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AFLATOXIN EXPOSURE AND RISK OF HEPATOCELLULAR CARCINOMA IN TAIWAN

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To investigate the carcinogenic effect of environmental aflatoxin exposure, 56 cases of hepatocellular carcinoma (HCC) diagnosed between 1991 and 1995 were identified and individually matched by age, sex, residence and date of recruitment to 220 healthy controls from the same large cohort in Taiwan. Blood samples were analyzed for hepatitis B and C viral markers and for aflatoxin-albumin adducts; urine was tested for aflatoxin metabolites. We obtained information about socio-demographic characteristics, habitual alcohol drinking, cigarette smoking and diet in a structured interview. Hepatitis B virus surface antigen (HBsAg) carriers had a significantly increased risk for HCC. After adjustment for HBsAg serostatus, the matched odds ratio (OR_m) was significantly elevated for subjects with high levels of urinary aflatoxin metabolites. When stratified into tertiles, a dose-response relationship with HCC was observed. The OR_m for detectable aflatoxin-albumin adducts was not significant after adjustment for HBsAg serostatus. HBsAg-seropositive subjects with high aflatoxin exposure had a higher risk than subjects with high aflatoxin exposure only or HBsAg seropositivity only. In male HBsAg-seropositive subjects, adjusted ORs were 2.8 (95% confidence interval [CI] = 0.9–9.1) for detectable compared with non-detectable aflatoxin-albumin adducts and 5.5 (CI = 1.3–23.4) for high compared with low urinary aflatoxin metabolite levels. Our results suggest that environmental aflatoxin exposure may enhance the hepatic carcinogenic potential of hepatitis B virus. A large-scale study will be needed to evaluate the effect of aflatoxin exposure on HBsAg non-carriers.

In Taiwan, hepatocellular carcinoma (HCC) is the leading cause of cancer deaths for males and the third highest for females (Department of Health, 1994). Clinico-epidemiological evidence suggests that hepatitis B virus (HBV) infection is the most important cause of HCC in the world (Feitelson, 1992; Robinson, 1994; Yu and Chen, 1994). In several countries, however, including Japan and Italy, about 50% of HCC cases are related to hepatitis C virus (HCV) (Colombo *et al.,* 1991; Saito *et al.,* 1990). With the ability to detect HCV infection by immunoassay or PCR techniques, HCV infection has been closely linked to the development of HCC, especially in hepatitis B virus surface antigen (HBsAg)–seronegative patients (Di Bisceglie, 1995; Yu and Chen, 1994).

In Taiwan, 70–90% of HCC patients are anti-HCV or HBsAg-seropositive, suggesting that current HBV or HCV infection is a major cause of HCC (Yu and Chen, 1994). Nonetheless, in 10–30% of HCC patients no defined cause is found. Moreover, at the township level, there is a 6-fold variation in average HCC mortality but only a 2-fold variation in HBsAg-seropositive rate. It seems therefore likely that risk factors other than viral infection also contribute to the development of HCC in Taiwan.

Aflatoxin B_1, a well-established animal hepatocarcinogen, is a suspected HCC risk factor in hyperendemic areas like sub-Saharan Africa, Southeast Asia and southern China, where dietary staples are highly contaminated (IARC, 1992). Studies carried out in Taiwan have also suggested that aflatoxin exposure may be associated with HCC. In 2 case-control studies, subjects who consumed more peanut products, frequently contaminated by aflatoxins, had an elevated risk of HCC (Chen *et al.,* 1991; Lu *et al.,* 1988). Other studies using biological markers of exposure have provided additional information directly implicating aflatoxin as a risk factor. A cross-sectional study in Taiwan using urinary aflatoxin metabolites as an exposure marker showed a significant correlation between an individual's aflatoxin level and age-adjusted HCC mortality for the township of residence (Hatch *et al.,* 1993). Immunohistochemical staining of HCC needle biopsy–smeared tissue samples also showed a high percentage (35/50, 70%) with detectable aflatoxin-DNA adducts (Chen *et al.,* 1992). A recent follow-up study in mainland China demonstrated that those with detectable urinary aflatoxin metabolites or guanine adducts had an elevated risk of HCC. Perhaps more significantly, there was a strong interaction, on the order of 60-fold, between chronic HBV infection and urinary aflatoxin exposure in determining liver cancer risk (Qian *et al.,* 1994; Ross *et al.,* 1992).

Measurement of protein adducts is an alternative to urinary excretion of aflatoxin as an exposure biomarker and, because of the 21-day half-life of albumin, should provide information on exposure over a longer period of time. We developed an ELISA for quantitation of aflatoxin-albumin adducts (Chen *et al.,* 1996*b*) and have applied it here in a follow-up study of cases and controls nested in a cancer-screening cohort to investigate the effect of environmental aflatoxin exposure. These studies were carried out in Taiwan, where HBV is hyperendemic and aflatoxin contamination is common.

MATERIAL AND METHODS

From July 1990 to June 1992, a community-based cancer-screening project was carried out in 7 townships. Ma-Kung, Hu-Hsi and Pai-Hsa are located on the Penghu Islets, the highest HCC incidence area in Taiwan. The other 4 townships are located on Taiwan Island. Initially, research assistants abstracted name, national identification number, sex, birthday, education and residence for those born between 1927 and 1961 from the records of local housing offices. There were 47,079 male and 42,263 female eligible individuals who were invited by letter to participate. A total of 12,024 males and 13,594 females enrolled. Duration of residence, time of screening, transportation and distance between residence and research center were all associated with response. Non-smokers, the elderly or those with more education had higher rates of response (Chen *et al.,* 1996, *a*).

All respondents were interviewed at local research centers by carefully trained personnel using a structured questionnaire to obtain information about socio-demographic characteris-

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tics; habits of alcohol drinking and cigarette smoking; health history; familial history of major diseases and consumption of pickled, salted, fermented or peanut products during early childhood. Habitual cigarette smoking was defined as having smoked more than 4 days a week for at least 6 months. Information about duration and intensity was obtained. Habitual alcohol drinking was defined as drinking alcohol-containing products more than 4 days a week for at least 6 months. The same day, 20 ml of fasting blood were collected into 1 heparinized vacutainer and 1 without anti-coagulant. Samples were centrifuged and blood components separated and stored at −70°C. Spot urine samples were also collected. Biospecimens were shipped to a central laboratory at National Taiwan University on dry ice and stored at −70°C or −30°C.

Serum samples were assayed for HBsAg and α-fetoprotein (AFP) by enzyme immunoassay (EIA) with commercial kits (Abbott, North Chicago, IL), anti-HCV by EIA with second-generation commercial kits (HCV, Abbott) and, for liver function markers, alanine transaminase (ALT) and aspartate transaminase (AST), with an auto-analyzer (Hitachi Model 736, Japan) and commercial kits (BioMerieux, Mercy l'Etoile, France). Anti-HCV and AFP were assayed in all males and females who resided in Hu-Hsi and Pai-Hsa on the Penghu Islets; the other assays were carried out on samples from all participants. Abdominal ultrasonography was administered by gastroenterologists (G.-T.H., P.-M.Y., H.-S.L. and S.-N.L.) using 2 apparatuses with real-time linear and sector probes (Toshiba SAL-38B and SSA-240A, Tokyo, Japan). Those eligible for scans were 3,711 males and 622 females who resided in Hu-Hsi or Pai-Hsa, who were either HBsAg carriers, anti-HCV-positive, ALT ≥ 45 IU/l, AST ≥ 40 IU/l or AFP ≥ 20 ng/ml or who had familial liver cirrhosis or a history of liver cancer in first-degree relatives. Participation rates for ultrasonographic scanning were 91.7% and 90.5% for males and females, respectively. Subjects with ultrasonographic images compatible with HCC were referred to teaching medical centers for confirmatory diagnosis by computerized tomography, digital subtracted angiogram, aspiration cytology and pathological examination. Intensive follow-up, including abdominal ultrasonographic scanning and AFP determination every 6–12 months, was carried out on those with ultrasonographic images compatible with hemangioma, pseudotumor, severe liver cirrhosis or AFP ≥ 20 ng/ml.

All participants were recontacted by letter between July 1992 and June 1994 and requested to visit local research centers, at which time a structured questionnaire soliciting information on occupation, change in smoking and drinking habits and health status was administered. Blood and urine samples were again obtained, separated, frozen and stored as for the screening samples. Telephone interviews were carried out to obtain information about health status and hospitalization for those who were unable to attend. Copies of death certificates in the study areas were obtained periodically from the local housing offices. In June 1995, intensive follow-up was accomplished through linkage with the national death certification and cancer registration data bases, using national identification number, sex and birth date. Overall follow-up rate was above 98%.

Between February 1991 and June 1995, 50 HCC cases were identified by expert review of medical records; 48 had complete data on ultrasonographic scanning. At the first scanning, 23 cases showed images compatible with HCC. Another 6 deaths with ICD9 code 155 were regarded as HCC cases. According to interview data, none of the HCC cases had been previously diagnosed with liver cancer but 3 had a history of cirrhosis. Twenty-two HCC cases were histologically/cytologically confirmed and the others had AFP > 400 ng/ml coupled with computerized tomography or digitally subtracted angiogram images consistent with HCC. Each HCC case was individually matched to 4 controls who were free of liver cancer or cirrhosis at the time of case identification. Controls were randomly selected from a pool of eligible subjects matched to cases by age (±5 years), sex, township of residence and date of recruitment (±3 months). There were 56 HCC cases and 220 controls in this analysis. Screening serum samples of 52 cases and 168 controls and urine samples of 38 cases and 137 controls were available and shipped to Columbia University on dry ice for aflatoxin exposure determination.

Albumin was prepared from serum as described previously (Wild *et al.,* 1990) and concentration determined with bicinchoninic (BCA Reagent, Pierce, Rockford, IL). Then 2 mg albumin in 500 μl PBS were digested with 0.5 mg proteinase K (Boehringer Mannheim, Indianapolis, IN) for 15 hr at 37°C and non-digested protein precipitated and washed with cold acetone. Acetone layers were combined and dried under vacuum, then redissolved in 0.5 ml PBS containing 1% FCS and 1 mM phenylmethylsulfonylfluoride to inhibit residual proteinase K activity. Digested albumin extracts were analyzed by competitive ELISA using a polyclonal anti-serum (number 7) as previously described (Chen *et al.,* 1996*b*). Briefly, 50 μl albumin extracts equivalent to 200 μg albumin were added to 96-well plates (Easywash, Corning, NY) previously coated with 3 ng AFB_1 epoxide-modified human serum. A standard curve was generated by serial dilution of enzymatically digested [^{3}H] AFB_1 epoxide-modified human serum albumin. Polyclonal antiserum 7 was used at $1:2 \times 10^5$ dilution and the secondary anti-serum, goat anti-rabbit IgG alkaline phosphatase (Boehringer Mannheim), was used at 1:750 dilution. The substrate was p-nitrophenyl phosphate (Sigma, St. Louis, MO), and absorbance at 405 nm was read on a Dynatech MR5000 96 well plate reader (Dynatech, Chantilly, VA). Samples with less than 20% inhibition were considered non-detectable. A positive control of serum from a rat treated with 1.5 mg AFB_1 and a negative control of pooled human sera from non-smoking US subjects were run with each assay. The detection limit of this assay (20% inhibition) was 0.01 fm/μg albumin. The coefficient of variation was 20% (n = 13). To reduce batch and plate variation, serum or urine samples of each case-control group were analyzed on the same plate. All samples were analyzed twice in duplicate wells with the laboratory blinded to case/control status.

Urinary aflatoxin metabolites were determined by competitive ELISA using monoclonal antibody AF8E11, with high affinity to AFB_1, and significant cross-reactivity with some aflatoxin derivatives, including AFB_2, AFM_1, AFG_1 and AFP_1 but no recognition of AFB_1-guanine (Hatch *et al.,* 1993). Briefly, 2.5 ml of urine were adjusted to pH 5.0, with 1 N HCl then digested with 500 U β-glucuronidase (Sigma). Urine was extracted by Sep-Pak C-18 cartridges (Waters, Milford, MA) previously washed with chloroform, methanol and water. After extraction, cartridges were rinsed with 10 ml water and 5 ml 5% methanol in water and eluted with 5 ml 80% methanol. Eluants were dried under vacuum and redissolved in 0.5 ml PBS. Concentrations of urinary aflatoxin metabolites were determined using a standard curve of serially diluted AFB_1. The coefficient of variation was 10% (n = 5).

Because not all data were available for all subjects, the reported percentages refer to the total number of subjects, with complete data for the relevant variables. Urinary aflatoxin metabolites were dichotomized using the median value for all samples. To evaluate the dose-response relationship between increasing aflatoxin exposure and HCC risk, levels were divided into tertiles, based on the total samples analyzed. Conditional logistic regression was employed to obtain the matched odds ratio (OR_m) and 95% confidence interval (CI) for each variable. Variables significantly associated with HCC ($p < 0.1$) and factors with other evidence suggesting an effect on HCC were included in the multivariate analysis. Un-

matched ORs were obtained when there would be severe loss of information using matched analysis. All analyses were based on screening data and were performed by SAS and EGRET software packages.

RESULTS

There were 50 male and 6 female HCC cases; 35 of the cases and 137 of the controls resided in the highest incidence area. Socio-demographic characteristics of the study subjects are given in Table I. Education, ethnicity and family monthly income were similar in cases and controls, as were the matching variables age, sex and residence.

Table II shows the frequency of HBsAg and anti-HCV, habits of cigarette smoking and alcohol drinking and levels of aflatoxin-albumin adducts and urinary aflatoxin metabolites among HCC cases and controls. After adjustment for anti-HCV status, the OR_m for HBsAg carriers compared with non-carriers was 45.5 (CI = 13.8–149.7). Smokers and drinkers had non-significantly elevated risks for HCC, as did subjects positive for anti-HCV.

HCC cases had a higher percentage of detectable aflatoxin-albumin adducts than controls (31/52, 59.6% *vs.* 62/180, 34.4%; $p < 0.01$). The OR_m for aflatoxin-albumin adducts was no longer statistically significant when adjustment was made for HBsAg carrier status. The OR_m for those with urinary aflatoxin metabolites in the upper 50th percentile compared to those with levels in the lower 50th percentile was significantly different from unity irrespective of adjustment for HBsAg carrier status. Moreover, when metabolite levels were stratified into tertiles, there was a dose-response relationship between aflatoxin and HCC risk (test for trend, $p < 0.05$), with adjusted OR_m values of 2.3 (CI = 0.6–9.2) and 7.2 (CI = 1.5–34.3) for subjects with middle and high levels, respectively, compared with those with a low level.

Although both aflatoxin-albumin adducts and urinary metabolites were correlated with HCC risk, the 2 exposure measures related only weakly; the correlation coefficient between aflatoxin-albumin adducts and urinary metabolites on a logarithmic scale was 0.11 ($p = 0.26$). The correlation was similar in HBsAg-negative and -positive subjects.

Anti-HCV was detected in 8.5% (4/47) of HBsAg-positive HCC cases, 6.7% (2/30) of HBsAg-positive controls, 25.0% (2/8) of HBsAg-negative HCC cases and 7.5% (4/47) of HBsAg-negative controls. Compared with those positive for neither viral markers, OR_m values were 86.1 (CI = 9.7–763.7) for subjects positive for both HBsAg and anti-HCV, 50.6 (CI = 14.2–179.8) for subjects positive for HBsAg only and 4.0 (CI = 0.5–29.9) for subjects positive for anti-HCV only. Based on the Mantel (1963) extension test for increased risk with neither marker positive as referent, the OR for developing HCC was found to increase in the presence of anti-HCV alone, HBsAg alone and both anti-HCV and HBsAg ($\chi = 10.32$, $p < 0.01$).

The combined effects of aflatoxin exposure and HBsAg carrier status on the development of HCC are shown in Table III. Among controls, aflatoxin-albumin adducts were not associated with HBsAg status: 34.6% (9/26) of carriers and 36.6% (52/142) of non-carriers had detectable adducts. Non-carrier controls had slightly higher urinary aflatoxin metabolite levels than carrier controls (53/114, 46.5% *vs.* 8/22, 36.4%; $p > 0.1$). OR_m values for HBsAg-seropositive subjects with high aflatoxin exposure were higher than for HBsAg-seropositive subjects with low aflatoxin exposure or HBsAg-seronegative subjects with high aflatoxin exposure. The increased risk ratios in HBsAg carriers were 4.02 (70.0/17.4) for detectable compared with non-detectable aflatoxin-albumin adducts and 4.91 (111.9/22.8) for high compared with low urinary aflatoxin metabolites.

In Table IV we illustrate the combined effects of aflatoxin exposure and HBV infection. The adjusted OR values for the development of HCC in male HBsAg-seropositive subjects are 2.8 (CI = 0.9–9.1) for detectable compared with non-detectable aflatoxin-albumin adducts and 5.5 (CI = 1.3–23.4) for high compared with low levels of urinary aflatoxin metabolites. There was a non-significant dose-response relationship between increasing urinary aflatoxin levels and HCC risk, with ORs of 2.1 (CI = 0.3–12.4) and 5.6 (CI = 0.8–38.1), respectively, for the middle and high levels compared with the low level (test for trend: $p = 0.06$).

TABLE I – SOCIODEMOGRAPHIC CHARACTERISTICS OF 56 HCC CASES AND 220 CONTROLS

Variable	Cases		Controls	
	Number	%	Number	%
Age at recruitment (yr)				
30–44	4	7.1	19	8.6
45–54	26	46.4	91	41.4
55–64	26	46.4	110	50.0
Gender				
Male	50	89.3	197	89.5
Female	6	10.7	23	10.5
Residence				
Taiwan Island	21	37.5	83	37.7
Penghu Islets	35	62.5	137	62.3
Education				
Illiterate	15	26.8	42	19.1
Elementary school	24	42.9	109	49.5
High school and above	17	31.3	69	31.4
Ethnicity				
Fukienese	43	76.8	174	79.1
Hakka	10	17.9	31	14.1
Others	3	5.4	15	6.8
Family income (NT$/month)				
< 15,000	14	26.9	57	27.0
15,000–29,999	14	26.9	70	33.2
≥ 30,000	24	46.2	84	39.8

DISCUSSION

Our study applies simultaneously 2 biological markers of exposure to aflatoxin, albumin adducts in blood and urinary excretion of metabolites, to investigate the relationship between aflatoxin, viral infection and HCC. Cases and controls were drawn from a cancer-screening cohort in several townships in the Penghu Islets of Taiwan, where aflatoxin exposure is high, and on Taiwan Island, where exposure is lower. The principal finding is the consistent demonstration of an enhanced effect of aflatoxin exposure on the risk of HCC in HBsAg carriers, regardless of which exposure marker is used.

Our results for aflatoxin and viral infection are comparable with those of a cohort study carried out in Shanghai, China, using urinary AFB_1, its metabolites and the N7 guanine adduct (Ross *et al.*, 1992). The Shanghai study found that those with detectable levels of any aflatoxin metabolites had a significantly higher OR_m than those with non-detectable aflatoxin and that increased risk of HBV infection on HCC was enhanced in aflatoxin-positive compared with -negative subjects. A follow-up of the same cohort confirmed the enhanced effect of aflatoxin exposure on the potential risk of HBV infection (increased risks of HBV infection were 7.3/1.0 and 59.4/3.4 for aflatoxin-negative and -positive subjects, respectively) (Qian *et al.*, 1994).

Several lines of evidence, including animal, epidemiological and molecular studies, suggest that aflatoxin exposure may amplify the hepatic carcinogenic potential of HBV infection. Higher levels of adduct formation in virus-infected animals exposed to AFB_1 have been reported (Cova *et al.*, 1990).

TABLE II – SEROPOSITIVITY OF HBsAg AND ANTI-HCV, HABITS OF CIGARETTE SMOKING AND ALCOHOL DRINKING, LEVELS OF AFLATOXIN-ALBUMIN ADDUCTS AND URINARY AFLATOXIN METABOLITES OF HCC CASES AND CONTROLS

Variable	Cases		Controls		Matched odds ratio[1]		Adjusted odds ratio[2]	
	Number	%	Number	%	OR_m	95% CI	OR_m	95% CI
HBsAg carrier status[3]								
Negative	8	14.3	188	85.8	1.0		1.0	
Positive	48	85.7	31	14.2	43.3[7]	13.4–140.3	45.5[2]	13.8–149.7
Anti-HCV status[4]								
Negative	49	89.1	201	92.6	1.0		1.0	
Positive	6	10.9	16	7.4	1.6	0.5–4.5	2.5	0.6–10.8
Habitual cigarette smoking								
Non-smokers	24	42.9	103	47.2	1.0		1.0	
Smokers[5]	32	57.9	117	55.8	1.2	0.6–2.3	2.2	0.8–5.7
Habitual alcohol drinking								
Non-drinkers	42	76.4	188	85.8	1.0		1.0	
Drinkers[6]	13	24.6	31	14.2	1.9	0.9–4.2	2.6	0.8–8.8
Serum level of aflatoxin-albumin adducts								
Nondetectable	21	40.4	118	65.6	1.0		1.0	
Detectable	31	59.6	62	34.4	4.6[7]	2.0–10.4	1.6	0.4–5.5
Urinary level of aflatoxin metabolites								
Low	12	31.6	76	55.5	1.0		1.0	
High	26	68.4	61	44.5	3.3[7]	1.4–7.7	3.8[7]	1.1–12.8

[1]Matched for sex, age, residence and date of recruitment.–[2]In addition to matched variables, the odds ratio associated with hepatitis B virus surface antigen (HBsAg) was adjusted for anti-HCV and odds ratios associated with cigarette smoking and alcohol drinking, anti-HCV, aflatoxin-albumin adducts and urinary aflatoxin metabolites were adjusted for HBsAg.–[3]HBsAg, hepatitis B virus surface antigen.–[4]Anti-HCV, antibody against hepatitis C virus.–[5]Includes current and ex-smokers.–[6]Includes current and former drinkers.–[7]$p < 0.05$.

TABLE III – COMBINED EFFECTS OF AFLATOXIN EXPOSURE MEASURED IN SERUM AND URINE AND HBsAg CARRIER STATUS ON THE DEVELOPMENT OF HCC

Aflatoxin exposure index	HBsAg[1]	Cases	Controls	OR_m[2]	95% CI
Aflatoxin-albumin adducts					
Non-detectable	Negative	7	101	1.0[3]	
Detectable	Negative	1	52	0.3	0.0–3.6
Non-detectable	Positive	14	17	17.4[5]	3.7–81.9
Detectable	Positive	30	9	70.0[5]	11.8–415.4
Urinary aflatoxin metabolites					
Low	Negative	2	61	1.0[4]	
High	Negative	4	53	1.7	0.3–10.8
Low	Positive	10	14	22.8[5]	3.6–143.4
High	Positive	22	8	111.9[5]	13.8–905.0

[1]HBsAg, hepatitis B virus surface antigen.–[2]OR_m, matched odds ratio adjusted for cigarette smoking and alcohol drinking.–[3]Mantel extension test for increased risk: $\chi = 9.29$, $p < 0.01$.–[4]Mantel extension test for increased risk: $\chi = 8.01$, $p < 0.01$.–[5]$p < 0.05$.

TABLE IV – AFLATOXIN EXPOSURE AND RISK OF HCC IN MALE HBsAg-POSITIVE SUBJECTS

Aflatoxin exposure index	Cases	Controls	OR[1]	95% CI
Aflatoxin-albumin adducts				
Non-detectable	13	15	1.0	
Detectable	27	9	2.8[2]	0.9–9.0
Urinary aflatoxin metabolites				
Low	9	14	1.0	
High	20	7	5.5[3]	1.3–23.4

[1]Unmatched odds ratio adjusted for age, residence (Penghu/Taiwan), cigarette smoking and alcohol drinking.–[2]$0.05 < p < 0.1$.–[3]$p < 0.05$.

Transgenic mice over-expressing the gene for the large envelope polypeptide of HBV who were exposed to AFB_1 had more rapid and extensive hepatocyte dysplasia and HCC than transgenic mice not exposed to AFB_1 (Sell *et al.*, 1991).

In human studies, Allen *et al.* (1992) found that mean aflatoxin-albumin adducts in HBsAg-positive children in Gambia, West Africa, were higher than in non-carrier children. An interaction between aflatoxin and HBV is also suggested by the early onset of HCC in cases with detectable aflatoxin-DNA in liver tissue. Chen *et al.* (1992) used a monoclonal antibody (6A10), generated against the persistent form of the major N7 guanine adduct of AFB_1, to stain needle biopsy smears of newly diagnosed HCC cases. Thirty-five (70%) of the HCC samples had detectable levels of adducts. When stratified by HBeAg and HBsAg serostatus, the onset age in adduct-positive subjects was 10 years younger than that in adduct-negative subjects who either had both HBV markers or were HBsAg-positive only. In subjects with neither marker positive, the onset ages were similar irrespective of DNA adduct status.

Additional evidence for aflatoxin as a hepatocarcinogen comes from the mutation spectrum of the *p53* tumor-suppressor gene in liver tumors. The mutation frequency at codon 249 in high aflatoxin exposure areas, such as South Africa, Qidong (China), Senegal and Taiwan, was significantly higher than that in low aflatoxin areas, like Japan, Europe, and the United States (Eaton and Gallagher, 1994). When stratified by HBV markers, mutation frequency at codon 249 was 52/143 (36.3%) for HBV-infected patients and 4/34 (11.7%) for patients without HBV markers in high aflatoxin exposure areas and 6/126 (4.8%) and 4/167 (2.4%) in low-exposure areas. This evidence also suggests that HBV infection may act synergistically with aflatoxin to induce a specific mutation in *p53*.

Although both aflatoxin-albumin adducts and urinary metabolites were related to HCC risk, there was a poor correlation between these 2 markers of exposure in the same controls. Urinary excretion monitors exposure over the past 24–48 hr while human serum albumin has a half-life of 21 days and thus should measure exposure over the past several months (Skipper and Tannenbaum, 1990). Since albumin adducts may reflect a longer term of environmental aflatoxin exposure, misclassification should be reduced. The association between HCC and albumin adducts should therefore be stronger than

that between HCC and urinary metabolites, but in this study both markers gave similar ORs.

We also found that co-infection by HBV and HCV resulted in higher risk than infection by HBV or HCV alone. Similar results were seen in several case-control studies in Taiwan (Yu and Chen, 1994). Patients co-infected by these 2 viruses have more severe liver disease and poorer prognosis than those infected with HBV alone, despite the fact that HBV replicative activity is suppressed by HCV (Liaw, 1995). The mechanism of dual hepatitis virus infection on the development of HCC needs further study.

Several methodologic issues need to be addressed. First, because 48% (23/48) of cases had ultrasonographic images compatible with HCC at first scanning, we further stratified HCC cases to scan positive or negative to examine the association among those clearly disease-free at the time of sample collection. The adjusted ORs for the comparisons between first-scan negative cases and controls were 3.8 (CI = 1.1–13.2) for detectable compared to non-detectable aflatoxin-albumin adducts and 4.8 (CI = 0.8–28.1) for high compared with low urinary aflatoxin metabolites. The corresponding figures for the comparisons between first-scan positive cases and controls were 2.9 (CI = 0.8–10.4) and 6.9 (CI = 1.3–35.1), respectively. The results for incident and prevalent cases were therefore similar.

Second, we used several follow-up methods, including personal and telephone interview, death certificate collection, data linkage to all study subjects, and abdominal ultrasonographic scanning and AFP determination in subjects who were positive for at least 1 of 6 screening markers. The national death data base is complete in Taiwan; however, there is an approximate 18 month time lag in data entry. Since the interval between the 2 interviews for all study subjects was much longer than between the 2 scannings for screening positive subjects (12–24 *vs.* 3–12 months), risk factors associated with abdominal ultrasonographic scanning may be over-estimated. To reduce this effect, we obtained copies of death certificates from the local housing offices in the study areas every 4–6 months. Data on clinico-pathological diagnosis were also collected for all HCC deaths, including the 6 cases identified from death certificate.

Third is the effect of missing data on aflatoxin exposure due to the lack of biospecimens from some study subjects. The average age at recruitment was identical for those with and without urinary aflatoxin metabolite levels, and HBsAg-seropositive rates were similar in both groups (31% *vs.* 25%). However females and residents of Penghu Islets had a higher percentage with missing urinary aflatoxin metabolite data than males and residents of Taiwan Island. Nonetheless, the effect of missing data was small when we compared the risk estimates for aflatoxin exposure in regression models with or without subjects with missing data (Table IV). The unmatched odds ratio for detectable compared to non-detectable aflatoxin-albumin adducts changed from 2.8 (CI = 0.9–9.0) to 2.9 (CI = 0.9–9.1) for models without and with subjects with missing data, and for high compared with low urinary aflatoxin metabolites level OR changed from 5.5 (CI = 1.3–23.4) to 4.1 (CI = 1.1–15.4).

Finally, there is concern that the small sample size in our study limits statistical power to detect a small increase in risk. Given a type I error (α) level of 0.05, type II error (β) level of 0.20, aflatoxin exposure rate among healthy controls of 0.40, detectable OR of 3.0, and a control-case ratio of 4.0, the minimum sample size required for cases is 36 (Schlesselman, 1982). Though data are missing for some study subjects, the statistical power to detect a significant association between aflatoxin exposure and HCC risk was >0.80 in our study.

In summary, our study provides further support for the multiple roles of aflatoxin, HBV, and HCV in HCC development. While elevated DNA or protein adducts are now an accepted measure of exposure, their relationship to cancer risk has been regarded as tentative; however, the study by Ross *et al.* (1992) using banked urine samples lent credence to the notion that these markers might be useful for estimation of risk. The findings presented here support the significance of the urine assay and extend it to albumin adduct analysis.

Although a nationwide vaccination program was implemented a decade ago, there are still 3 million HBV adult carriers in Taiwan. Since exposure to aflatoxin is common, its effects on the development of HCC make it an important public health concern. An active food surveillance program to reduce or eliminate aflatoxin exposure is warranted.

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