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Polycyclic Aromatic Hydrocarbon- and Aflatoxin-albumin Adducts, and Hepatitis B Virus Infection and Hepatocellular Carcinoma in Taiwan

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Abstract

To determine the association between polycyclic aromatic hydrocarbon (PAH) exposure and risk of hepatocellular carcinoma, a case-control study nested within a community based cohort was conducted in Taiwan. Baseline blood samples, collected from a total of 174 HCC cases and 776 matched controls, were used to determine the level of PAH-albumin adducts by competitive enzyme-linked immunosorbent assay. Conditional logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence intervals (CI) to assess the effect of PAH-albumin adducts on risk of HCC. When compared to subjects in the lowest quantile, there was an increase in risk of HCC, with adjusted ORs of 1.0 (95% CI 0.5-2.0), 1.2 (95% CI 0.6-2.4) and 2.0 (95% CI 1.0-4.2) for subjects in the 2nd, 3rd, and 4th quantile, respectively. In addition, significantly increased risk was observed among cases with high AFB_1-albumin adducts with adjusted ORs of 1.9 (95% CI 0.6-6.1), 1.7 (95% CI 0.6-4.9) and 2.1 (95% CI 0.5-8.2) subjects in the 2nd, 3rd, and 4th quantile, respectively, compared with subjects in the 1st quantile. The combination of PAH- and AFB_1-albumin adducts above the mean and hepatitis B virus infection resulted in OR of 8.2 (95%CI=3.6-19.0), compared to those with low adducts and virus negative. These results demonstrate that PAH-albumin adducts are associated with an increased risk of HCC, especially among those with high aflatoxin exposure and that environmental PAH exposure may enhance the hepatic carcinogenic potential of hepatitis B virus infection.

Keywords

Aflatoxin-albumin adducts; PAH-albumin adducts; Hepatocellular carcinoma; Hepatitis B virus

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Introduction

In Taiwan, primary hepatocellular carcinoma (HCC) is the leading cause of cancer death for males and the second for females. Epidemiological evidence suggests that HCC is a complex and multifactorial disease related to both viral infection and chemical carcinogens[1]. Hepatitis B virus (HBV) infection is an important risk factor for HCC in Taiwan[2]; 70-90% of HCC patients have chronic HBV infection as measured by HBV surface antigen (HBsAg)[3]. However, viral infection, including hepatitis C virus, cannot explain the overall risk of developing HCC suggesting that exposure to environmental carcinogens may also be important.

In a previous prospective study in Taiwan, using biomarkers to assess exposure to aflatoxin B_1 (AFB_1), a dietary mold contaminant, we demonstrated that the presence of aflatoxin-albumin adducts as well as urinary aflatoxin metabolites were associated with increased risk of HCC[4]. A viral-chemical interaction was also observed[5]. Similar results were observed in a study carried out in China[6].

PAHs are ubiquitous environmental pollutants produced during all types of combustion of organic material and are found in cigarette smoke[7], polluted air[8] and the diet[9;10]. Exposure to high levels of environmental PAHs, using DNA adducts as a biomarker of exposure as well as the body's metabolic response to exposure, is associated with increased risk of lung and breast cancer[11-15]. More recently, we have demonstrated that higher levels of PAH-DNA adducts are present in adjacent nontumor tissues of HCC cases compared to tissues obtained from surgical controls[16].

PAH exposure that occurs years before disease onset may have a more important impact on hepatocarcinogenesis than exposure around the time of diagnosis, especially for people living in high aflatoxin exposure areas. However, no long-term follow-up study has investigated the carcinogenic effect of environmental PAH exposure on the development of HCC. We previously used a Cancer Screening Program (CSP) cohort to investigate aflatoxin exposure [17]. However, insufficient baseline blood was collected for analysis of PAH-DNA adducts in white blood cells by ELISA. While there are limited data on the relationship between PAH exposure and PAH-albumin adducts, several studies have used PAH-albumin adducts as a surrogate for DNA adduct measurement[18-21]. Unlike DNA, there is no repair mechanism for protein. Thus, chronic low level exposure may be more sensitively detected by monitoring albumin adducts than DNA adducts. We previously measured PAH-DNA in white blood cells and PAH-albumin adducts in foundry workers and found that PAH-albumin adducts were higher in the foundry workers than in the reference group; there was a weak correlation between the two adduct values in the same individual[22].

The specific aim of this study was to assess whether PAH exposure, as assessed by albumin adduct levels in baseline blood samples, is associated with HCC risk. We hypothesized that cases would have higher PAH-albumin adduct levels than controls and that there would also be an interaction between PAH- and AFB_1-albumin adducts, as well as between adducts and HBV infection.

Materials and methods

Study cohort

Subjects are from a community-based CSP cohort recruited in Taiwan. This study was approved by Columbia University's Internal Review Board as well as the research ethnics committee at the College of Public Health, National Taiwan University, Taipei, Taiwan; written informed consent was obtained from all subjects, and strict quality controls and

safeguards were used to protect confidentiality. The cohort characteristics and methods of screening and follow up have been described in more detail previously[23;24]. Briefly, individuals who were between 30 and 64 years old and lived in seven townships in Taiwan, three located on Penghu Islets with the highest HCC incidence in Taiwan, and the other four from Taiwan Island were recruited between July 1990 and June 1992 with a total of 12,020 males and 11,923 females. Participants were personally interviewed based on a structured questionnaire and donated a 20 ml blood sample at recruitment. Aliquots of serum, buffy coat, plasma and red blood cells were separated and stored at -70°C. Biospecimens were transported on dry ice to a central laboratory at the National Taiwan University and were kept at -70°C until transport on dry ice to Columbia University for analysis.

Blood samples were screened by serological markers, including alanine transaminase (ALT), aspartate transaminase (AST), α-fetoprotein (AFP), HBsAg and anti-HCV. Anti-HCV and AFP were assayed in all males and females who resided in Hu-His and Pai-Has on Penghu. The other markers were assayed in samples from all participants. Any participant who had an elevated level of ALT (≥45 IU/ml), AST (≥40 IU/ml), or AFP (≥20 ng/ml), was positive for HBsAg or anti-HCV or had a family history of HCC or liver cirrhosis among first degree relatives was referred for upper abdominal ultrasonography examination. Suspected HCC cases were referred to teaching medical centers for confirmatory diagnosis by computerized tomography, digital subtracted angiogram, aspiration cytology and pathological examination. The criteria for HCC diagnosis included: a histo-pathological examination; a positive lesion detected by at least two different imaging techniques (abdominal ultrasonography, angiogram or computed tomography); or by one imaging technique and a serum AFP level >400 ng/ml.

Intensive follow-up, including abdominal ultrasonographic screening and serum AFP level determination every three months, was carried out on those with ultrasonographic images indicative of liver cirrhosis whereas others were examined annually. Any suspected HCC cases identified during follow-up were referred for confirmatory diagnosis as described above.

Study subjects

A total of 242 cases were newly diagnosed with HCC between February 1991 and June 2004. A total of 1266 controls were randomly selected from cohort subjects who were not affected with HCC through the follow-up period by matching to each case by age (±5 years), gender, residential township and date of recruitment (±3 months). The number of matched controls per case varied depending on the number of eligible controls with available specimens and ranged from 2-6. Baseline blood samples were available from 174 cases and 776 controls and shipped to Columbia University on dry ice for determination of PAH- and AFB_1-albumin adducts. In their distributions by gender, age, residential area and smoking status, subjects for whom specimens were available were similar to those for whom specimens were unavailable.

PAH- and AFB_1-albumin adducts in blood

Albumin was prepared from baseline blood essentially as described [25]. For determination of PAH-albumin, aliquots were treated with acid and heat to release benzo(a)pyrene (BP) tetrols and possibly other PAH, that were then extracted with ethyl acetate. The organic layer was dried and redissolved in phosphate buffered saline (PBS) before analysis by competitive ELISA with monoclonal antibody 8E11 as previously described[26-28]. Briefly, 50 l of albumin extracts, equivalent to 200 g of albumin, were added to 96-well plates (Easywash, Corning, NY) previously coated with 5 ng of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo (a)pyrene (BPDE-I)-DNA by drying a PBS solution. The standard curve was generated by adding serial dilutions of BPDE-I-tetrols in PBS from 5 to 2500 fmol in 50 l into the coated wells followed by 50 l of antibody. The values for each subject were expressed as fmol of PAH

per microgram albumin. Samples with less than 15% inhibition were considered undetectable and assigned a value of 150 fmol/mg albumin.

AFB_1-albumin adduct data was available from our prior study on 73 cases and 185 controls (ref3). Additional samples (101 cases and 591 controls) were assayed by ELISA as previous described[29]. Briefly, enzyme digested albumin was extracted and the equivalent of 200 g of albumin added to 96-well plates previously coated with 3 ng AFB_1 epoxided-modified human serum albumin. Polyclonal antiserum 7 was used and samples with <20% inhibition were considered non-detectable and assigned a value of 1 fmol/mg.

In both assays, samples were assayed by duplicate analysis in duplicate wells. Samples were grouped into case-control sets and assayed on the same day to minimize any effects of day-to-day laboratory variation but with laboratory personnel blinded to case or control status.

Statistical methods

The χ^2 test was used to examine differences in the distributions of variables between cases and controls. A *t*-test was used to evaluate the difference in mean PAH-albumin levels (In fmol/mg albumin) by disease status. PAH and AFB_1-albumin adduct levels were natural log-transformed to normalize the distribution. To examine the independent and combined effects of the formation of PAH-albumin adducts on HCC among subjects with and without chronic HBV infection, the level of PAH-albumin adducts was analyzed as a categorical variable rather than a continuous variable. PAH-albumin adducts were used to divide subjects into different exposure groups: those with detectable versus undetectable adducts and those with adducts levels above the mean value for all controls samples versus those below the mean. To evaluate the dose-response relationship between PAH-albumin adducts and HCC risk, subjects with detectable PAH-albumin adducts were divided into quartiles. The lowest quantile consisted of subjects with nondetectable adducts plus those in the lowest quartile of detectable adducts.

HBsAg, smoking, and alcohol consumption-adjusted ORs and 95% CIs were derived from conditional logistic regression models to indicate the magnitude of the association between levels of PAH-albumin adducts and HCC risk. This relationship was further examined through stratified analyses for the different groups, categorized by aflatoxin exposure. Subjects were divided into two different groups: those with AFB_1-albumin adducts levels above the mean level for all controls and those below the mean. The test for trend of adjusted ORs across strata was based on Wald's test with consecutive scores 1, 2, 3, and 4 assigned to the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} quantile of PAH-albumin adducts. All analyses were performed with SAS software 9.0 (SAS Institute, Cary, NC). All statistical tests were based on two-tailed probability.

Results

The study included 174 (146 males and 28 females) cases and 776 (650 males and 126 females) controls (Table 1). The mean age of both case and control subjects was 53±8. The frequency of positive HBsAg status was significantly higher in cases compared to controls (66.1% versus 24.1%) as was that of positive anti-HCV antibody (21.1% versus 8.1%). The distributions of habitual smoking were similar for cases and controls (50.6% and 49.7%, respectively). There was no significant difference in frequency of alcohol consumption between cases and controls (22.4% and 18.4%, respectively).

The association of PAH-albumin adducts with HCC risk is given in Table 2. The percentage of subjects with detectable PAH-albumin adducts did not significantly differ between cases (48.9%) and controls (47.3%). However, the mean level of ln PAH-albumin adducts was significantly higher in cases than in controls (5.74±0.83 versus 5.59±0.74 ln fmol/mg albumin, $p<0.05$).The mean levels of ln PAH-albumin adducts were highest in male HCC cases (5.76

±0.84 fmol/mg albumin), following by female cases (5.65±0.81 fmol/mg albumin), female controls (5.64±0.81 fmol/mg albumin), and male controls (5.58±0.72 fmol/mg albumin). After adjustment for HBsAg status, smoking and alcohol drinking, the OR for those with PAH-albumin levels above the mean compared to those with levels below the mean was 1.1 (95% CI=0.7-1.8). When PAH-albumin adduct levels were stratified into quantiles, HCC risk increased with adjusted ORs of 1.0 (95%CI=0.5-2.0), 1.2 (95% CI=0.6-2.4) and 2.0 (95% CI=1.0-4.2) for subjects with adducts in the 2nd, 3rd, and 4th quantile, respectively, compared with those in the lowest quantile (p_{trend}=0.08).

To assess the relationship between PAH- and AFB_1-albumin adducts on HCC risk, subjects were divided into two groups based on the mean value of AFB_1-albumin for the controls group: those with high AFB_1-albumin adducts verse those with low AFB_1-albumin adducts. The effect of PAH exposure, comparing subjects with level of PAH–albumin above the mean verse level below the mean, was more pronounced among subjects with high AFB_1-albumin (OR=1.7, 95%CI=0.8-3.3) compared to those with AFB_1-albumin below mean (OR=0.6, 95% CI=0.2-2.1) (Table 3). Similarly, among those with high AFB_1-albumin adducts, there was a dose-response relationship between PAH-albumin adducts and HCC with ORs of 1.9 (95% CI=0.6-6.1), 1.7 (95%CI=0.6-4.7), and 2.1 (95%CI=0.5-8.2), respectively, for adduct levels in the 2nd, 3rd, and 4th quantile of adducts compared to the lowest quantile (p_{trend}<0.05) but not for those with low AFB_1-albumin adducts. In the combined effect analysis, the odds ratio was 1.4 (0.7-2.6) for those with levels of PAH and AFB_1-albumin adducts above the mean compared to those with levels below the mean. The combined effect of PAH- and AFB_1-albumin adduct was more pronounced among cases diagnoses with HCC within the first 5 years of follow up (OR=3.3 95%CI=1.1-9.9, p<0.05) compared to those diagnosed after 5 years (OR=0.7, 95%CI=0.3-1.7) (data not shown).

The combined effect of PAH-, AFB_1-albumin adducts, and HBsAg is given in Table 4. Subjects positive for HBsAg and with both PAH- and AFB_1-albumin adducts above the mean had a significantly increased HCC risk (OR=8.2, 95%CI=3.6-19.0) compared to subjects negative for HBsAg and with both adducts below the mean (*p* for linear trend <0.0001).

Discussion

This case-control study nested within a cancer screening program cohort, demonstrated that PAH-albumin adducts are associated with increased risk for development of HCC, with an OR of 2.0 (95% CI=1.0-4.2) for adduct levels in the highest quantile. In addition, risk was highest in those with the combination of higher PAH-, and AFB_1-albumin adducts and HBV infection. There are more than 100 PAHs and they are well recognized carcinogens in humans[30]. Since PAHs are ubiquitous environmental pollutants, exposure is imprecise using questionnaire assessment. Moreover, misclassification of individual exposure could result in a bias away from the null for putative risk factors. The development of biomarkers measuring levels of the covalent adducts of PAH on DNA and protein, and urinary excretion of PAH metabolites [31] provides a more accurate means of determining exposure on an individual basis. Several studies have supported an association between PAH-DNA adducts and risk of cancer[11; 32-35]. A meta-analysis of six case-control and one prospective study showed that the presence of a high level of bulky DNA adducts is associated with increased risk of lung, oral, and bladder cancer[36]. Two case-control studies found an association of PAH-DNA adducts and risk of breast cancer[11;37]. We also showed, using an immunohistochemical staining assay, that PAH-DNA adducts were higher in surgically removed or needle-biopsy liver tissues of HCC cases than controls in Taiwan[38]. Risk was higher in the highest tertile of tissue level of adducts compared to those in the lowest tertile (OR=3.9, 95%CI=1.0-14.9)[39].

The availability of blood albumin from serum or plasma banks from clinical and epidemiological studies presents unique opportunities for molecular dosimetry that will probably stimulate further studies on PAH-albumin adducts. Albumin adducts have been used to assess exposure to PAH [40;41]and aflatoxin [42] in both the general population and in occupationally exposed individuals. The use of albumin adducts as a biomarker of long-term exposure to PAHs has several advantages: (1) albumin adducts may primarily reflect damage in hepatocytes since albumin synthesis only takes place in the liver, a major site of metabolism [43]; (2) a single measurement of albumin adducts may provide a representative average exposure from all exposure routes over several months; and (3) albumin adducts may account for inter-individual differences in uptake, metabolism, distribution, and elimination. However, in order to justify the measurement of PAH-albumin adducts in large-scale epidemiological studies, the relationship of protein adducts in blood to DNA adducts in target tissue should be determined. In a small study of paraffin tumor tissues and paired plasma samples from 39 HCC patients, we found that the highest PAH-albumin adducts were present in those with the highest mean PAH-DNA adducts in liver, although the results were not statistically significant[44].

The mechanism of the effects of PAH on hepatocarcinogenesis is not fully understood. One hypothetic mechanism is increased production of oxidative stress. A study comparing biomarkers of PAH exposure and oxidative stress found a significant correlation between levels of PAH-albumin and urinary 8-oxo-7′8-dihydro-2′-deoxyguanosine(8-oxodG), a promutagenic lesion[45]. Oxygen radicals can cause extensive damage to DNA, resulting in further cancer development.

Dietary aflatoxin exposure as measured by AFB_1-albumin adducts has been reported as an HCC risk factor in Taiwan[46;47] in our previous studies. There are no prior data on the association between combined PAH and aflatoxin exposure on HCC risk. To the best of our knowledge, this is the first study to use long-term follow-up data to evaluate the joint effect of PAH and AFB_1 on the risk of developing HCC. In the stratified analysis, the association between PAH-albumin adducts and HCC was stronger among subjects with a higher level of AFB_1-albumin adducts (Table 3). Similarly, the biological gradient between PAH-albumin adducts and HCC risk was observed among subjects with higher AFB_1-albumin adducts. In the combined effect analysis, the odds ratio was higher in subjects with both adduct levels above mean, especial for cases diagnosis with HCC within 5 years of blood collection, compared to those with both adduct level below mean (data not shown).

However, our data provide only limited evidence to support the hypothesis that people with high aflatoxin exposure are more vulnerable to the effects of PAH exposure. A limitation of our study was the small cell counts in the stratified analyses that can lead to chance significance or an unstable risk estimate. Although caution should be exercised when interpreting our stratified results, we cannot exclude the possibility that this statistically trend test reflects an underling biological interaction between PAH and aflatoxin exposure in HCC risk. Our results warrant confirmation in larger studies.

Taiwan is an area of hyperendemic HBV infection. About 21.5% of our controls were HBsAg positive, similar to the general population. In addition, environmental PAH exposure is very common; apart from occupational exposure, the general population is mainly exposed from dietary sources[48] and cigarette smoke. It is well know that HBV is not directly cytopathic, and the development of liver cirrhosis and HCC in patients with chronic HBV infection is a multistage process with a multifactorial etiology including interactions between host and environmental factors[49;50]. In Taiwan, the role of PAH exposure in risk of viral-related HCC may be more important among people with aflatoxin exposure since PAHs are widely distributed in the environment and exposure is virtually unavoidable. A significant combined effect of HBV and PAH-, and AFB_1-albumin adducts was observed (Table 4). The precise

molecular mechanism for the enhanced effect of PAH and aflatoxin exposure on the risk of HCC in those with viral infection is unclear. However, liver injury resulting from infection may lead to enhanced cell proliferation that, in the presence of DNA damage from PAH or aflatoxin, results in increased mutations.

This study has several limitations. Study subjects participated in the screening program on a voluntary basis, and there may exist some self-selection bias. The effect of missing data on PAH exposure due to the lack of biospecimens from some study subjects is unclear. However, since both cases and controls were selected from the same cohort, it is likely that the physical and psychological characteristics related to participation are comparable. In addition, the average age of recruitment and the distribution of habitual smoking were similar in those with and without PAH-albumin adducts data.

A limitation of case-control studies in which cases are recruited at the time of diagnosis is that the biomarker may reflect the disease rather than the etiology. In contrast, prospective cohort studies rely on measurements in blood or urine samples collected prior to diagnosis. This rules out the possibility that the biomarker levels were due to metabolic changes associated with an already existing cancer. Several cohort studies have investigated the relationship between adducts and cancer risk. Urinary AFB-N^7-guanine, a biomarker of aflatoxin exposure, was associated with a relative risk of 5.0 (95%CI=2.1-11.8) in samples collected 3-6 years prior to diagnosis [51] while our previous study of the CSP cohort found that aflatoxin metabolites in urine collected up to 5 years prior to diagnosis were associated with an OR of 5.5 (95% CI=1.3-23.4) among male HBsAg positive subjects[52].

In summary, our study provides support for the role of PAH exposure in the development of HCC, especially people with high aflatoxin exposure. It also provides important clues for prevention of HCC. While universal immunization of all newborns is reducing HBV infection rates in Taiwan[53], reduction of exposure to chemical hepatocarcinogens may be another effective way to prevent HCC among those who are infected.

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Abbreviations

Anti-HCV
: antibodies to hepatitis C virus

CSP
: cancer screening program

ELISA
: enzyme-linked immunosorbent assay

HBsAg
: hepatitis B virus surface antigen

HBV
: hepatitis B virus

HCC
: hepatocellular carcinoma

PAH
: polycyclic aromatic hydrocarbon

RR
: relative risk

Table 1

Sociodemographic characteristics of HCC cases and controls

Variable	Cases(N=174) No.	%	Controls(N=776) No.	%	P-value
Age, y (Mean, SD)	53.8(7.9)		53.4(7.9)		
Gender					
Female	28	16.1	126	16.2	0.96
Male	146	83.9	650	83.8	
HBsAg					
Negative	59	33.9	589	75.9	
Positive	115	66.1	187	24.1	<0.0001
AntiHcv†					
Negative	131	78.9	680	91.9	
Positive	35	21.1	60	8.1	<0.0001
Habitual cigatette smoking					
No	86	49.4	390	50.3	0.84
Yes	88	50.6	386	49.7	
Habitual alcohol drinking‡					
No	135	77.6	633	81.6	0.41
Yes	39	22.4	142	18.4	
ln PAH-Albumin, fmol/mg (Mean, SD)	5.74(0.83)		5.59(0.74)		0.02*

p values for the chi-square test for categorical variables except for * indicating *p* value for t-test for continuous variable

†: Anti-HCV data missing for 44 subjects

‡: Habitual alcohol drinking data missing for 1 control

Table 2

PAH-albumin adducts and risk of HCC

PAH_Albumin	Cases		Controls		OR(95% CI)	OR(95% CI)*
	No.	%	No.	%		
Non-detectable	89	51.2	409	52.7	1.0	1.0
Detectable	85	48.9	367	47.3	1.0(0.6-1.5)	0.9(0.5-1.4)
Below mean	93	53.5	467	60.1	1.0	1.0
Above mean	81	46.6	309	39.9	1.3(0.8-2.0)	1.1(0.7-1.8)
Quantile 1‡	97	55.8	501	64.6	1.0	1.0
Quantile 2	16	9.2	90	11.5	1.1(0.6-2.0)	1.0(0.5-2.0)
Quantile 3	23	13.2	93	12.0	1.5(0.8-2.8)	1.2(0.6-2.4)
Quantile 4	38	21.8	92	11.9	2.6(1.3-5.3)†**	2.0(1.0-4.2)§

**:p value <0.01

§: p<0.05

†: p for linear trend<0.05

*: adjusted for HBsAg status, habitual smoking, and alcohol drinking

‡: Subjects with detectable adducts were quartiled and those in the lowest quartile combined with those with nondetectable adducts for Quantile 1

Table 3

PAH-albumin adducts and risk of HCC by AFB_1 exposure

	High AFB_1-albumin				Low AFB_1-albumin			
PAH_Albumin	**Cases No.**	**Controls No.**	**OR(95% CI)**	**OR(95% CI)***	**Cases No.**	**Controls No.**	**OR(95% CI)**	**OR(95% CI)***
Non-detectable	40	233	1.0	1.0	49	176	1.0	1.0
Detectable	46	193	1.4(0.7-2.8)	1.1(0.5-2.3)	39	174	0.7(0.3-1.4)	0.7(0.3-1.4)
Below mean	44	281	1.0	1.0	49	186	1.0	1.0
Above mean	42	145	2.0(1.0-4.3)	1.7(0.8-3.8)	39	164	0.8(0.4-1.7)	0.8(0.3-1.6)
Quantile 1‡	47	301	1.0	1.0	50	200	1.0	1.0
Quantile 2	11	51	1.8(0.6-4.9)	1.9(0.6-6.1)	5	39	0.7(0.2-2.2)	0.6(0.2-2.1)
Quantile 3	12	38	2.2(0.8-6.3)	1.7(0.6-4.9)	11	55	0.7(0.2-2.0)	0.7(0.2-2.1)
Quantile 4	16	36	4.0(1.1-13.8) †**	2.1(0.5-8.2)	22	56	1.4(0.5-3.8)	1.5(0.6-4.0)

**: *p* value <0.01

†: *p* for linear trend<0.05

*: adjusted for HBsAg status, habitual smoking, and alcohol drinking

‡: Subjects with detectable adducts were quartiled and those in the lowest quartile combined with those with nondetectable adducts for Quantile 1

High aflatoxin exposure: subjects with AFB_1-albumin adducts higher than mean of AFB_1-albumin adducts among controls

Low aflatoxin exposure: subjects with AFB_1-albumin adduct lower than mean of AFB_1-albumin adducts among controls

Table 4

The combined effects of HBsAg, PAH and AFB_1-albumin adduct on HCC risk

HBsAg	PAH and AFB_1-Albumin	Cases No.	Controls No.	OR(95% CI)
Negative	Both below mean	15	135	1.0
Negative	One above mean	30	342	0.8(0.4-1.7)
Negative	Two above mean	14	112	1.1(0.5-2.8)
Positive	Both below mean	34	51	5.1(2.5-10.8)*
Positive	One above mean	53	103	4.9(2.5-9.9)*
Positive	Two above mean	28	33	8.2(3.6-19.0)*

ORs adjusted for habitual smoking, and alcohol drinking

*:*p* value <0.0001

p for linear trend<0.0001. Test for trend was done by Wald's test