol intake was defined as drinking alcohol-containing products more than 4 d a week for at least 6 mo.

At enrollment, blood samples were tested in Taiwan for serologic markers, including alanine transaminase, aspartate transaminase, α -fetoprotein (AFP), HBsAg, and antibody against hepatitis C virus (anti-HCV). HBsAg was tested by RIA (Abbott Laboratories). Anti-HCV and AFP were tested by enzyme immunoassay using commercial kits (Abbott Laboratories). Both alanine transaminase and aspartate transaminase levels were determined with a serum chemistry autoanalyzer (model 736; Hitachi Co.) using commercial reagents (Biomerieux). 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 alanine transaminase (≥ 45 IU/mL), aspartate transaminase (≥ 40 IU/mL), or AFP (≥ 20 ng/mL), was positive for HBsAg or anti-HCV, or had a family history of HCC or liver cirrhosis among

first-degree relatives was referred for upper abdominal ultrasonography examination. Suspected HCC cases were referred to teaching medical centers for confirmatory diagnosis by computerized tomography, digital subtracted angiogram, aspiration cytology, and pathologic examination. The criteria for HCC diagnosis included a histopathologic examination and a positive lesion detected by at least two different imaging techniques (abdominal ultrasonography, angiogram, or computed tomography) or by one imaging technique and a serum AFP level of >400 ng/mL.

Intensive follow-up, including abdominal ultrasonographic screening and serum AFP level determination every 3 mo, was carried out on those with ultrasonographic images indicative of liver cirrhosis, whereas others were examined annually. Any suspected HCC cases identified during follow-up were referred for confirmatory diagnosis as described above. Ninety-seven cases were diagnosed by histopathologic examination, 146 cases by at least two different imaging techniques (abdominal ultrasonography, angiogram, computed tomography, or magnetic resonance imaging), and 74 cases by one imaging technique and an AFP level of ≥ 400 ng/mL.

Study Subjects. Between February 1991 and June 2004, a total of 241 cases were newly diagnosed with HCC. Cases were primarily identified through linkage to the National Cancer Registry and death certification systems. Of these, 11 cases were excluded because of nonavailability of stored blood. A total of 1,052 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 y), gender, residential township, and date of recruitment (± 3 mo). The number of matched controls per case varied depending on the number of eligible controls with available specimens and ranged from two to six. Data on AFB₁ biomarkers were available from our prior study on 56 cases and 220 controls (11). None of these controls became a case during the additional follow-up. Baseline blood samples (174 cases and 832 controls) and urine samples (142 cases and 684 controls) from new subjects were available and shipped to Columbia University on dry ice for determination of AFB₁-albumin adducts as well as urinary AFB₁ metabolites.

AFB₁-Albumin Adducts in Blood and AFB₁ Metabolites in Urine. Additional samples were assayed for albumin adducts by ELISA as previously described (11). Briefly, 50 μL of albumin extracts, equivalent to 200 μg of albumin, were added to plates previously coated with 3 ng of AFB₁ epoxide-modified human serum albumin. Polyclonal antiserum 7 was used at a $1:2 \times 10^5$ dilution and the secondary antiserum, goat anti-rabbit IgG-alkaline phosphatase conjugate, was used at a 1:750 dilution. Concentrations of AFB₁-albumin adducts were determined using a standard curve of serially diluted AFB₁ epoxide-modified human serum albumin that had been enzymatically digested. Samples with $<20\%$ inhibition were considered undetectable and assigned a value of 0.01 fmol/ μg . Two control samples were analyzed with each batch of test samples: a pooled sample of plasma from nonsmoking subjects from the United States and a positive control of serum from a rat treated with

1.5 mg AFB₁. The coefficient of variation of the mean of two controls was 20% ($n = 13$).

Urine samples were assayed essentially as described previously (11). Sep-Pak urine extracts (50 μ L) were added to plates previously coated with 3 ng of AFB₁ epoxide-modified bovine serum albumin. AF8E11 was used at 1:1,500 dilution and the secondary antiserum, goat anti-mouse alkaline phosphatase, was used at 1:1,000 dilution. AF8E11 mainly reacts with AFB₁, but there is significant cross-reactivity with several aflatoxin derivatives, including AFB₂, AFM₁, AFG₁, and AFP₁ (12). Concentrations of urinary metabolites were determined using a standard curve of serially diluted AFB₁. Samples with <20% inhibition were considered undetectable and assigned a value of 1 fmol/mL. A pooled sample of urine from five controls was used as a quality control and analyzed with each batch of test samples. The coefficient of variation was 10% ($n = 5$).

Statistical Methods. The χ^2 test was used to examine differences in the distributions of variables between cases and controls. Levels of AFB₁-albumin adducts and urinary AFB₁ metabolites were natural log transformed to normalize the distribution. All statistical analyses used data after log transformation. The cutpoint values were based on the distribution of log-transformed data among controls; however, for ease of interpretation, we present the values as arithmetic data. The distributions of the two biomarkers are higher in the more recently assayed samples compared with prior data. Initially in the data analysis, we used different cutoff values for the two batches of aflatoxin biomarker assays. However, the results were not significantly different between these two rounds. In the final data analysis, we combined both batches and adjusted for the batch effect on the values of the biomarkers by creating a new variable indicating round 1 or 2. A Pearson partial correlation coefficient was used to determine the correlation between AFB₁-albumin adducts and urinary AFB₁ metabolites adjusted by age and gender. To examine the independent and combined effects of the levels of AFB₁ exposure on HCC among subjects with and without chronic HBV infection,

the levels of biomarkers were analyzed as a categorical variable rather than a continuous variable. Subjects were divided into different exposure groups: those with biomarker levels above the mean value (59.8 fmol/mg for AFB₁-albumin adducts and 55.2 fmol/mL for urinary AFB₁ metabolites) for all controls samples versus those below the mean. To evaluate the dose-response relationships between biomarkers and HCC risk, subjects were divided into quartiles based on control values (<26.9, ≥ 26.9 to <43.5, ≥ 43.5 to <71.35, and ≥ 71.35 fmol/mg for AFB₁-albumin adducts; <25.7, ≥ 25.7 to <48.5, ≥ 48.5 to <77.35, and ≥ 77.35 fmol/mL for urinary AFB₁ metabolites). HBsAg, anti-HCV, assay batch, smoking, alcohol consumption, and body mass index (BMI)-adjusted ORs and 95% CIs were derived from conditional logistic regression models stratified on the matching factors to estimate the association between levels of AFB₁ exposure and HCC risk. The test for trend of adjusted ORs across strata was based on Wald's test with consecutive scores of 1, 2, 3, and 4 assigned to the first, second, third, and fourth quartiles of the biomarker. All analyses were done with Statistical Analysis System software 9.0 (SAS Institute). All statistical tests were based on two-tailed probability.

Results

The sociodemographic characteristics of HCC cases and controls among all subjects and those with data on both biomarkers are given in Table 1. The distributions of characteristics were similar for both groups. There were no differences in mean ages and BMI between cases and controls. Significantly more controls (64.4%) were negative for both HBV and HCV biomarkers compared with cases (15.0%). The distributions of habitual smoking were similar for cases and controls (50.0% and 45.3%, respectively), but there was a significant difference in frequency of alcohol consumption (22.8% and 17.0%, respectively).

The mean levels of AFB₁-albumin adducts were not statistically significantly different between cases and

Table 1. Sociodemographic characteristics of HCC cases and controls

Variable	Total subjects		<i>P</i>	Subjects with available data on both biomarkers		<i>P</i>
	Cases ($n = 230$) %	Controls ($n = 1,052$) %		Cases ($n = 198$) %	Controls ($n = 904$) %	
Mean age (y)*	53.9 (7.8)	53.7 (7.6)	0.68	53.8 (7.5)	53.6 (7.4)	0.95
BMI (mean \pm SD)*	24.4 (3.4)	24.5 (3.6)	0.77	24.3 (3.6)	24.3 (3.4)	0.33
Gender, n (%)						
Female	46 (20.0)	207 (19.7)	0.91	34 (17.2)	150 (16.6)	0.84
Male	184 (80.0)	845 (80.3)		164 (82.8)	754 (83.4)	
HBsAg/anti-HCV, n (%)						
-/-	34 (15.0)	676 (64.4)	<0.0001	25 (12.9)	581 (64.5)	<0.0001
+/-	143 (63.3)	271 (25.8)		131 (67.5)	241 (26.7)	
-/+	39 (17.3)	72 (6.9)		28 (14.4)	52 (5.8)	
+/+	10 (4.4)	30 (2.9)		10 (5.2)	27 (3.0)	
Smoking, n (%)						
No	115 (50.0)	575 (54.7)	0.20	98 (49.5)	477 (52.8)	0.4
Yes	115 (50.0)	477 (45.3)		100 (50.5)	427 (47.2)	
Alcohol, n (%)						
No	176 (77.2)	872 (83.0)	0.04	151 (77.0)	741 (82.1)	0.01
Yes	52 (22.8)	179 (17.0)		45 (23.0)	162 (17.9)	

NOTE: Anti-HCV data missing for seven subjects. Alcohol data missing for three subjects.

**P* value for *t* test for continuous variable; all other *P* values for the χ^2 test for categorical variables.

controls (56.5 ± 53.5 versus 59.8 ± 59.5 fmol/mg, respectively; $P = 0.45$; Table 2). The mean level of urinary AFB₁ metabolites, however, was significantly higher in cases than in controls (71.3 ± 54.9 versus 55.2 ± 41.9 fmol/mL, respectively; $P < 0.0001$). There was a weak linear correlation between levels of AFB₁-albumin adducts and urinary AFB₁ metabolites, with a partial Pearson correlation coefficient of 0.15 ($P < 0.0001$) among controls (data not shown). The association of AFB₁ exposure with HCC risk is given in Table 2. AFB₁-albumin adducts were detectable in >93% of subjects. After multivariable adjustment, the OR for those with AFB₁-albumin adducts levels above the mean compared with those with levels below the mean was 1.54 (95% CI, 1.01-2.36). When AFB₁-albumin adduct levels were divided into quartiles based on control values, there was a nonstatistically significant increased risk for those in the higher quartiles, with adjusted ORs (95% CI) of 1.11 (0.69-1.83), 0.18 (0.69-2.03), and 1.47 (0.83-2.58) for the second, third, and fourth quartiles relative to the lowest, respectively ($P_{\text{trend}} = 0.19$). The adjusted OR for those with urinary AFB₁ metabolite levels above the mean compared with those with levels below the mean was 1.76 (95% CI, 1.18-2.58). When urinary AFB₁ metabolite levels were divided into quartiles, HCC risk increased, with adjusted ORs (95% CI) of 0.98 (0.54-1.78), 0.95 (0.55-1.69), and 1.99 (1.18-3.36) for subjects with AFB₁ metabolites in the second, third, and fourth quartiles, respectively, compared with those in the lowest quartile ($P_{\text{trend}} = 0.008$).

To assess the effect of HBV infection on the relationship between biomarkers and HCC, subjects were stratified into two groups based on HBsAg status (Table 3). The effect of AFB₁ exposure, comparing subjects with AFB₁-albumin adduct levels above the mean versus below the mean, was not different between carriers and noncarriers of HBsAg (OR, 1.43; 95% CI, 0.76-2.71 and OR, 1.65; 95% CI, 0.63-4.33, respectively).

Similarly, among carriers of HBsAg, the ORs (95% CI) were 0.75 (0.38-1.46), 1.14 (0.53-2.46), and 1.46 (0.66-3.24), respectively, for adduct levels in the second, third, and fourth quartiles of adducts compared with those in the lowest quartile ($P_{\text{trend}} = 0.33$). The corresponding ORs (95% CI) were 1.87 (0.67-5.21), 1.03 (0.32-3.31), and 1.70 (0.46-6.28; $P_{\text{trend}} = 0.64$) among noncarriers.

The OR for those with urinary AFB₁ metabolite levels above the mean compared with those with levels below the mean was 4.29 (95% CI, 1.43-12.85) among noncarriers. There was a statistically significant dose-response relationship between urinary AFB₁ metabolites and HCC with ORs (95% CI) of 0.57 (0.14-2.43), 1.43 (0.32-6.42), and 4.91 (1.18-20.48; $P_{\text{trend}} = 0.02$), respectively, for adduct levels in the second, third, and fourth quartiles of adducts among noncarriers compared with those in the lowest quartile. There were no significant associations among carriers of HBsAg.

The combined effect of HBV infection and AFB₁ exposure is shown in Table 4. Adjusted OR (95% CI) was 7.49 (5.13-10.93) for carriers of HBsAg compared with noncarriers, regardless of AFB₁ status. The ORs (95% CI) were 10.38 (5.73-18.82) and 15.13 (7.83-29.25) for HBsAg carriers with levels of albumin adduct and urinary metabolites above the mean compared with noncarriers with values below the mean, respectively. Values were similar when cases were stratified into those diagnosed within 3 years ($n = 83$) or more than 3 years after ($n = 147$) sample collection (Table 4). Carriers of HBsAg and HBsAg carriers with both albumin adducts and urinary metabolite levels above the mean had a significantly increased HCC risk (OR, 17.29; 95% CI, 6.98-42.82) compared with noncarriers and those with both biomarkers below the mean ($P_{\text{trend}} < 0.0001$; Table 4). The combined effect of HBV or AFB₁ did not significantly differ based on the age of onset of HCC. The ORs (95% CI) for HBsAg carriers with levels of albumin adducts above the mean compared with noncarriers with values

Table 2. Biomarkers of AFB₁ exposure and risk of HCC

AFB ₁ exposure	Cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR (95% CI)	OR (95% CI)*
AFB₁-albumin adducts (fmol/mg)				
Mean (SD)	56.5 (53.5)	59.8 (59.5)		
Nondetectable	15 (6.5)	57 (5.4)	1.0	1.0
Detectable	215 (93.5)	995 (94.6)	0.92 (0.49-1.74)	0.99 (0.48-2.02)
Below mean (<59.8)	155 (67.4)	708 (67.3)	1.0	1.0
Above mean (≥59.8)	75 (32.6)	344 (32.7)	1.10 (0.76-1.60)	1.54 (1.01-2.36) [†]
<26.9	66 (28.7)	263 (25.0)	1.0	1.0
≥26.9 to <43.5	58 (25.2)	262 (24.9)	0.92 (0.61-1.41)	1.11 (0.69-1.83)
≥43.5 to <71.35	49 (22.3)	264 (25.1)	0.76 (0.48-1.21)	1.18 (0.69-2.03)
≥71.35	57 (24.8)	263 (25.0)	0.94 (0.58-1.51)	1.47 (0.83-2.58)
			$P_{\text{trend}} = 0.58$	$P_{\text{trend}} = 0.19$
Urinary AFB₁ metabolites (fmol/mL)				
Mean (SD)	71.3 (54.9)	55.2 (41.9)		
Nondetectable	23 (11.6)	107 (11.9)	1.0	1.0
Detectable	175 (88.4)	797 (88.1)	1.07 (0.59-1.94)	1.70 (0.89-3.25)
Below mean (<55.2)	85 (42.9)	509 (56.3)	1.0	1.0
Above mean (≥55.2)	113 (57.1)	395 (43.7)	1.77 (1.27-2.47) [†]	1.76 (1.18-2.58) [†]
<25.7	45 (22.7)	225 (24.9)	1.0	1.0
≥25.7 to 48.5	31 (15.7)	223 (24.7)	0.76 (0.45-1.27)	0.98 (0.54-1.78)
≥48.5 to 77.35	45 (22.7)	230 (25.4)	0.97 (0.59-1.59) [†]	0.95 (0.55-1.69) [†]
≥77.35	77 (38.9)	226 (25.0)	1.91 (1.21-3.01) [†]	1.99 (1.18-3.36) [†]
		$P_{\text{trend}} = 0.0009$	$P_{\text{trend}} = 0.008$	

*Adjusted for batch of aflatoxin biomarker assay, HBsAg, anti-HCV status, habitual smoking, alcohol drinking, and BMI.

[†] $P < 0.01$.

Table 3. Biomarkers of AFB₁ exposure and risk of HCC by HBsAg status

	HBsAg positive			HBsAg negative		
	No. HCC	No. controls	OR (95% CI)	No. HCC	No. controls	OR (95% CI)
AFB ₁ -albumin adducts (fmol/mg)						
Nondetectable	10	15	1.0	5	42	1.0
Detectable	145	286	0.58 (0.18-1.91)	70	709	1.17 (0.27-5.13)
Below mean (<59.8)	111	219	1.0	44	489	1.0
Above mean (≥59.8)	44	82	1.43 (0.76-2.71)	31	262	1.65 (0.63-4.33)
<26.9	50	95	1.0	16	168	1.0
≥26.9 to <43.5	35	79	0.75 (0.38-1.46)	23	183	1.87 (0.67-5.21)
≥43.5 to <71.35	35	69	1.14 (0.53-2.46)	14	195	1.03 (0.32-3.31)
≥71.35	35	58	1.46 (0.66-3.24)	22	205	1.70 (0.46-6.28)
			<i>P</i> _{trend} = 0.33			<i>P</i> _{trend} = 0.64
Urinary AFB ₁ metabolites (fmol/mL)						
Nondetectable	19	50	1.0	4	57	1.0
Detectable	124	218	1.31 (0.64-2.67)	51	579	3.00 (0.36-24.90)
Below mean (<55.2)	63	139	1.0	22	370	1.0
Above mean (≥55.2)	80	129	1.19 (0.72-1.98)	33	266	4.29 (1.43-12.85)*
<25.7	33	69	1.0	12	156	1.0
≥25.7 to 48.5	22	50	0.93 (0.41-2.16)	9	173	0.57 (0.14-2.43)
≥48.5 to 77.35	36	71	0.65 (0.29-1.45)	9	159	1.43 (0.32-6.42)
≥77.35	52	78	1.23 (0.62-2.41)	25	148	4.91 (1.18-20.48)*
			<i>P</i> _{trend} = 0.54			<i>P</i> _{trend} = 0.02

NOTE: ORs were adjusted for batch of aflatoxin biomarker assay, anti-HCV status, habitual smoking, alcohol drinking, and BMI.

**P* < 0.01.

below the mean were 11.94 (5.00-12.85) and 10.87 (5.32-22.20) for cases diagnosed with HCC at ages below and above 50, respectively (data not shown). The corresponding ORs (95% CI) were 15.94 (5.00-28.82) and 16.76 (7.63-36.81) for HBsAg carriers with levels of urinary metabolites above the mean compared with noncarriers with values below the mean (data not shown).

Discussion

In this study, with a longer follow-up period and more HCC cases than our prior study in this cohort, we applied two biological markers of AFB₁ exposure, AFB₁-albumin adducts and urinary AFB₁ metabolites, to investigate the relationship between AFB₁, HBV infection, and HCC. Consistent with our previous study that included 56 cases diagnosed with HCC within 4 years of follow-up (11), we found a statistically significant elevated risk of HCC with increasing levels of AFB₁ exposure. This is also in agreement with another nested case-control study of 18,244 men in Shanghai, People's Republic of China, among whom there were 22 incident cases of liver cancer. Analysis of urine samples banked 1 to 4 years before diagnosis gave a relative risk of 2.4 (95% CI, 1.0-5.9) for any of the AFB₁ metabolites and 4.9 (95% CI, 1.5-16.3) for detectable AFB₁-N⁷-Gua adducts (13). A subsequent follow-up study from this cohort showed that the presence of any urinary AFB₁ biomarker significantly predicted liver cancer (relative risk, 5.0; 95% CI, 2.1-11.8), with a relative risk of 9.1 (95% CI, 2.9-29.2) for the presence of AFB₁-N⁷-Gua adducts (3). Although Gan et al. (14) used a different assay for the measurement of AFB₁-albumin adducts, we used their data correlating adducts to chronic intake of AFB₁ to estimate median intake of AFB₁ from median levels of albumin adducts among our controls. These estimations suggest that ~30 µg/d increases the risk of developing HCC by 1.5-fold.

Our previous report on this cohort, with urinary AFB₁ metabolite data on 38 HCC cases and AFB₁-albumin data on 52 cases, reported a 111-fold increased risk of HCC among HBsAg carriers with high urinary AFB₁ metabolites and 70-fold for detectable AFB₁-albumin adducts compared with those with low/nondetectable levels and those negative for HBsAg (11). A synergistic interaction between the presence of urinary AFB₁ biomarkers and HBV seropositive status was also observed in the cohort study conducted in China with 50 HCC cases (relative risk, 59.4; 95% CI, 16.6-212.0; ref. 3). In the present study, with a much larger sample size (230 cases with AFB₁-albumin adducts and 198 with AFB₁ metabolite data), we did not observe a significant synergistic interaction between HBV infection and AFB₁ exposure among cases diagnosed with HCC between 1 and 13 years after sample collection. Stratifying subjects by years between sample collection and diagnosis did not alter these results. This result is in contrast with our earlier study due to the larger number of cases (*n* = 83) diagnosed within 3 years as a result of the more complete case identification. Thus, the primary reason for these discrepant results may be the small sample sizes in prior studies. To date, there are no other data on the long-term combined effect of AFB₁ with HBV on HCC.

It is difficult to accurately assess AFB₁ exposure at the individual level either by questions about consumption of foods suspected to be major sources of exposure or by direct analysis of foods. Epidemiologic studies using urine and blood samples banked several years before diagnosis have shown that the presence of AFB₁ metabolites in urine is associated with a significantly elevated risk of HCC (11, 13, 14). In general, AFB₁-albumin adducts have been recognized as a somewhat longer-term markers of AFB₁ exposure. Because albumin adducts are as long lived as albumin, which has a half-life of 21 days in humans, they provide information on accumulated exposure over a period of 2 to 3 months. The long-term stability of human AFB₁-albumin adducts

Table 4. The combined effect of HBV infection and AFB₁ exposure and risk of HCC

HBsAg		HCC/controls	OR (95% CI)	Diagnosis with HCC			
				Within 3 y of follow-up	OR (95% CI)	More than 3 y	OR (95% CI)
AFB ₁ -albumin adducts							
Positive vs negative							
Negative	Below mean (<59.8)	44/489	7.49 (5.13-10.93)*	12/174	1.0	32/315	1.0
Negative	Above mean (≥59.8)	31/262	1.64 (0.89-3.03)	7/67	2.58 (0.78-8.59)	24/195	1.42 (0.68-2.99)
Positive	Below mean (<59.8)	111/219	7.03 (4.45-11.09)*	46/119	4.32 (2.12-8.79)*	65/100	11.22 (6.05-20.82)*
Positive	Above mean (≥59.8)	44/82	10.38 (5.73-18.82)*	18/39	9.87 (3.70-26.39)*	26/43	10.71 (4.88-23.50)*
			<i>P</i> _{trend} < 0.0001				<i>P</i> _{trend} < 0.0001
Urinary AFB ₁ metabolites							
Negative	Below mean (<55.2)	22/370	1.0	7/150	1.0	15/220	1.0
Negative	Above mean (≥55.2)	33/266	2.84 (1.44-5.57)*	10/83	4.18 (1.24-14.15) [†]	23/183	2.34 (0.99-5.53)
Positive	Below mean (<55.2)	63/139	11.01 (5.79-20.92)*	27/85	7.09 (2.41-20.86)*	36/54	20.95 (8.52-51.55)*
Positive	Above mean (≥55.2)	80/129	15.13 (7.83-29.25)*	36/68	12.72 (4.29-37.75)*	44/61	19.80 (7.88-49.75)*
			<i>P</i> _{trend} < 0.0001				<i>P</i> _{trend} < 0.0001
Negative	Both below mean	15/240	1.0				
Negative	Either one above mean	28/304	2.01 (0.92-4.39)				
Negative	Both above mean	12/92	2.84 (1.06-7.62) [†]				
Positive	Both below mean	47/104	9.22 (4.39-19.34)*				
Positive	Either one above mean	73/126	12.87 (6.20-26.72)*				
Positive	Both above mean	23/38	17.29 (6.98-42.82)*				
			<i>P</i> _{trend} < 0.0001				

NOTE: ORs were adjusted for batch of aflatoxin biomarker assay, anti-HCV status, habitual smoking, alcohol drinking, and BMI.

**P* < 0.0001.†*P* < 0.05.

has been confirmed (15), as well as their significant correlation ($r = 0.69$) with AFB₁ intake (14). Studies in rats showed that AFB₁-albumin adducts reflect the formation of DNA damage occurring in the liver (16). In humans, AFB₁-albumin adducts are correlated with the prevalence of a specific codon 249 mutation in *p53* in liver tumors (17). With the larger sample size in the present study, we are able to observe a statistically significant increased HCC risk with an OR of 1.54 (95% CI, 1.01-2.36) among subjects with AFB₁-albumin adduct levels above the mean compared with those with AFB₁-albumin adduct levels below the mean, although the test of a dose-response effect did not reach statistical significance.

Urinary AFB₁ metabolites also provide a reliable assessment of dietary AFB₁ intake (18, 19), with a correlation of 0.65 found between total dietary AFB₁ intake and urinary AFM₁ excretion (19). Both nested case-control studies of HCC, including ours (11) and the one in China (13, 14), suggest that measurement of urinary AFB₁ metabolites is also a useful biomarker to evaluate HCC risk. Our present results suggest that both AFB₁ biomarkers are valuable biomarkers for predicting HCC risk even in cases diagnosed more than 13 years after blood and urine collection.

In our study, levels of AFB₁-albumin adducts and urinary AFB₁ metabolites show only a weak although statistically significant linear correlation. This is not unexpected because there is a complex balance of cytochrome P450-mediated, epoxide hydrolase-mediated, and glutathione S-transferase-mediated metabolism of AFB₁, which results in various metabolites, including the reactive epoxide that is responsible for albumin adduct formation (20).

Although the formation of AFB₁-guanine adducts through interaction between AFB₁-8,9-epoxide and hepatic DNA is critical for the carcinogenesis induced by AFB₁ (21), exposure to AFB₁ can also induce oxidative DNA damage in liver tissue (22, 23). We previously observed positive correlations between urinary AFB₁ metabolites and biomarkers of oxidative damage (8-oxodeoxyguanosine and 15-F_{2t}-isoprostane) in urine (24, 25), suggesting that levels of urinary AFB₁ metabolites might partly represent the amount of oxidative damage produced by exposure to AFB₁. An elevated level of urinary 15-F_{2t}-isoprostane was also associated with an increased risk of HCC (24).

Previously, the concept of a causative role for AFB₁ in human liver was not universally accepted because of the presence of endemic HBV in high-risk populations (26). In the present study, we found urinary AFB₁ metabolites independently and significantly associated with increased risk of HCC. In prior studies (3, 11, 13), the small number of cases without chronic HBV infection limited our ability to evaluate the effect of AFB₁ exposure on HCC risk among this subgroup. In the larger present study, we observed a statistically significant individual effect of AFB₁ exposure on HCC risk among noncarriers, with an OR of 4.29 for those with urinary AFB₁ metabolites above versus below the mean. This provides evidence that not only HBV carriers but also noncarriers are vulnerable to the carcinogenic effect of AFB₁. It is unclear why the effect of AFB₁ on risk of HCC was somewhat weaker among carriers of HBV than in

noncarriers. With respect to AFB₁-albumin, in cases with serious liver disease, such as liver cirrhosis and hepatitis virus infection, the turnover of albumin might be much more variable, affecting adduct formation. In addition, HCV infection might also affect liver function and our data analysis may not fully adjust for this. Unfortunately, we do not have sufficient HCV-infected cases to stratify by HCV status.

A limitation of the study is that biomarker samples were assayed in two batches 12 years apart. The distributions of two biomarkers are higher in the more recently assayed samples compared with prior data. Due to sample availability, we could not reanalyze the prior samples. In the data analysis, we adjusted for the batch of aflatoxin biomarker assay effects on the biomarker values. Because samples were run in case-control sets and conditional logistic regression models were used to analyze data, cases and their controls are still comparable. Another limitation is the smaller number of subjects with urine compared with plasma. However, in their distributions by gender, age, BMI, hepatitis virus infection, smoking status, and alcohol consumption, subjects with available data on both biomarkers were similar to the total study population.

In summary, we found a significant positive association between AFB₁ exposure, measured as AFB₁-albumin adducts and urinary AFB₁ metabolites, and risk of HCC. But we no longer observed a synergistic interaction between HBV infection and AFB₁ exposure on HCC risk. The effect of combined AFB₁ exposure and HBV infection is more consistent with an additive than a multiplicative model.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Appendix A.

Using the mean value of AFB₁-albumin adducts among controls and Fig. 1 from Gan et al. (14), we converted adduct levels to dietary intake of AFB₁, resulting in a value of ~30 µg/d. As shown in Table 2, this level of intake corresponds to an OR of 1.5. However, Gan et al. used a RIA with a different antibody than used in this article, which may lead to differences in quantitative values of adduct levels and, thus, errors in the estimation.

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Hui-Chen Wu, Qiao Wang, Hwai-I Yang, et al.

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