# Effects of Multiple Risk Factors for Hepatocellular Carcinoma on Formation of Aflatoxin $B_1$-DNA Adducts[1]

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## Abstract

**Covalent binding of aflatoxin $B_1$ ($AFB_1$) with hepatic DNA may be a critical step in hepatocarcinogenesis. The extent of the $AFB_1$ binding to DNA may depend on various endogenous factors and concurrent exposure to other environmental agents. This study was performed to determine whether any individual characteristics correlated with the formation of $AFB_1$-DNA adducts. The major $AFB_1$-DNA adduct, $AFB_1$-$N^7$-guanine, was measured using a high performance liquid chromatographic assay in urine samples from 43 asymptomatic hepatitis B virus surface antigen carriers and 43 noncarriers randomly selected from a cohort study in Taiwan. The total aflatoxin metabolite level was associated with the detection rate of urinary $AFB_1$-$N^7$-guanine adducts in a dose-dependent manner. The $AFB_1$-DNA adduct excreted in the urine was detectable in 60% of individuals who smoked cigarettes but abstained from alcohol, 64% of individuals who had a habit of drinking alcohol but not smoking cigarettes, and only 29% of those who neither smoked nor drank alcohol. The association between urinary $AFB_1$-DNA adduct level and habits of smoking cigarettes and drinking alcohol remained statistically significant when adjustment was made for potential confounders. There was a significant increase with age for the detection rate of urinary $AFB_1$-$N^7$-guanine adducts. Age and habits of cigarette smoking and alcohol drinking were also found to be associated with a higher percentage of $AFB_1$-$N^7$-guanine in total $AFB_1$ metabolite excretion, indicating an increased activation of $AFB_1$. No significant association with the $AFB_1$-DNA adduct level was observed for hepatitis B virus surface antigen carrier status, educational level, and ethnicity. These data suggest a potential role of age, cigarette smoking, and alcohol drinking in $AFB_1$-induced hepatocarcinogenesis.**

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## Introduction

Considerable evidence indicates that HCC[3] is multifactorial in origin. Our previous epidemiological studies have linked many risk factors to the development of HCC (1–8). One of the most striking epidemiological characteristics of HCC is its remarkable geographic variation. High-incidence areas cluster in the Far East and tropical Africa, where the annual incidence ranges up to 500 cases per 100,000 population (9). Although chronic HBV infection has been well documented as the most important risk factor for HCC in these areas, ingestion of $AFB_1$ has long been implicated as another major cause (10). This mycotoxin is the most potent hepatocarcinogen in a variety of animal species (11). Field studies in Africa and Southeast Asia have shown a strong correlation between high incidence of HCC and contamination of foodstuffs by AFs (12, 13).

$AFB_1$ is metabolized by the microsomal mixed-function oxygenase enzyme system to a variety of reduced and oxidized derivatives, including an unstable reactive epoxide, $AFB_1$-8,9-epoxide, which can form adducts with nucleophilic sites in DNA (14). The latter process has been shown to be critical for the carcinogenesis induced by $AFB_1$ in animals (15).

The major product formed by the interaction of $AFB_1$ with hepatic DNA *in vivo* is $AFB_1$-$N^7$-guanine (16). This compound is lost rapidly from DNA and excreted in the urine of $AFB_1$-treated animals. The quantity of the adducts excreted in the urine following $AFB_1$ administration was shown to be proportional to the amount of $AFB_1$-$N^7$-guanine initially formed in the liver DNA (17). The measurement of these adducts in human urine provides a noninvasive method of estimating DNA binding by $AFB_1$ at the target site and a useful surrogate dosimeter for HCC risk following $AFB_1$ exposure. The impact of chemoprotection on urinary levels of $AFB_1$-$N^7$-guanine adducts in rats has been demonstrated to be comparable to that on the hepatic DNA modification levels by $AFB_1$ (18). A case-control study nested in a cohort study indicated that the presence of urinary $AFB_1$-DNA adducts was the most important predictor of developing $AFB_1$-related HCC among urinary AF biomarkers examined (19).

The complete elimination of exposure to $AFB_1$ is not possible in high HCC risk areas. Recent experimental works have focused on the chemopreventive effects of a number of synthetic and natural compounds that may modulate the $AFB_1$ binding to DNA *in vitro* and *in vivo* (15, 18, 20, 21). On the other hand, there may be considerable interindividual variation in the DNA adduct levels formed by $AFB_1$ for a given dietary exposure to this mycotoxin. There is a strong interaction between chronic HBV infection and AF exposure on the devel-

[3] The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; AF, aflatoxin; $AFB_1$, aflatoxin $B_1$; $AFB_1$-$N^7$-guanine, 8,9-dihydro-8-($N^7$-guanyl)-9-hydroxyaflatoxin $B_1$; HBsAg, HBV surface antigen; anti-HCV, antibodies against hepatitis C virus; HPLC, high performance liquid chromatography; CI, confidence interval.

opment of HCC (19). One possible mechanism responsible for this interaction, among several that have been proposed, is that chronic HBV infection may alter AF metabolism, either the activation to the reactive epoxide or the detoxification. However, epidemiological data on the association between HBsAg carrier status and the serum level of the $AFB_1$-albumin adduct, another surrogate estimator of hepatic DNA damage induced by $AFB_1$, have been inconsistent (22–25). Other factors that may influence the binding of $AFB_1$ to hepatic DNA have yet to be identified. Cigarette smoking and alcohol drinking have also been linked to the development of HCC (2, 4, 8). They may interact with other HCC risk factors, such as $AFB_1$, in the pathogenesis of HCC. This study was carried out to evaluate the correlations of multiple HCC risk factors with the formation of $AFB_1$-DNA adducts, using urinary $AFB_1$-$N^7$-guanine as a biomarker for assessing $AFB_1$-DNA binding in the liver.

## Materials and Methods

**Subjects.** This study included a total of 43 male HBsAg carriers and 43 male age-matched (within 5 years) noncarriers who were randomly selected from the cohort members without HCC in a prospective study on the multifactorial etiology of HCC in Taiwan. The cohort characteristics and method of follow-up have been described in a previous study (8). At entry into this cohort study, all the study participants were tested for HBsAg by RIA (Abbott Laboratories, North Chicago, IL). Those who were positive for HBsAg were scheduled to undergo both ultrasonography and $\alpha$-fetoprotein measurement every 6–12 months. Individuals with ultrasonographic images compatible with liver cirrhosis or HCC and/or elevated $\alpha$-fetoprotein levels of more than 20 ng/ml were referred to hospitals for further confirmatory examinations, including fine-needle aspiration cytology and image diagnosis. All the chronic HBsAg carriers included in this study remained asymptomatic throughout a 5-year follow-up period, except two who were subsequently affected with liver cirrhosis. This study was conducted with the approval of the Department of Health and in compliance with regulations for the protection of human research subjects. Urine samples were collected in disposable containers from study subjects at the initial recruitment examination during a period from August 1988 through June 1992. The urine samples were stored at −30°C until they were used for analysis of AF metabolites. At the time of urine collection, each study subject was also personally interviewed to obtain information on demographic characteristics, habits of cigarette smoking and alcohol drinking, and the frequency (meals per month) of consuming peanuts and fermented bean products, which were thought to be major AF-contaminated foodstuffs in Taiwan within the 6 months before questionnaire interview, as well as personal and family history of various chronic diseases. A habit of cigarette smoking was defined as having smoked cigarettes more than 4 days a week for at least 1 year. Alcohol drinking was defined as having consumed alcohol more than 1 day a week for at least 1 year. All but seven study subjects were also tested for anti-HCV. Anti-HCV was examined by a second-generation enzyme immunoassay (Abbott Laboratories, North Chicago, IL).

**Analysis of Urinary AF Metabolites.** Authentic $AFB_1$-$N^7$-guanine was synthesized according to the method of Martin and Garner (26). Standards of $AFB_1$, $AFM_1$, and $AFP_1$ (Sigma Chemical Co., St. Louis, MO) were prepared in bezene:acetonitrile (98:2, v/v), and $AFB_1$-$N^7$-guanine was prepared in methanol.

For extraction of $AFB_1$ metabolites in urine, an aliquot of 5 ml urine sample was incubated with 5 ml of 0.1 M $Na_2SO_4$ and 5 ml of 0.1 M acetic acid containing 710 units $\beta$-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 was 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 and 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 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 HPLC analysis.

$AFB_1$ metabolites excreted in the urine were analyzed by reversed-phase HPLC. HPLC was performed with a Waters system incoporating two M510 pumps, an M680 system controller, an M717 autoinjector, and an M470 scanning fluorescence detector. Quantitation of various $AFB_1$ metabolites was accomplished with a Millennium 2010 chromatography manager (Waters Associates, Milford, MA). The HPLC column used was a $C_{18}$ 10-$\mu$m (300 × 3.9 mm) $\mu$Bondpak column (Waters). Gradient elution was used to improve the resolution of very similar structures of $AFB_1$ 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, 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 a 430-nm emission wavelength for $AFM_1$ and with 500 nm emission for $AFB_1$-$N^7$-guanine and $AFB_1$. $AFM_1$, $AFB_1$-$N^7$-guanine, and $AFB_1$ eluted at 8.9, 21.5, and 36.4 min, respectively. The HPLC analysis for $AFP_1$ was done with a 30-min elution with 12% acetonitrile:water, followed by a 12–22% acetonitrile 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. $AFP_1$ chromatographed with a retention time of 43.1 min by monitoring the fluorescence emission at 500 nm with the excitation wavelength at 365 nm. All aqueous mobile phases before use were adjusted by orthophosphoric acid and triethylammonium formate buffer to pH 3.0.

**Statistical Methods.** The relationship between monthly consumption frequency of peanuts and fermented bean products and the excretion of total AF metabolites (including $AFM_1$, $AFP_1$, $AFB_1$-$N^7$-guanine, and $AFB_1$) in urine was examined by using Pearson's correlation coefficient. Odds ratios and their 95% CIs were computed to examine the associations of the positivity of urinary $AFB_1$-$N^7$-guanine with various variables. Urinary levels of total AF metabolites were trichotomized according to the tertile distribution of the levels of all study subjects. Mantel's $\chi^2$ test for a trend was performed to examine the dose-response relationship for the odds ratios of the positivity of $AFB_1$-$N^7$-guanine in relation to age and the total AF metabolites excreted. Multivariate-adjusted odds ratios were estimated by modeling the data through logistic regression. In the analysis, consecutive scores of 1, 2, 3, and 4 were assigned to the age groups of 30–39, 40–49, 50–59, and ≥60 years. All

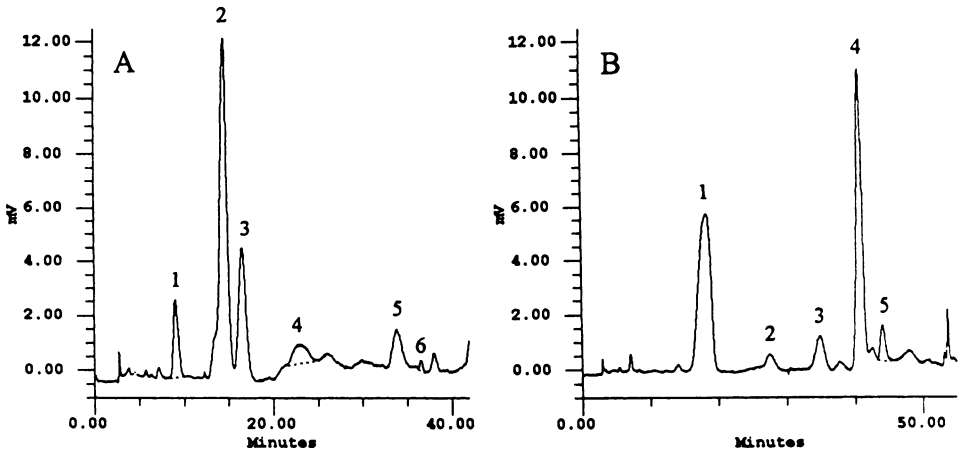


*Fig. 1.* Representative HPLC profile of the $AFB_1$ metabolites detected from a human urine sample. Details of the extraction of $AFB_1$ metabolites in urine and HPLC analysis are described in "Materials and Methods." The minimum detectable concentration of AFs in the urine samples analyzed was 0.05 ng/ml. *A*, elution profile of $AFM_1$, $AFB_1$-$N^7$-guanine, and $AFB_1$ was obtained by elution for 12 min with 15% acetonitrile and then at 22% acetonitrile. Peak identification: *1*, $AFM_1$; *2* and *3*, unknown; *4*, $AFB_1$-$N^7$-guanine; *5*, unknown; *6*, $AFB_1$. *B*, elution profile of $AFP_1$ was obtained by elution for 30 min with 12% acetonitrile followed by a 12–22% acetonitrile:water linear gradient generated over 12 min, then elution at 22% acetonitrile. Peak identification: *1–4*, unknown; *5*, $AFP_1$.

*P* values for tests of statistical significance were based on two-tailed probability.

## Results

The ages of study subjects ranged from 33 to 66 years. Fifty-one percent of them had ages between 40 and 59 years. Among the study subjects, 48.8% were Fukien Taiwanese, 37.2% were mainland Chinese who or whose parents migrated to Taiwan after the World War II, and the remaining 14.0% were Hakka Taiwanese. Most study subjects (88.4%) had an educational level of senior high school or above. There were only two HBsAg carriers and two HBsAg noncarriers who were positive for anti-HCV.

We measured urinary levels of $AFB_1$, two of its main metabolites ($AFM_1$ and $AFP_1$), and the $AFB_1$-$N^7$-guanine adducts. Representative chromatograms of the $AFB_1$ metabolites detected from a human urine sample are shown in Fig. 1. $AFM_1$ was observed as the most abundant $AFB_1$ metabolite excreted. All study subjects were positive for $AFM_1$. A total of 34 (39.5%) of the urine samples contained a detectable level of $AFB_1$-$N^7$-guanine adducts. The range of $AFB_1$-$N^7$-guanine adducts was 0.10–6.06 ng/ml in the positive samples. $AFP_1$ was detectable in 81.4% of the study subjects, and $AFB_1$ was detectable in only 20.9%. Most study subjects (93.0%) had urinary $AFB_1$-$N^7$-guanine adduct levels of less than 10% of the amount of total AF metabolites excreted in the urine.

There was a significant correlation between the excretion of total AF metabolites in urine and the monthly consumption frequency of peanut and fermented bean products ($r = 0.39$; $P = 0.0002$). Because we cannot estimate the level of exposure to $AFB_1$ from all possible sources precisely by using a questionnaire, the urinary level of total AF metabolites was used as a surrogate dosimeter for $AFB_1$ exposure in this study.

Table 1 shows a significant dose-response relationship between the urinary level of total AF metabolites and the detection rate of $AFB_1$-$N^7$-guanine adducts in urine (trend test, $P = 0.005$). The odds ratios associated with the positivity of urinary $AFB_1$-$N^7$-guanine adducts from the first tertile (<3.4 ng/ml) to the third tertile (≥6.9 ng/ml) of the urinary total AF metabolite level were 1.0, 4.4 (95% CI, 1.3–15.4), and 5.4 (95% CI, 1.7–17.8), respectively.

*Table 1* Association between total AF metabolite excretion in urine and the detection rate of urinary $AFB_1$-$N^7$-guanine adducts

| Total urinary AF metabolites (ng/ml) | Total *n* | Detection rate of $AFB_1$-$N^7$-guanine adducts (%) | Odds ratio (95% CI) |
|---|---|---|---|
| <3.4[a] | 29 | 17.2 | 1.0[b] |
| 3.4–6.8 | 25 | 48.0 | 4.4 (1.3–15.4) |
| ≥6.9 | 32 | 53.1 | 5.4 (1.7–17.8) |

[a] The urinary level of total AF metabolites was trichotomized according to the tertile distribution of the levels of all study subjects.
[b] Test for trend, $P = 0.005$.

Table 2 shows the univariate analysis of the relationship of the positivity of urinary $AFB_1$-$N^7$-guanine adducts with sociodemographic characteristics, HBsAg carrier status, and habits of alcohol drinking and cigarette smoking. The odds ratio associated with the positivity of urinary $AFB_1$-$N^7$-guanine increased monotonically with increasing age (trend test, $P = 0.026$). Hakka Taiwanese had a higher detection rate of the $AFB_1$-DNA adducts than other ethnic groups. However, the ethnic difference in urinary $AFB_1$-$N^7$-guanine positivity was not statistically significant. The detection rate of urinary $AFB_1$-$N^7$-guanine adducts for individuals with an educational level of senior high school or above was similar to that for those who had an educational level of junior high school or below.

$AFB_1$-$N^7$-guanine was detectable in 34.9% (15 of 43) of HBsAg noncarriers and 44.2% (19 of 43) of HBsAg carriers. The difference in the detection rate was not significant between the two groups. The detection rate of $AFB_1$-$N^7$-guanine in urine was much lower among individuals who neither smoked nor drank alcohol (28.6%) than those who smoked but abstained from alcohol (60.0%) and the habitual alcohol drinkers who did not smoke (63.6%). This detection rate was slightly higher in the study subjects who smoked cigarettes and consumed alcohol (36.4%) than in those who neither smoked nor drank alcohol. Compared with those who neither smoked cigarettes nor

*Table 2* Associations of the detection rate of urinary $AFB_1$-$N^7$-guanine adducts with selected sociodemographic variables, HBsAg carrier status, and habits of cigarette smoking and alcohol drinking

| Variable | Total *n* | Detection rate of $AFB_1$-$N^7$-guanine adducts (%) | Odds ratio (95% CI) |
|---|---|---|---|
| Age (yr) | | | |
| 30–39 | 15 | 20.0 | 1.0[a] |
| 40–49 | 25 | 32.0 | 1.9 (0.4–8.6) |
| 50–59 | 19 | 47.4 | 3.6 (0.8–17.0) |
| ≥60 | 27 | 51.9 | 4.3 (1.0–18.8) |
| Ethnic group | | | |
| Fukien Taiwanese | 42 | 40.5 | 1.0 |
| Mainland Chinese | 32 | 31.3 | 0.7 (0.3–1.8) |
| Hakka Taiwanese | 12 | 58.3 | 2.1 (0.6–7.6) |
| Educational level | | | |
| Senior high school and above | 76 | 39.5 | 1.0 |
| Junior high school and below | 10 | 40.0 | 1.0 (0.3–3.9) |
| HBsAg carrier status | | | |
| Negative | 43 | 34.9 | 1.0 |
| Positive | 43 | 44.2 | 1.5 (0.6–3.5) |
| Habitual alcohol drinking/cigarette smoking | | | |
| No/no | 49 | 28.6 | 1.0 |
| No/yes | 15 | 60.0 | 3.8 (1.1–12.5) |
| Yes/no | 11 | 63.6 | 4.4 (1.1–17.3) |
| Yes/yes | 11 | 36.4 | 1.4 (0.4–5.7) |

[a] Test for trend, $P = 0.026$.

drank alcohol, the odds ratios associated with the positivity of urinary $AFB_1$-$N^7$-guanine adducts were 3.8 (95% CI, 1.1–12.5) for individuals who smoked but abstained from alcohol, 4.4 (95% CI, 1.1–17.3) for habitual alcohol drinkers who did not smoke, and 1.4 (95% CI, 0.4–5.7) for individuals who smoked cigarettes and drank alcohol.

Results of the logistic regression analysis of multiple variables associated with urinary level of $AFB_1$-$N^7$-guanine are shown in Table 3. The detection rates of urinary $AFB_1$-$N^7$-guanine among habitual alcohol drinkers who did not smoke and cigarette smokers who abstained from alcohol were almost identical. To get a greater statistical power, the two groups were combined in the multivariate analysis. Urinary levels of total AF metabolites still significantly associated with the positivity of $AFB_1$-$N^7$-guanine in a dose-response manner. The odds ratio of the positivity of urinary $AFB_1$-$N^7$-guanine was 1.6 (95% CI, 1.0–2.7) for an increase in 10 years of age. Compared with those who neither smoked cigarettes nor drank alcohol, the multivariate-adjusted odds ratio for individuals who smoked cigarettes but abstained from alcohol and habitual alcohol drinkers who did not smoke was 3.5 (95% CI, 1.1–11.0). The odds ratio was 1.6 (95% CI, 0.4–7.5) for those who smoked cigarettes and drank alcohol. No significant association with the $AFB_1$-$N^7$-guanine adducts was observed for chronic HBsAg carrier status, ethnicity, and level of education. The odds ratios in relation to age and habits of cigarette smoking and alcohol drinking did not materially change after the removal of the three nonsignificant variables from the logistic regression analysis.

We further examined the dose-response association between the detection rate of urinary $AFB_1$-$N^7$-guanine adducts and cumulative exposure to cigarette smoke and alcohol. Cumulative cigarette smoking in pack-years was calculated by multiplying the number of packs of cigarettes smoked per day by the duration in years of cigarette smoking. Consecutive scores of 0, 1, 2, 3, and 4 were assigned to the cumulative

*Table 3* Logistic regression analysis of multiple factors on the positivity of urinary $AFB_1$-$N^7$-guanine adducts

| Variable | Adjusted odds ratio (95% CI) | *P* |
|---|---|---|
| Total urinary AFs (ng/ml) | | |
| <3.4 | 1.0 | |
| 3.4–6.8 | 3.9 (1.0–15.7) | 0.0554 |
| ≥6.9 | 5.2 (1.4–20.0) | 0.0155 |
| Age[a] | 1.6 (1.0–2.7) | 0.0426 |
| HBsAg carrier status | | |
| Negative | 1.0 | |
| Positive | 1.2 (0.4–3.7) | 0.6970 |
| Habitual alcohol drinking/cigarette smoking | | |
| No/no | 1.0 | |
| No/yes or yes/no | 3.5 (1.1–11.0) | 0.0317 |
| Yes/yes | 1.6 (0.4–7.5) | 0.5234 |
| Educational level | | |
| Senior high school and above | 1.0 | |
| Junior high school and below | 1.6 (0.3–9.1) | 0.6002 |
| Ethnic group | | |
| Hakka Taiwanese | 3.0 (0.7–13.1) | 0.1510 |
| Other | 1.0 | |

[a] Consecutive scores of 1, 2, 3, and 4 were assigned to the age groups of 30–39, 40–49, 50–59, and ≥60 years, respectively.

cigarette smoking of 0, >0 and ≤5.2, 5.3–16.3, 16.4–24.0, and >24.0 pack-years. Because there was no synergistic interaction between cigarette smoking and alcohol drinking on the formation of $AFB_1$-DNA adducts, the association of cumulative cigarette smoking with the urinary $AFB_1$-DNA adducts was analyzed separately for nondrinkers and drinkers. Among nondrinkers, there was a positive dose-response relationship between pack-years of cigarette smoking and the detection rate of urinary $AFB_1$-$N^7$-guanine after adjustment for age (trend test, $P = 0.048$). However, this association was not statistically significant among drinkers. The measure of cumulative alcohol intake was defined as the cross-product of the quantity of ethanol the subject usually consumed each week and the duration in years of alcohol drinking. Consecutive scores of 0, 1, 2, 3, and 4 were assigned to the cumulative alcohol drinking of 0, >0 and ≤1321.9, 1322.0–5229.9, 5223.0–8058.6, and >8058.6 gram-years. Among those who never smoked, the age-adjusted odds ratio associated with the positivity of the $AFB_1$-DNA adducts in urine increased with increasing cumulative intake of alcohol (trend test, $P = 0.075$). Among smokers, no notable association between cumulative alcohol drinking and the $AFB_1$-$N^7$-guanine adduct levels was observed.

$AFB_1$ is metabolized through various biotransformation pathways to the highly reactive 8,9-epoxide metabolites and a variety of derivatives that have lower carcinogenicity (including $AFM_1$ and $AFP_1$). Whether an association exists between the increased metabolic activation of $AFB_1$ to the DNA adducts and age and habits of cigarette smoking and alcohol drinking was further analyzed in Table 4. The proportion of study subjects who had a high percentage of the amount of $AFB_1$-$N^7$-guanine adducts in total urinary $AFB_1$ metabolites, defined as a percentage greater than the median of study subjects with detectable concentrations of $AFB_1$-$N^7$-guanine adducts in urine, increased with age. This proportion was higher among individuals who smoked but abstained from alcohol and the habitual alcohol drinkers who never smoked when compared with those who neither smoked nor drank. However, the proportion was slightly lower in subjects who smoked and drank than in those who neither smoked nor drank alcohol.

*Table 4* Frequency distribution of the percentage of the amount of $AFB_1$-$N^7$-guanine adducts in total AF metabolites excreted in urine, by age, cigarette smoking, and habitual alcohol drinking

| Variable | Group | $AFB_1$-$N^7$-guanine in total urinary AF metabolites (%) | | | Adjusted-Odds ratio[b] |
|---|---|---|---|---|---|
| | | None | Low | High[a] | |
| Age (yr) | 30–39 | 80.0 | 13.3 | 6.7 | 1.0[c,d] |
| | 40–49 | 68.0 | 20.0 | 12.0 | 3.0 |
| | 50–59 | 52.6 | 21.1 | 26.3 | 6.6 |
| | ≥60 | 48.2 | 22.2 | 29.6 | 7.5 |
| Habitual alcohol drinking/ cigarette smoking | No/no | 71.4 | 14.3 | 14.3 | 1.0[e] |
| | No/yes | 40.0 | 33.3 | 26.7 | 2.7 |
| | Yes/no | 36.4 | 18.2 | 45.4 | 6.6[f] |
| | Yes/yes | 63.6 | 27.3 | 9.1 | 0.9 |

[a] High percentage was defined as a percentage greater than the median (4.3%) of the study subjects with detectable concentration of $AFB_1$-$N^7$-guanine adducts in urine.

[b] Adjusted-odds ratios associated with a high percentage of $AFB_1$-$N^7$-guanine in total AF metabolites excretion were derived from a logistic regression model including age and habits of alcohol drinking and cigarette smoking as covariates.

[c] Individuals aged 30–39 years who had undetectable concentration of urinary $AFB_1$-$N^7$-guanine adducts were used as reference group.

[d] Test for trend, $P = 0.053$.

[e] Individuals who neither smoked nor drank alcohol and had undetectable concentrations of urinary $AFB_1$-$N^7$-guanine adducts were used as reference group.

[f] $P = 0.03$.

## Discussion

The carcinogenic potency of $AFB_1$ has been correlated with the extent of its covalent binding to hepatic DNA in various animal models (15, 20). The formation of $AFB_1$-DNA adducts can lead to mutations of proto-oncogenes and tumor suppressor genes (27, 28). Many studies have thus focused on the DNA adduct formed by $AFB_1$ because of its role in the initiation process of HCC development. Our previous studies using an indirect immunofluorescence assay demonstrated that 50–70% of the liver tissues from HCC patients in Taiwan had detectable levels of $AFB_1$-DNA adducts, suggesting a possible role of the adduct in hepatocarcinogenesis (5, 6).

For a given level of exposure to $AFB_1$, the extent of the formation of the $AFB_1$-DNA adduct may vary with age, genetic variation in metabolic capacity for $AFB_1$ activation and/or detoxification, nutritional status, and concurrent exposure to other environmental agents (*e.g.*, HBV, cigarette smoking, and alcohol drinking). We have shown the effects of various vitamins on the formation of $AFB_1$-DNA adducts in cultured woodchuck hepatocytes (21). This study attempted to determine whether any individual characteristics correlated with this adduct formation. The presence of $AFB_1$-DNA adducts in liver specimens is readily detected by immunohistochemical methods (5, 6). This method can be applied to quantitate the extent of binding of $AFB_1$ to DNA in target cells. However, liver tissues are usually obtained from patients with HCC. The patients may change their dietary exposure to AFs and habits of cigarette smoking and alcohol drinking after the diagnosis of HCC. Their capacities for metabolic activation and/or detoxification of $AFB_1$ may also have been altered due to the pathological change of the liver and thus may affect the formation of $AFB_1$-DNA adducts. This study was thus performed in persons without HCC using $AFB_1$-$N^7$-guanine excreted in the urine as a biomarker for the ultimate carcinogenic form of $AFB_1$, which has reacted with hepatic DNA.

The interaction between AF exposure and chronic HBV infection has long been suspected to play an important role in the development of HCC in high-risk areas. A strong interaction between the two factors in HCC induction has been reported from a recent nested case-control study carried out within a large-scale cohort study (19). In animal models, the synergistic interaction between $AFB_1$ and HBV in hepatocarcinogenesis has been reported in woodchucks (29) and in transgenic mice carrying the human *HBsAg* gene (30). However, the biological mechanism that underlies the interaction requires elucidation.

The biotransformation of environmental carcinogens occurs mainly in the endoplasmic reticulum, which in virus-infected hepatocytes was observed to be hyperplasic (31). Because metabolic mechanisms, either activation or deactivation, are well known to play a crucial role in chemical carcinogenesis, there were numerous studies to examine whether alterations in the metabolism of $AFB_1$ to the DNA adducts resulting from chronic HBV infection may contribute to explaining the interaction between HBV and $AFB_1$ exposure (32, 33). Increased metabolic activation of chemical hepatocarcinogens, including $AFB_1$, has been observed in woodchucks naturally infected with woodchuck hepatitis virus (32). An analysis of liver preparations from a limited number of human liver biopsies also reported enhanced activation of the tryptophan pyrolysate Try-P-2, a hepatocarcinogen, in HBsAg carriers, irrespective of histological diagnosis. However, a significant stimulation of the activation of $AFB_1$ was observed only in cases with mild chronic active hepatitis (33).

The possible association between chronic HBV infection and the increased activation of $AFB_1$ has also been examined in epidemiological studies, using $AFB_1$-albumin adducts or the $AFB_1$-$N^7$-guanine adducts excreted in the urine as the surrogate dosimeter for estimating hepatic DNA binding by $AFB_1$. Two studies on children in Gambia reported higher $AFB_1$-albumin adduct levels in HBsAg carriers than in noncarriers (22, 23). However, the dietary intake of $AFB_1$ can be extremely variable between HBsAg carriers and noncarriers. No food intake data were available for the subjects in these studies. In another study in Gambia, a detailed analysis of dietary intake of AF over a 7-day period was carried out on a total of 20 individuals aged 15–56 years. After adjustment for dietary $AFB_1$ exposure, no significant difference in both the level of $AFB_1$-albumin adducts and the urinary $AFB_1$-$N^7$-guanine was found between the HBsAg carriers and noncarriers (24, 25). In the present study, the consumption frequency of peanut and fermented bean products correlated well with the urinary level of total AF metabolites. Measurement of the excretion of total AF metabolites may provide a means to estimate the level of $AFB_1$ ingestion. We also failed to find a significant association between the detection rate of $AFB_1$-$N^7$-guanine adducts in urine and chronic HBsAg carrier status when taking into account the total AF metabolite excretion and other variables. Changes in the expression of various forms of cytochrome P450 during the development of hepatitis have been shown in a recent animal study (34). In this study, only two of the HBsAg carriers were found to be affected with liver cirrhosis. Most HBsAg carriers remained asymptomatic through the follow-up period. Whether chronic HBV infection may be indirectly related to the metabolism of $AFB_1$ through chronic hepatitis merits further studies.

Many recent studies have implicated that cigarette smoking is a major nonviral risk factor for HCC (2, 4, 8). However, the role of cigarette smoking in hepatocarcinogenesis is not well understood. We have found that cigarette smoking is related to the increased expression of the *neu* oncogene in the development of HCC (4). It was also observed that the low

serum level of retinol was correlated with cigarette smoking. This has been implicated as an alternative mechanism responsible for the action of cigarette smoking in the pathogenesis of HCC (7). In this study, cigarette smoking was observed to be positively associated with the urinary $AFB_1$-$N^7$-guanine adducts. This finding suggests that cigarette smoking may interact with $AFB_1$ in the initiation process of carcinogenesis and thus may result in an increased risk of $AFB_1$-related HCC.

Our results also suggest that habitual alcohol drinking may augment the $AFB_1$ binding to DNA. Habitual alcohol drinking has long been postulated as a risk factor for HCC because of its hepatotoxic effects and its relationship to liver cirrhosis (35). However, it may also increase HCC risk without the presence of liver cirrhosis through a variety of mechanisms (36). The average quantity of alcohol consumed by Chinese in Taiwan is not large. A moderate excess risk of HCC associated with alcohol drinking has been consistently observed in Taiwan (2). Researchers have suggested that alcohol consumption may cause carcinogenesis by inducing microsomal enzymes involved in the metabolism of carcinogens. Alcohol drinking may also cause an increased risk of cancer through exacerbation of nutritional deficiency, which may modulate the metabolism of carcinogens (36). Many nutritional elements have been demonstrated to act as blocking agents for the interaction of the activated (electrophilic) forms of carcinogens with cellular DNA in chemically induced carcinogenesis (37).

The detection rate of urinary $AFB_1$-$N^7$-guanine was slightly higher in individuals who smoked cigarettes and drank alcohol than in those who neither smoked nor drank. However, this detection rate was much lower than the corresponding figures in cigarette smokers who abstained from alcohol and habitual alcohol drinkers who did not smoke. The lack of the synergism between cigarette smoking and alcohol drinking seems to be perplexing. In addition to alcohol drinking, cigarette smoking has also been linked to the development of liver cirrhosis (38). Whether cigarette smoking may augment the toxic effects of alcohol on hepatocytes among habitual alcohol drinkers and thus may result in an impaired $AFB_1$ metabolism remains to be studied.

AFs are among the few widely disseminated environmental carcinogens. The complete elimination of exposure to $AFB_1$ is too expensive and not feasible in areas such as Taiwan, where the climate is warm and humid, which is good for fungal growth. Although the underlying mechanism for the effects of cigarette smoking and alcohol drinking on the formation of $AFB_1$-DNA adducts remains to be elucidated, the results of this study suggest that intervention against cigarette smoking and alcohol drinking may be important for lowering the $AFB_1$-DNA formation in people living in areas with heavy AF contamination of food.

The HCC incidence increases with age (9). This phenomenon may be explained by the accumulation of exposure to hepatocarcinogens with increasing age and/or the effect of aging itself. Our finding of a significant linear increase with aging for the detection rate of urinary $AFB_1$-DNA adducts suggests that age may modify the hepatocarcinogenesis induced by $AFB_1$. Species susceptibility to $AFB_1$ carcinogenesis has been linked with genetically determined differences in the biotransformation of $AFB_1$ (39). The carcinogenic metabolite of $AFB_1$ generated from cytochrome P450-dependent epoxidation may be subject to metabolic conjugation and other kinds of detoxification. Glutathione *S*-transferase M1 and microsomal epoxide hydrolase are both involved in $AFB_1$ detoxification (40). A significant association has been reported between glutathione *S*-transferase M1 and epoxide hydrolase genotypes and the presence of $AFB_1$-albumin adducts (41). In this study, the detection rate of urinary $AFB_1$-$N^7$-guanine was observed to be higher in Hakka Taiwanese than in other ethnic groups, but the difference was not statistically significant. Whether the ethnic difference in the formation of $AFB_1$-DNA adducts might be due to different exposures to other dietary factors and/or different genetic compositions associated with $AFB_1$ metabolism remain to be determined.

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