A Follow-Up Study of Urinary Markers of Aflatoxin Exposure and Liver Cancer Risk in Shanghai, People's Republic of China[1]

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Abstract

A cohort of 18,244 mostly middle-aged (45–64 years) men residing in four small geographically defined areas of Shanghai was accrued between January 1986 and September 1989. In addition to an in-person interview regarding dietary and other past exposures, each subject donated a single void urine sample at recruitment so that the presence of aflatoxins in urine could be assessed. In addition, a 1-year survey of market foods in Shanghai was conducted to quantitatively estimate the extent of aflatoxin exposure in the study population. After close to 70,000 person-years of follow-up, 55 incident cases of hepatocellular carcinoma (HCC) had been identified. Levels of urinary aflatoxin B_1 and the oxidative metabolites, including the major aflatoxin nucleic acid adduct, aflatoxin-N^7-guanine, were determined for 50 of the 55 identified cases of HCC. Two hundred sixty-seven controls were chosen randomly from the cohort; they were matched to the 50 cases by age (within 1 year), time of specimen collection (within 1 month), and residence. After integrating the high-pressure liquid chromatography chromatograms to measure aflatoxin-N^7-guanine, aflatoxin M_1, aflatoxin P_1, and aflatoxin B_1, 49, 67, 53, and 71 of the urine samples had detectable levels of these compounds, respectively. The aflatoxin metabolite detected at the highest concentration was aflatoxin P_1; the range was 0.59–16.0 ng/ml. The range of aflatoxin M_1 in the urine was 0.17–5.2 ng/ml. The aflatoxin-N^7-guanine adduct range was 0.3–1.81 ng/ml in the 49 positive samples. A nested case-control analysis showed highly significant associations between the presence of urinary aflatoxins, serum hepatitis B surface antigen positivity, and HCC risk. Risk was especially elevated in individuals who were positive for both of these biomarkers (relative risk = 59.4; 95% confidence limit, 16.6, 212.0 after adjustment for cigarette smoking, a potential confounder). On the other hand, a cohort analysis using all 55 cases of HCC revealed no strong or statistically significant association between HCC risk and dietary aflatoxin consumption as determined from the in-person food frequency interview combined with the survey of market foods in the study region. Our results underline the importance of biomarker measurements in assessing the aflatoxin-HCC association in epidemiological studies.

Introduction

A goal of molecular epidemiology is to identify those individuals within populations who are at greatest risk of developing cancer from exposure to known etiological agents. Chronic infection with HBV[4] and ingestion of aflatoxin-contaminated foods are considered major risk factors for HCC, which causes at least 250,000 deaths annually worldwide. Both exposures are common in high risk areas for HCC, but the likelihood that a given individual so exposed will develop HCC is small (1–4). HBV status can be determined through immunological detection of antibodies against viral gene products in the blood of infected individuals, and the importance of HBV as a risk factor for HCC has been established through prospective studies utilizing this biomarker (4). In contrast, evidence that aflatoxins play an etiological role in the development of HCC has been based largely to date on estimates of aflatoxin ingestion in various populations, obtained through population-based estimates of food intake combined with food sampling and analysis. Analytical methods for the detection of aflatoxin metabolites, DNA, and protein adducts in urine and blood have been developed recently (5, 6), and studies designed to validate these parameters as biomarkers of aflatoxin ingestion by individuals within exposed populations are in progress.

HCC is the third leading cause of cancer mortality in the PRC, resulting in more than 100,000 deaths annually, comprising nearly 15% of the total cancer mortality burden. Nationwide cancer mortality surveys in the PRC have shown large geographical variations in liver cancer mortality. Generally, high incidence regions correlate with specific climatic conditions. High liver cancer rates occur in coastal

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[4] The abbreviations used are: HBV, hepatitis B virus; AFB-N^7-gua, aflatoxin-N^7-guanine; AFM_1, aflatoxin M_1; AFP_1, aflatoxin P_1; AFB_1, aflatoxin B_1; AFQ_1, aflatoxin Q_1; AFG_1, aflatoxin G_1; PRC, People's Republic of China; HPLC, high-pressure liquid chromatography; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline; HBsAg, hepatitis B surface antigen; RR, relative risk; CL, confidence limits; HCC, hepatocellular carcinoma.

areas or in inland provinces where average summer temperatures are greater than 30°, where heavy rains occur from June to September, and where average humidity is high. These climatic conditions are known to be conducive to mold spoilage of foods and therefore to aflatoxin contamination (7–9). Even in urban areas of the PRC, HCC can be a substantial public health problem. In Shanghai, for example, HCC incidence rates are exceeded only by lung cancer and stomach cancer in men, and only by breast, stomach, and lung cancer in women. HCC incidence rates in Shanghai are 5- to 10-fold higher than those in the United States (10). We have undertaken molecular epidemiological studies in high risk areas of the PRC in order to determine directly the relationship of aflatoxin exposure to HCC risk.

In these studies, we have employed aflatoxin metabolites, nucleic acid adducts, and serum albumin adducts as measures of aflatoxin ingestion. These biomarkers are based on the knowledge that the parent compound AFB_1 is converted to its carcinogenic forms through metabolism by members of the cytochrome P-450 enzyme superfamily to a reactive 8,9-epoxide. These enzymes also oxidize AFB_1 to various other derivatives, including AFM_1, AFQ_1, AFP_1, as well as a reduced aflatoxin species, aflatoxicol. Details of the pathways involved have been comprehensively reviewed (11). The reactive electrophilic epoxide covalently binds to DNA (12) and serum albumin (13), forming AFB-N^7-gua and lysine adducts, respectively. Both of these adducts have been validated as molecular dosimeters in experimental animals and in humans (14–18). The role of the major aflatoxin DNA adduct, AFB-N^7-gua, in the cancer initiation process provides a mechanistic rationale for the study of this adduct as a molecular dosimeter in humans (15, 16).

Aflatoxin biomarkers are being used now to prospectively evaluate the relationship between aflatoxin exposure and HCC risk in a large cohort of middle-aged men in Shanghai, China. Recently, we reported that the presence of urinary AFB_1, its metabolites AFM_1 or AFP_1, or AFB-N^7-gua was associated with a statistically significant 4-fold increase in HCC risk after adjustment for HBsAg positivity and other potential confounders (19). In this cohort study, we also have attempted to quantitatively estimate aflatoxin ingestion in subjects by means of a food frequency questionnaire coupled with a determination of aflatoxin levels in selected market foods. The present paper focuses on this dietary assessment of aflatoxin exposure and its relationship with HCC risk and levels of urinary aflatoxin metabolites. Expanded findings relating HCC risk to the presence of specific aflatoxin biomarkers in the urine are also presented.

Materials and Methods

General Study Design. The design of the cohort study has been described in detail previously (19). Briefly, a cohort of 18,244 mostly middle-aged (45–64 years) men residing in four small geographically defined areas of Shanghai was accrued between January 1986 and September 1989. In addition to an in-person interview regarding dietary and other past exposures, each subject donated a 10-ml blood sample and a single void urine sample at recruitment. The interviews and specimen collections were administered by nurses employed by the Shanghai Cancer Institute, typically at the homes of the subjects several hours after the evening meal. Follow-up of this cohort is accomplished through routine review of death certificates, routine review of the population based Shanghai Cancer Registry records, and annual recontact of each cohort member.

A structured questionnaire was employed for the in-person interview of each subject. The respondent was asked to indicate the frequency (in number of times per day, week, month, or year) with which he usually consumed each of 45 food items as an adult. For seasonal foods, we obtained the frequency of consumption when the food was in season. The amounts of various cooking oils used each month by the household were also indicated.

In order to establish a standard portion size for each of the food items investigated, 100 subjects randomly selected from all cohort members were asked to indicate, using standard measuring instruments and food models, their normal serving size of each of the foods under study. For each food, the mean weight of all recorded servings was taken as the weight of the standard portion.

As of February 1, 1992, the 18,244 participants in the cohort had contributed 69,393 man-years of observation. Three hundred sixty-four incident cancer cases had been identified, including 55 cases of liver cancer. The basis for the diagnosis of these 55 cases was as follows: biopsy confirmed ($n = 9$), elevated serum α-fetoprotein with consistent clinical and radiologic history ($n = 25$), positive computerized axial tomography scan and/or ultrasonography with consistent clinical history ($n = 19$), and death certificate only ($n = 2$).

Urine samples from 50 of the 55 identified cases of HCC and 267 controls selected from the remaining cohort were analyzed. Multiple controls were matched to each case by age (within 1 year), time of sample collection (within 1 month), and neighborhood of residence. The controls were selected randomly from among all cohort members who were enrolled in the study and who had no history of liver cancer on the date of cancer diagnosis of the index case. Ten controls were matched to each of the first six identified cases of HCC. For the remaining 44 cases, the matching ratio was 5:1 for 38 cases, 4:1 for 2 cases, and 3:1 for 4 cases.

Determination of Aflatoxins in Human Urine. Urine samples were collected in disposable containers in the homes of participants usually between 7 and 9 p.m. Immediately after collection, the samples were taken to the Shanghai Cancer Institute and stored at 4°. Within 48 h, the urine was centrifuged and two 25-ml aliquots were loaded onto individual C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA) that had previously been washed sequentially with 5 ml each of 5% methanol:water, 80% methanol:water, 100% methanol, and 5% methanol:water. Urine was applied to the Sep-Pak at a flow rate of 2–3 ml/min and the effluent was discarded. The Sep-Pak was washed sequentially with 10 ml of 5% methanol:water and 10% methanol:water, the ends were sealed with parafilm, and the sealed cartridge was stored until analysis. Prior to elution of the aflatoxins, 5 ml of 5% methanol:water was passed through the cartridge. Aflatoxin metabolites were eluted with 10 ml 80% methanol:water, which was evaporated to dryness under reduced pressure. The residue was reconstituted in 0.3 ml of 0.1 N hydrochloric acid with heating at 50° for 10 min to ensure that relatively insoluble aflatoxin-DNA adducts were dissolved. After cooling to room temperature, 0.5 ml 1 M ammonium formate, pH 4.5, was added. The volume was adjusted to 10 ml with water and the sample was applied to a 4-ml preparative monoclonal antibody affinity column at a flow rate of about 0.3 ml/minute (16, 20, 21). The effluent from the loading procedure was saved. The affinity column was washed twice with 7 ml PBS, pH 7.4, and

once with 7 ml water to remove nonspecifically bound material. Aflatoxin derivatives were eluted from the preparative monoclonal antibody affinity column with 7 ml 70% DMSO:water. The column was washed twice with 7 ml PBS, and the DMSO eluate was combined with the two PBS washes. The DMSO concentration in the combined fractions was reduced to less than 3.5% by the addition of water. This sample was then applied to a C_{18} Sep-Pak, prepared as described above to remove the DMSO, and eluted with 80% methanol:water, which was then reduced to dryness under vacuum. The residue was redissolved in 100 μl 0.1 N hydrochloric acid with heating at 50° for 10 min, and adjusted to about pH 3.0 by adding an equal volume of 1 M ammonium formate, pH 4.5.

In our earlier study of 22 HCC cases and their matched controls (19), immunoaffinity columns that contained antibodies recognizing AFB-N^7-gua, AFP_1, AFM_1, and AFB_1 were used. For the more recently identified 28 additional cases of HCC and their matched controls, an immunoaffinity column containing two additional aflatoxin-specific antibodies recognizing AFQ_1 and AFG_1 was used (20). These antibodies were not available at the time of the first study.

Aflatoxin derivatives were analyzed by reversed phase HPLC using a Beckman Model 160 fixed wavelength detector set at 365 nm, 0.001 absorbance units, full scale, connected in series with a Hewlett-Packard Model 1040A diode-array detector to quantify aflatoxin metabolites as previously described (16, 20, 21). The HPLC column used was a C_{18} 5 μm (25-cm) Ultrasphere analytical column (Rainin Instrument Co., Woburn, MA) and chromatographic separation was obtained by elution for 20 min with 13% ethanol:water, followed by a 13–25% ethanol:water linear gradient generated over 25 min, and then isocratic elution with 25% ethanol:water. All mobile phases were buffered with 0.01 M triethylammonium formate, pH 3.0, and the column temperature was maintained at 35°. The flow rate was 1 ml/minute. Authentic aflatoxin standards were used to determine chromatographic retention times.

The limit of detectability for aflatoxin derivatives in this HPLC procedure was about 1.0 ng. Thus, in a 25-ml urine sample, the minimum concentration of aflatoxins detectable was 0.04 ng/ml. We had previously shown (20, 21) that the average recovery of aflatoxin derivatives from urine was about 65%. Thus, the minimum detectable concentration of aflatoxins in the samples analyzed was about 0.07 ng/ml.

Analysis of Aflatoxin Levels in Foods. In conjunction with the cohort study, a survey of 23 different types of foods commonly consumed by Shanghai residents was conducted over the course of a calender year from April 1, 1988 to March 31, 1989. Food samples were collected from many small markets in several defined urban Shanghai areas in which members of the study cohort resided. The survey was designed to ensure that seasonal foods were included in order to obtain as complete a record of aflatoxin contamination as possible. A total of 1827 different food samples were collected and analyzed, representing by far the most comprehensive of such surveys conducted to date in urban Shanghai. These analytical data, together with the dietary histories, enable an estimate of dietary intake of aflatoxins to be calculated for each individual.

Aflatoxin levels in certain foods [dried green bean, dried soybean, dried red bean, bean curd (tofu), partially-dried bean curd (tofu gann), vegetarian chicken (made from soy bean), soy sauce, fermented bean curd, fermented bean pastes, peanut butter, soybean oil and rapeseed oil] were determined following extraction with 2 ml methanol:water (55:45, v/v) (22). Extracted aflatoxins were partitioned into chloroform, which was evaporated to dryness. The residue was redissolved in 0.5 ml methanol and then adjusted to 10 ml 5% methanol:water by the addition of water. This mixture was loaded onto a monoclonal antibody affinity column of 3 ml volume as previously described (23). The affinity column was washed twice with 3 ml PBS, pH 7.4, and then with 4 ml distilled water to remove nonspecifically bound materials. AFB_1 was eluted from the column with two consecutive 3-ml portions of 70% DMSO:water. Eluates were pooled and diluted with 114 ml distilled water to a final DMSO concentration of 3.5%; this solution was passed through a prewashed C_{18} Sep-Pak cartridge. Aflatoxin was eluted from the Sep-Pak with 10 ml of 80% methanol:water and evaporated to dryness. Aflatoxin levels were quantified by thin layer chromatography and fluorodensitometry. The recovery of AFB_1 (10 ppb) in soy sauce was 81% and the limit of detection was 0.2 ppb. The identity of AFB_1 was confirmed by trifluoroacetic acid derivitization on the thin layer chromatography plate (22). Aflatoxin contamination of rice, wine, beer, pig liver, corn, and wheat flour was determined by a slightly modified method of Pons (24). Samples were extracted with methanol:10% NaCl (4:1, v/v), and the extract was passed through a 2-g silica gel column. AFB_1 levels were determined by normal phase chromatography-HPLC with fluorescence detection. The fluorescence detector flow cell was packed with silica gel to enhance detection.

AFM_1 in fresh milk and milk powder was analyzed by the method of Winterlin and Hsieh (25). Ten ml or 10 g of milk were diluted to 25 ml with distilled water and transferred to a C_{18} Sep-Pak. AFM_1 was eluted from the Sep-Pak with 4 ml 30% acetonitrile in water and analyzed by reversed phase HPLC.

Data Analysis. For each subject, we computed a quantitative index of aflatoxin exposure (in μg/year) by summing across 14 contaminated foods the cross-product of intake frequency (in number of times per year), standard portion weight (in grams), and level of aflatoxin contamination (in μg/kg of food). Tertiles of exposure categories were constructed based on the distribution of this index across the entire cohort. Subjects were then classified into the categories of low, medium, or high exposure.

Standard methods of cohort analysis were used to examine HCC risk in relation to levels of dietary aflatoxin exposure. Incidence rates were adjusted by age (up to 54, 55–59, and 60+ years) by direct standardization using the person-year distribution of the entire cohort as an internal standard (26). The method of Breslow et al. (27) was used to compute RR and associated 95% CL. The statistical package GLIM was used to perform these calculations.

The conditional logistic regression methodology was used in examining the relationship between urinary biomarkers of aflatoxin exposure and HCC risk, based on data from 50 cases of HCC and their 267 matched controls (28). The computations were performed using the statistical package EPILOG. All quoted p values are two sided.

Results

Human Urine Analysis for Aflatoxin Biomarkers. From a total of 18,244 urine samples collected during the 3-year period, 317 (50 HCC cases and 267 controls) were analyzed for aflatoxins by the analytical method described above. These analyses revealed that AFB-N^7-gua, AFM_1, AFP_1, and

Table 1 Presence of urinary aflatoxin biomarkers among 50 cases of hepatocellular carcinoma and 267 matched controls

	Cases/controls	RR (95% CL)	Adj. RR (95% CL)[a]
No biomarker	14/158	1.0	1.0
Presence of			
Any biomarker[b]	36/109	4.0 (2.0, 7.8)	5.0 (2.1, 11.8)
AFB_1-N^7-Gua (adduct)	18/31	7.6 (3.2, 18.0)	9.1 (2.9, 29.2)
AFP_1	14/39	4.5 (2.0, 10.4)	5.1 (1.7, 15.3)
Without adduct	6/26	2.8 (1.0, 7.9)	3.1 (0.8, 11.9)
With adduct	8/13	10.3 (3.2, 33.3)	11.0 (2.4, 50.9)
AFM_1	18/49	4.4 (2.1, 9.6)	5.8 (2.2, 15.2)
Without adduct	10/39	3.1 (1.3, 7.5)	4.0 (1.4, 11.9)
With adduct	8/10	9.7 (3.2, 29.9)	16.1 (3.6, 72.5)
AFB_1	15/56	3.4 (1.5, 7.7)	3.5 (1.2, 9.9)
Without adduct	9/43	2.6 (1.0, 6.5)	2.8 (0.9, 9.0)
With adduct	6/13	6.3 (2.0, 20.1)	5.7 (1.3, 26.0)
AFQ_1[c]	7/24	3.4 (1.0, 11.6)	7.6 (1.2, 85.2)
Without adduct	3/20	1.8 (0.4, 8.2)	3.1 (0.3, 40.5)
With adduct	4/4	17.7 (1.8, 174.9)	9.9 (1.1,134.7)
AFG_1[c]	9/26	3.8 (1.2, 12.3)	3.8 (0.8 ,20.3)
Without adduct	5/22	2.7 (0.7, 9.8)	2.7 (0.5, 16.1)
With adduct	4/4	11.1 (2.0, 61.3)	6.0 (0.5, 95.3)

[a] Adjusted for HBsAg positivity and cigarette smoking.
[b] AFB_1, AFP_1, AFM_1, or AFB_1-N^7-Gua.
[c] These assays were performed on only 28 HCC cases and their matched controls (see text for details). The reference category of no AFB biomarkers had five cases and 63 controls.

AFB_1 were detected in 49, 67, 53, and 71 of the urine samples, respectively (Table 1). The aflatoxin metabolite detected at the highest concentration in urine samples was AFP_1 with a range of 0.59–16.0 ng/ml. The range of AFM_1 in the urine was 0.17–5.2 ng/ml. The AFB-N^7-gua adduct level range was 0.3–1.81 ng/ml in the 49 positive samples. All of these values are similar to previous studies of aflatoxin levels in human urine samples (16, 21). One significant difference between these data in Shanghai and previous observations made in Guangxi Province, PRC and The Gambia, West Africa, was that in Shanghai, about 50% of the urine samples contained no detectable aflatoxins, whereas in the other studies less than 15% of the urine samples had no detectable levels of aflatoxin metabolites (16, 21). This is presumed to reflect more prevalent dietary exposures to aflatoxins in these rural areas compared with the urban Shanghai area.

Table 1 shows the importance of AFB-N^7-gua relative to other aflatoxin biomarkers as a predictor of HCC risk in this cohort. In the absence of AFB-N^7-gua, the RRs associated with the presence of AFM_1, AFB_1, or AFP_1 ranged from 2.6 to 3.1, and were not statistically significant for AFP_1 and AFB_1. In contrast, when AFB-N^7-gua also was present, the RRs associated with positivity for AFB_1, AFM_1, or AFP_1 ranged from 6.3 to 10.3 and all were statistically significant. Comparable results were obtained when we repeated the analysis with adjustment for potential confounders, which included HBsAg positivity and cigarette smoking.

The initial study of urinary aflatoxins in this cohort examined 22 HCC cases and their matched controls (19). The immunoaffinity column used to isolate the urinary aflatoxins did not contain antibodies that recognized AFQ_1, an oxidative metabolite produced by the same cytochrome P-450 as the aflatoxin-8,9-epoxide (29) and AFG_1. In the interim between the first set of samples and the data reported in this paper, monoclonal antibodies specific for AFQ_1 and AFG_1 have been made (20); this new immunoaffinity procedure was applied to the latest 28 HCC cases and their matched controls. Similar to the results with AFP_1, AFM_1, and AFB_1, the presence of AFB-N^7-gua adduct with either AFQ_1 or AFG_1 also led to higher risk than did the absence of the adduct (Table 1).

Aflatoxin Levels in Foods. The major staple foods consumed in urban Shanghai were long-grain rice, round-grain rice, and wheat flour. As shown in Table 2, the levels of aflatoxins found in rice and wheat flour samples were low, generally less than 1 μg/kg. Thirty percent of tested samples were found to contain AFB_1 and the range was 0.06–4.11 μg/kg. In contrast to the above foods, peanuts contained much higher levels of aflatoxins; 23% of tested samples contained more than 20 μg/kg and four samples had levels greater than 100 μg/kg. The maximum level detected in the peanut samples was 820 μg/kg and the average for all contaminated peanut samples was 39.6 μg/kg. In addition, 65% of the peanut butter samples tested were positive for aflatoxin; 8% of the peanut butter samples contained more than 20 μg/kg. The maximum level was 54.6 μg/kg and the average for all peanut butter samples was 7.1 μg/kg.

Aflatoxin contamination was not limited to the staple foods described above. A number of sauces and pastes used in cooking were also found to be contaminated. Fifty-seven percent of soy sauce samples contained detectable quantities of AFB_1; 16% of samples contained levels higher than 20 μg/kg. Two soy sauce samples were contaminated at levels higher than 100 μg/kg, one of which had a level of 188 μg/kg. The mean aflatoxin content of soy sauce products was 16.6 μg/kg. Aflatoxin contamination also was found in two fermented foods, fermented bean curd and fermented bean paste. However, the mean contamination levels of these fermented products were relatively low, 1.10 and 1.26 ng/kg of food, respectively.

Rapeseed and soybean oils were the most common cooking oils sold in the marketplace. A total of 73 soybean

Table 2 Aflatoxin (AF) contents in selected market foods in Shanghai[a]

Food	No. of samples	% Positive	Mean (range) among positive samples (μg AF/kg)
Long rice	60[b]	30.0	0.28 (0.06–1.15)
Round rice	60[b]	15.0	0.19 (0.06–0.31)
Wheat flour	60[b]	31.7	0.64 (0.11–4.11)
Corn	39[b]	69.2	1.14 (0.13–5.13)
Peanuts	62[b]	69.4	39.6 (0.5–820.0)
Peanut butter	62	64.5	7.10 (0.5–54.6)
Dried green beans	10	0.0	
Dried soybeans	8	0.0	
Dried red beans	20	30.0	9.93 (3.6–21.2)
Bean curd (tofu)	23	0.0	
Partially-dried tofu	24	8.3	4.03 (1.7–6.3)
Vegetarian chicken (soybean product)	24	0.0	
Soy sauce	56	57.1	16.6 (0.4–188)
Fermented bean curd	45	33.3	1.10 (0.3–4.3)
Fermented bean paste	10	50.0	1.26 (0.2–2.8)
Soybean oil	73	30.1	1.10 (0.25–4.62)
Rapeseed oil	70	22.9	1.53 (0.3–3.75)
Milk (AFM_1)	57	73.7	0.08 (0.025–0.95)
Milk powder (AFM_1)	15	26.7	0.20 (0.1–0.35)
Pig liver	47	36.2	0.43 (0.2–0.87)
Yellow rice wine	56	3.6	7.50 (5–10)
Fruit wine	5	0.0	
Local beer	65	0.0	

[a] Unless specified otherwise, all aflatoxin values represent AFB_1 levels.
[b] Each of these samples was derived from five separate samples of foods that were then pooled for analysis.

oil and 70 rapeseed oil samples were tested. About 30% and 23% of soybean and rapeseed oil samples, respectively, had detectable levels of aflatoxin, with average contamination being 1.10 μg AFB_1/kg and 1.53 μg AFB_1/kg, for rapeseed and soybean oil, respectively. Peanut oil was rarely consumed in Shanghai.

Milk and milk powder samples were analyzed for AFM_1. A total of 57 milk and 15 milk powder samples were obtained and 74% and 27%, respectively, were found to be contaminated. The ranges of AFM_1 in milk and milk powder were 0.025–0.95 μg/L and 0.1–0.35 μg/L, respectively. Despite the high frequency of samples contaminated with AFM_1, approximately 98% of samples analyzed did not exceed the current United States Food and Drug Administration action level for this contaminant, namely, 0.5 μg AFM_1/L.

Table 2 also contains data on a variety of additional food products, including 47 samples of pig liver, 56 of yellow rice wine, 65 of local beers, and 5 of fruit wine. None of the samples of beer or fruit wine contained detectable amounts of aflatoxin, but 36% of pig liver and 4% of yellow wine samples were contaminated. In general, no significant differences in the level or frequency of aflatoxin contamination were observed in samples collected during different seasons of the year.

Corn is a major source of aflatoxin exposure in Guangxi Autonomous Region and other rural areas of the PRC (5, 16), but it has not been used widely as a staple food in Shanghai for several decades (8). Nonetheless, samples of corn collected in Shanghai markets were analyzed for aflatoxin contamination to compare frequency and levels of contamination with those observed in other regions. Detectable levels of AFB_1 were found in 69% of 39 samples, but the average level of contamination was only 1.14 μg/kg, which was much lower than that found in Guangxi.

Table 3 shows the relative contribution of various AFB-contaminated foods to the total dietary AFB exposure among cohort subjects. Peanuts and soy sauce seem to be the most important sources of dietary AFB exposure in Shanghai, responsible for over 80% of total exposure among cohort subjects.

Table 3 Percent contribution of various foods to the dietary aflatoxin exposure in cohort subjects

Food	%
Peanuts	54.7
Soy sauce	26.6
Rice	9.3
Wheat flour	4.1
Rice wine	1.4
Rapeseed oil	1.7
Soybean oil	1.0
Other miscellaneous foods	1.2

Table 4 provides the distribution of dietary aflatoxin levels among the 267 cohort control subjects whose urinary biomarker status has been determined. There was little difference in estimated dietary aflatoxin intake between the 158 subjects who tested negative for any aflatoxin biomarkers and the 109 subjects who tested positive for one or more aflatoxin biomarkers.

There was no significant association between dietary AFB_1 level and HCC risk among our cohort members (Table 5). We also compared the dietary AFB_1 levels of the 50 HCC cases and 267 matched controls whose HBsAg status had been determined. After adjustment for HBsAg positivity and cigarette smoking (potential confounders), the RRs for mid and upper tertiles relative to the lowest tertile of AFB_1 consumption were 1.5 (95% CL = 0.6, 4.1) and 1.1 (95% CL = 0.4, 2.9), respectively.

Hepatitis B Virus and Aflatoxin Interaction. Table 6 shows the independent and combined effects of HBsAg positivity

Table 4 Dietary aflatoxin level by urinary aflatoxin marker status in 267 control subjects

	Percentiles and mean dietary intake (μg/month)				
	n	P_{25}	P_{50}	P_{75}	Mean
No	158	6.2	8.0	10.9	10.8
Yes[a]	109	5.7	7.9	11.2	10.9
AFB_1-N^7-gua negative	78	6.0	8.1	13.1	11.5
AFB_1-N^7-gua positive	31	4.8	7.0	9.5	9.1

[a] Presence of AFB_1, AFP_1, AFM_1, or AFB_1-N^7-gua.

Table 5 Risk of hepatocellular carcinoma by estimates of dietary aflatoxin B_1 exposure in cohort subjects

Dietary aflatoxin B_1 exposure (μg/yr)	Total person-years	No. of cases	Age- and smoking-adjusted RR (95% CL)
Low (<71)	21,833	14	1.0
Medium (71–113)	23,547	25	1.6 (0.8, 3.1)
High (113+)	24,013	16	0.9 (0.4, 1.9)

and presence of urinary aflatoxins in determining HCC risk among our cohort subjects. HBsAg positivity alone and presence of urinary aflatoxins alone were significantly associated with 7.3- and 3.4-fold increases in HCC risk, respectively. Similar to our previously published results based on a smaller data set (19), there was a strong interaction of these two risk factors on HCC risk; individuals positive for both biomarkers exhibited a 59.4-fold elevation in HCC risk (95% CL = 16.6, 212.0) compared with those who were negative for both markers.

Discussion

In 1989, Yeh et al. (3) evaluated the roles of HBV and AFB_1 in the development of liver cancer in a cohort of 7917 men aged 25–64 years old in Guangxi Autonomous Region, PRC. After 30,188 man-years of observation, 149 deaths were observed, 76 (51%) of which were due to liver cancer. Ninety-one percent (69 of 76) of liver cancer patients were HBsAg-positive at recruitment in contrast to 23% of all members of the study cohort. In addition, the authors observed a 3.5-fold difference in liver cancer mortality among the four communities from which the cohort was drawn, although the population prevalence of HBsAg positivity in these four communities was very similar. When mean AFB_1 ingestion levels in the four subpopulations, estimated through analysis of market samples of commonly eaten foods, were plotted against the corresponding mortality rates of liver cancer, a positive and almost perfectly linear relationship was found. These data indicate that aflatoxin exposure may be an important risk factor for liver cancer in this high-risk population and emphasize the need for improved methods for assessment of individual exposure to characterize and quantify the exposure-risk relationship.

The data described in this and a previous paper (19) provide the first direct support of the notion that AFB-N^7-gua formation provides a very useful marker for assessing individual exposure and is an important step in aflatoxin-induced human hepatic carcinogenesis. Our results clearly indicate that of all urinary aflatoxin biomarkers examined, the presence of this nucleic acid adduct was the best predictor of HCC risk.

Using a structured questionnaire to assess usual dietary intake and a market survey to determine aflatoxin levels in Shanghai foods, we assigned a quantitative aflatoxin exposure level to each study subject and related this variable to urinary aflatoxin biomarker status and to liver cancer risk. We failed to find a dose-dependent association between the dietary index and either liver cancer risk or biomarker status. This stands in marked contrast to the very strong association observed between the presence of urinary aflatoxin biomarkers, especially the AFB-N^7-gua, and risk of liver cancer in the same study population. We think that the poor correlation between urinary aflatoxin biomarker status as determined from a single void urine sample and usual dietary exposure level as computed using interview data probably reflects the large day-to-day variation in urinary aflatoxin levels within a given individual, and the high rate of misclassification in assessing diet by means of a semiquantitative interview instrument. We have shown previously that urinary levels of aflatoxins accurately reflect intake levels of the past 24 hours, at least in areas where exposure to dietary aflatoxin is both common and intense (16, 21). Our data highlight the inadequacy of examining the aflatoxin-liver cancer association strictly by means of dietary assessment and indicate the importance of using biomarkers in determining exposure status of study subjects.

Our group had previously conducted an investigation of molecular dosimetry of AFB-N^7-gua, AFM_1, AFP_1, and AFB_1 in a rural population residing in Guangxi Autonomous Region, PRC (16). An objective of that study was to determine the relationship between accurately quantified aflatoxin intake and total urinary AFB-N^7-gua excretion (measured by the method used in the present study) by individual male and female subjects over a 1-week period. Data obtained in that study integrated day-to-day variations in both intake and excretion of AFB-N^7-gua, and revealed a correlation coefficient of 0.80 ($p < 0.1 \times 10^{-6}$) between dietary intake and urinary DNA adduct excretion. We also showed a relationship between dietary exposure to aflatoxins and urinary excretion of the major aflatoxin-DNA adduct, AFB-N^7-gua, and other metabolites in a similar study of male and female subjects in The Gambia, West Africa (21). These findings support the concept that quantitative measurement of the AFB-N^7-gua adduct in urine provides a reliable biomarker for AFB_1 exposures.

The prevalence of several urinary aflatoxin biomarkers is higher than we previously reported in this population (19), as we have continued to adjust the sensitivity and improve the methodology for detecting these compounds. Such fine-tuning does not affect the validity of the case-control comparisons reported here or previously. An unexplained observation, however, is the rather marked decline in the prevalence of unmetabolized AFB_1 with longer follow-up. Nonetheless, the prevalence of urinary AFB_1 remains relatively high in comparison to previous studies in which we have been involved in the PRC (16) and in The Gambia (21). The decline in the prevalence of AFB_1 in this second data set has occurred among both HCC patients and controls, so that urinary AFB_1 remains a strong predictor of HCC risk. To our knowledge, no systematic investigations of the pharmacokinetics of ingested aflatoxins in humans have been conducted and there is a great need for such evaluations in future studies.

Recent work relating to genetic alterations in human liver tumors has revealed exciting new data about the possible role of AFB_1 in the etiology of HCC. A large proportion

Table 6 Combined effects of HBsAg positivity and presence of urinary aflatoxin biomarkers[a] on risk of hepatocellular carcinoma in Shanghai

HBsAg	Aflatoxin negative			Aflatoxin positive		
	Cases[a]	Controls	RR[b] (95% CL)	Cases	Controls	RR[b] (95% CL)
Negative	5	134	1.0	13	102	3.4 (1.1, 10.0)
Positive	9	24	7.3 (2.2, 24.4)	23	7	59.4 (16.6, 212.0)

[a] AFB_1, AFP_1, AFM_1, and AFB_1-N^7-Gua.
[b] Adjusted for cigarette smoking.

of human liver tumors from China and Southern Africa contain a hotspot guanine to thymine transversion mutation in the tumor suppressor gene, p53, at codon 249. Because this is the predominant mutation induced by activated AFB_1 in both bacterial and mammalian species (30, 31), these observations have led to the suggestion that AFB_1 may have been responsible for the mutation (32, 33). This conjecture was further supported by one recent report indicating that mutations in p53 at codon 249 were prevalent only in liver tumors from persons living in regions of the world where high aflatoxin exposure is known to occur (34). Recent data examining the spectrum of codon 249 mutations in the p53 gene of tumors obtained in Shanghai also find a pattern of transversion mutations that is consistent with the aflatoxin exposure hypothesis (35). On the other hand, other recent studies have disputed the above hypothesis (36–39). The molecular dosimetry methods described in this paper for AFB-N^7-gua in urine may prove useful in elucidating the molecular basis for the role of aflatoxins in HCC pathogenesis.

The field of molecular dosimetry is rapidly expanding. Studies such as this one demonstrate the extraordinary potential of molecular biomarkers for individual risk quantification. The use of this methodology to identify high risk groups will contribute to efficient allocation of resources for reducing exposure and ultimately preventing cancer.

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