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Genetic Variations of Hepatitis B Virus and Serum Aflatoxin-Lysine Adduct on High Risk of Hepatocellular Carcinoma in Southern Guangxi, China

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Abstract

Background & Aims—Southern Guangxi area is one of the endemic areas for hepatocellular carcinoma (HCC) in China. This study evaluates the roles of genetic variations of hepatitis B virus (HBV) and aflatoxin B₁ (AFB₁) exposure in the formation of HCC in this high-risk area.

Methods—The study recruited 60 HCC patients and 120 age-, gender-, residency-matched controls. HBV genotype and basic core promoter (BCP) mutations were determined by nested-PCR/direct sequencing. Serum AFB₁-lysine adduct was measured by high performance liquid chromatography-fluorescence detection.

Results—HBV Genotype C was predominant in 75.0% of cases and 84.2% of controls. The 1762^T/1764^A double mutations, 1753^V mutations, and 1752^V mutations were associated with HCC risk evidenced by the adjusted odds ratio (OR) [95% confidence interval (95% CI)] of 3.89 (1.40–10.77), 2.87 (1.49–5.49), and 5.96 (1.75–20.25), respectively. The adjusted OR (95% CI) was 6.94 (1.68–27.78) for subjects with 1762^T/1764^A double mutations and high AFB₁-lysine adduct level; 2.01 (0.24–14.29), for those with only 1762^T/1764^A double mutations; and 4.26 (1.16–15.38) for those with only high AFB₁-lysine adduct level, respectively. The adjusted OR was 5.13 (1.79–14.71) for subjects with 1753^V mutations and high AFB₁-lysine adduct level; 1.20 (0.47–3.08) for those with only 1753^V mutations, and 2.28 (1.01–5.31) for those with high AFB₁-lysine adduct level, respectively.

Conclusions—These data confirmed the association of BCP mutations with HCC risk and the additive effects of 1762^T/1764^A double mutations and 1753^V mutations with dietary AFB₁ exposure in this high-risk area for HCC.

Keywords

hepatitis B virus; genotype; BCP mutations; aflatoxin-lysine adduct; hepatocellular carcinoma

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Introduction

Chronic hepatitis B virus (HBV) infection is the major cause of hepatocellular carcinoma (HCC) worldwide [1]. Although the mechanism is not well defined, variations in the viral genome, including specific genotypes and mutations, are thought to contribute to the formation of HCC [2,3]. To date, eight HBV genotypes are classified (A–H) based on an inter-group divergence of equal or greater than 8% in the complete nucleotide sequence [4]. Infection with the genotype C was associated with greater severity of liver diseases compared to genotype B, although this is still controversial [2,5]. The basic core promoter (BCP) resides in the overlapping HBV functional X gene domain and controls the transcription activity of procure RNA [6]. Mutations in this region were anticipated to influence HBV-induced chronic infection and hepatocarcinogenesis [7,8]. The 1762^T/1764^A double mutations (1762A-to-T and 1764G-to-A) in BCP region were commonly found to be borne by HCC patients in some high-risk populations and were thus suggested as potential biomarkers for hepatocarcinogenesis [3,9–13]. Also, the 1753^V mutations (1753T-to-C/A/G) were associated with the progression of liver disease [14,15]; nevertheless, their role in HCC development, especially in high risk populations, remains controversial [9,14,16].

The Southern Guangxi area is one of the areas in China with endemic HCC, reporting an incidence and mortality rate of more than 50/100,000 people per year [17]. Chronic HBV infection and dietary aflatoxin (AF) exposure are the two major risk factors for HCC in this area [18], while hepatitis C virus (HCV) infection is extremely uncommon [19]. AFB₁-lysine adduct in serum has been considered the most reliable biomarker in monitoring long-term human AFB₁ exposure [20]. The association of this adduct with HCC has been well established in high-risk populations in China [21], while its application in assessing HCC risk in southern Guangxi high-risk population has not yet been reported.

To this end, a case-control study was conducted to assess the role of HBV genetic variations, including HBV genotypes and BCP region mutations, dietary AFB₁ exposure measured by serum AFB₁-lysine adduct level, and their interactions in HCC risk in the Southern Guangxi population.

Materials and Methods

Study subjects, data collection and sample collection

A total of 60 HCC patients were recruited at Guangxi Medical University Cancer Hospital from August 2004 to August 2005. The eligibility criteria for cases were: (1) permanent resident in southern Guangxi area; (2) confirmed diagnosis of HCC as primary tumor by histological (surgical biopsy) or non-histological criteria (positive imaging test results, serum α -fetoprotein greater than 400 ng/ml, and clinical features); (3) no previous diagnosis of other cancers; (4) positive HBV surface antigen (HBsAg) test result when admitted into the hospital, using an enzyme-linked immunosorbent assay (ELISA) kit (Kehua Bio-engineering, Shanghai, China), and (5) negative HCV infection demonstrated by anti-HCV test using a ELISA kit (Kehua Bio-engineering, Shanghai, China). The 120 population-based controls were recruited from an ongoing cohort study conducted in Southern Guangxi area and were frequency-matched to cases on gender, age (in 10-year age groups) and place of

residence (township). The inclusion criteria for these controls were: (1) normal liver and renal function tests as well as electrocardiograms; (2) negative for serum α -fetoprotein; (3) no cancer history; (4) no use of prescribed medications; (5) no pregnancy or lactation for female volunteers; (6) positive HBsAg test, and (7) negative HCV infection by anti-HCV test. Demographic information of cases was obtained from the case history in the hospital archive. Demographic information of controls was collected through in-person interviews by trained interviewers who administered structured questionnaires. Additionally, whole blood samples (7 ml) were taken from cases at the time of admission to hospital and collected from controls at the time of recruitment. Serum samples were separated and stored frozen (-20°C) for shipping to the US laboratory for further analysis. The study protocol was approved by the Institutional Review Board for human subject protections at Texas Tech University and Guangxi Cancer Institute.

Determination of HBV genotypes and mutations

HBV DNA was extracted from 100 μl serum using a commercial viral extraction kit (Qiagen, Valencia, CA). A nested polymerase chain reaction (nPCR) was designed to amplify the major part of HBV X gene including BCP region. First round amplification was carried out using the sense primer H1 5' - GCT TTC ACT TTC TCG CCA AC -3' and antisense primer RH1 5' - TGG AGG CTT GAA CAG TAG GAC -3' with 767bp of PCR product. Second round amplification used first round PCR product as a template and was processed using the sense primer HBX1 5' - GCC AAG TGT TTG CTG ACG C -3' and antisense primer RHBX1 5' - AAA GTT GCA TGG GGC TGG TG -3' with 648 bp of PCR product. HBV amplicons were cleaned-up by a commercial gel purification kit (Qiagen, Valencia, CA) and sequenced subsequently in the ABI 3730XL sequencers (MCLAB, South San Francisco, CA). HBV genotypes were determined by the web based program-Viral Genotyping Tool [22]. For mutation analysis, the sequences generated were compared with non-mutational reference sequence (AY641563) using the BLAST program.

Measurement of serum AFB₁-lysine adduct

Levels of serum AFB₁-lysine adduct were measured by a high performance liquid chromatography (HPLC)-fluorescence detection [23] with modifications. In short, 150 μl of the serum sample was digested by Pronase (Calbiochem, San Diego, CA) for 3 hours at 37 $^{\circ}\text{C}$ and purified with Oasis Max cartridge (Water Co., Milford, MA). After elution with 2% formic acid in methanol, the eluents containing AFB₁-lysine adduct were evaporated until dry and reconstituted with 10% methanol in PBS.

HPLC analysis was carried out on an 1100 liquid chromatography system (Agilent, Wilmington, DE). Chromatographic separation was performed on an Agilent C18 column (5 μM , 250 $\times$ 4.6 mm). The mobile phase consisted of 20 mM ammonium phosphate monobasic (pH 7.2) and methanol in a linear gradient profile. The concentration of AFB₁-lysine adduct was monitored at the wavelength 405 nm (excitation) and 470 nm (emission). The typical retention time of authentic AFB₁-lysine adduct was 12.7 minutes. The results of AFB₁-lysine adduct concentration was adjusted for serum albumin level. The limit of detection was 0.5 pg/mg albumin.

Statistical Analysis

Fisher's exact test, Chi-square test, t-test, or Wilcoxon test were used as appropriate to examine difference between cases and controls. Odds ratio (OR) and 95 percent confidence intervals (95% CI) for HCC risk were calculated using unconditional logistic regression, and a full assessment of potential confounding factors was conducted. First-degree family history of cancer was added as a confounding factor into the final logistic regression model since it was unevenly distributed in cases and controls (Table 1). Age and gender were fitted into the final model too. Other variables were included in the final model if they changed the target odds ratio by 10 percent or more when added to the unadjusted model [24]. Stratified analyses were performed on HBV genotypes, BCP mutations, and AFB₁-lysine adduct levels to evaluate whether HCC risk differed with these factors. Considering the distribution frequency in each subgroup, the mean level of AFB₁-lysine adduct in the control group was used as a cut-off point in the stratification of low or high AFB₁-lysine levels. A *p*-value of less than 0.05 (two-tailed) was considered statistically significant. All data were analyzed by using SAS software version 9.1.5 (SAS Institute Inc., Cary, NC, USA).

Results

As shown in Table 1, the cases and controls, taking into account the frequency-matched age, gender and residency, had similar demographic characteristics except for their first-degree familial history of cancer. Among 14 cases with first-degree family history of cancer, there were 12 liver cancers (85.7%), one esophageal cancer (7.1%), and one rectum cancer (7.1%). Among 13 controls with first-degree family history of cancer, there were 10 liver cancers (76.9%), one esophageal cancer (7.7%), one lung cancer (7.7%), and one gastric cancer (7.7%), respectively.

Genotypes B, C, A+C and B+C were detected with genotype C predominant in both cases and controls (75% and 84.2%) (Table 2). No significant difference was found in the distribution of genotypes between the cases and controls (*p* = 0.280).

The distribution of BCP mutations is presented in Table 3. Detectable 1762^T/1764^A double mutations was found in 91.7% (55/60) cases and 72.5% (88/120) controls with significant difference (*p* = 0.007). The 1762^T single mutation was rare and only found in one genotype C-control. When stratified by HBV genotypes, 1762^T/1764^A double mutations/1762^T single mutation still showed significant difference between cases and controls. In genotype B-cases, the detectable rate of 1762^T/1764^A double mutations was 69.2% (9/13), which was significantly higher than the rate of 23.5% (4/17) in genotype B-controls after adjustment of age, gender, first-degree family history of cancer (*p* = 0.017). The detectable rate of 97.8% (44/45) in genotype C-cases was also significantly higher than the rate in genotype C-controls (83.2%, 84/101) after adjustment for age, gender, first-degree family history of cancer (*p* = 0.044). Of particular interest, a significantly higher rate of 1762^T/1764^A double mutations was found in genotype C-subjects compared to genotype B-subjects (case group: 97.8% vs. 69.2%, *p* = 0.007; control group: 82.2% vs. 23.5%, *p* < 0.001).

The rate of 1753^V mutations was more prevalent in cases than in controls (50.0% vs. 25.8%, *p* = 0.001) (Table 3). Subjects having 1753^V mutations had an increased risk for HCC

(adjusted OR = 2.78, 95% CI: 1.43–5.40). After stratifying by HBV genotype, the rates of 1753^V mutations were significantly higher in genotype C-cases compared to genotype C-controls (60.0%, 27/45 vs. 29.7%, 30/101, $p < 0.001$), whereas these types of mutations were rarely detected in genotype B-cases (2/13) and -controls (1/17), which showed no significant difference ($p = 0.565$). In all 1753^V mutations, 1753^C mutation was the major type detected in both cases (83.3%) and controls (90.3%). There were five other 1753^G mutations (4 cases and 1 control) and three 1753^A mutations (1 case and 2 controls).

Moreover, the co-existence of 1753^V mutations and 1762^T/1764^A double mutations was usually detected. Among 30 cases with 1753^V mutations, 29 (96.7%) had 1762^T/1764^A double mutations; and of the 31 controls with 1753^V mutations, 29 (93.5%) had the double mutations. In addition, combinations of 1753^C mutation and 1762^T/1764^A double mutations were detected in 40.0% (24/60) of cases and 22.5% (27/120) of controls (age-, gender-, family history of cancer-adjusted OR: 2.35, 95% CI: 1.18–4.70, $p = 0.016$).

Moreover, several other mutations in BCP region were detected in study subjects at low frequencies. 1752^V mutation (A-to-T/C/G) was found in 10 (16.7%) cases and 4 (3.3%) controls, showing a significant increase of HCC risk ($p = 0.004$) (Table 3). Mutation at position 1751 (G-to-A) was detected in 1 (1.7%) case. Mutation at position 1757 (G-to-A) was detected in 3 (5.0%) cases and 5 (4.2%) controls. Also, mutation at position 1768 (T-to-A) was found in 3 (5%) cases, and 1 of them was accompanied by a point mutation at position 1766 (C-to-T).

The distribution of serum AFB₁-lysine adduct in HCC cases and controls was illustrated in Figure 1. The average level of serum AFB₁-lysine adduct in cases was 11.10 ± 30.13 (mean $\pm$ SD) pg/mg albumin, with the median level of 4.84 pg/mg albumin (range: 0.54–227.13). The average level of AFB₁-lysine adduct in matched control group was 6.46 ± 8.34 (mean $\pm$ SD) pg/mg albumin with a median level of 3.77 pg/mg albumin (range: 0.79–51.93). Although levels of AFB₁-lysine adduct were higher in cases than controls, no statistically significant difference was found ($p = 0.115$).

Data for both AFB₁-lysine adduct levels and HBV BCP mutations on the risk of developing HCC are presented in Table 4. It was found that there was additive effects of 1762^T/1764^A double mutations and 1753^V mutations, and dietary AFB₁ exposure: the adjusted OR (95% CI) was 6.94 (1.68–27.78) for subjects with 1762^T/1764^A double mutations and high AFB₁-lysine adduct level; it was 2.01 (0.24–14.29) for those with only high AFB₁-lysine adduct level, and 4.26 (1.16–15.38) for those with only 1762^T/1764^A double mutations, respectively. The adjusted OR was 5.13 (1.79–14.71) for subjects with 1753^V mutations and high AFB₁-lysine adduct level; 1.20 (0.47–3.08) for those with only a high AFB₁-lysine adduct level, and 2.28 (1.01–5.31) for those with only 1753^V mutations, respectively.

Discussion

Our study showed a significant association of increased HCC risk with HBV 1762^T/1764^A double mutations, 1753^V mutations, and 1752^V mutations in the Southern Guangxi area, a high-risk area for HCC in China. In addition, we found additive effects of HBV BCP

mutations (1762^T/1764^A double mutations and 1753^V mutations) and high serum AFB₁-lysine adduct level on the risk of developing HCC. To our knowledge, there has been no previously published data exploring the interactions between HBV genomic variations (HBV genotypes, BCP mutations) and AFB₁ exposure biomarkers on HCC risks in the literature.

HBV genotype B and C are the two common genotypes in China [25–27] and Vietnam [28,29], a neighboring country of Southern Guangxi, where more than 80% of subjects have genotypes B or C. A previous study in Guangxi area reported 100% of genotype C in 20 asymptomatic HBV carriers [30], which was higher but still comparable to 84.2% of genotype C in our controls.

Several studies reported that genotype C was associated with a higher HCC risk as compared to genotype B [2,5]. However, the lack of such association was also reported by several other studies. A cohort study following up HBV infected children for 15 years in Taiwan found no increased HCC risk in genotype C-children, and instead, genotype B was the predominant type (74%) in HCC patients [31]. Also, in Qidong, another HCC high-risk area in China, genotype C was found to be predominant but with no difference between HCC patients and controls [32]. Similarly, our study showed this lack of association. Additionally, we found a significantly higher frequency of 1762^T/1764^A double mutations in genotype C-subjects than genotype B-subjects, which is consistent with previous studies [32,33] suggesting a possible linkage between 1762^T/1764^A double mutations and genotype C.

The 1762^T/1764^A double mutations and 1753^V mutations are the two most common mutations in the HBV BCP region. Although the pathogenic mechanism remains unclear, an *in vitro* study found that 1762^T/1764^A double mutations and 1753^V mutations could suppress the expression of HBeAg and induce a modest increase in viral replication rate [7,8]. The association of 1762^T/1764^A double mutations with the increased HCC risk was well established in high-risk populations in China and South Africa [9,10,34,35]. The relationship between 1753^V mutations and HCC development is less studied [14], and remains controversial, especially in high risk populations [9,14,16]. Our study supports the association of both 1762^T/1764^A double mutations and 1753^V mutations with HCC risk. The noteworthy fact is that 1753^V mutations presented at a lower frequency in both cases (50.0%) and controls (25.8%) than 1762^T/1764^A double mutations (91.7% and 74.1%, respectively), and always occurred along with the double mutations. Therefore, it is reasonable to hypothesize that mutations in the BCP region may appear in a time-dependent manner: 1762^T/1764^A double mutations may be involved in the early stage of hepatocarcinogenesis and 1753^V mutations may occur at a later stage. A previous study found that 1762^T/1764^A double mutations were persistently detected in patients over a period of four years before the diagnosis of HCC [3]. Although there is no such longitudinal study for 1753^V mutations, a study reported that the prevalence rate of 1753^V mutations dramatically increased with the severity of liver diseases: 0% in asymptomatic HBV carriers, 9% in chronic hepatitis patients, and 47% in liver cirrhosis or HCC patients [15]. Therefore, 1762^T/1764^A double mutations and 1753^V mutations may serve as valuable biomarkers for HCC risk among individuals with HBV chronic infection, especially in high risk areas.

Besides, our study found that 1752^V mutations in BCP region were also associated to HCC risk. There is no previously published data indicating such an association. Based on the low frequency of 1752^V mutations detected in study subjects (3.3%–16.7%), it seems that these mutations appear later after 1753^V mutations, which suggests these mutations as possible biomarkers for monitoring high-risk population. In a previous study conducted in Qidong, China, 1766^T or 1768^A mutation accompanying 1762^T/1764^A double mutations was associated with increased HCC risk [32]. However, the point mutation at position 1766 and 1768 was rarely found in our study subjects.

The contributing role of AFB₁ to HCC risk is confirmative in many populations [36]. Combined dietary AF exposure and chronic HBV infection were correlated with HCC risk in high-risk areas in China. In a nested case-control study from a Shanghai cohort, the relative risk for HCC in HBsAg and urinary AFB₁ metabolites positive subjects was found 59 times higher than those negative in HBsAg and urinary AFB₁ metabolites [37]. Two other studies in Taiwan confirmed the combinative effect of HBV infection and AFB₁ exposure on HCC risk [38, 39]. As proposed [40], it is possible that chronic HBV infection could fix and enhance DNA damage and oxidative stress induced by AFB₁, and vice versa. Since BCP mutations could alter HBV function/toxicity [7,8], we anticipated that the interaction between HBV and AF on HCC could be aggravated by BCP mutations. Our study indicated the additive effects of serum AFB₁-lysine adduct level with both 1762^T/1764^A double mutations and 1753^V mutation on the risk of developing HCC.

Exposure to AF-contaminated food in Southern Guangxi residents was repeatedly reported over the past several decades [17,18,41,42]. This study confirmed the high AF-exposure in both cases and controls, which were matched with residence to ensure similar dietary intake background. Also, to avoid temporal variation as a modifying factor, the blood samples were collected within the same season of the year in case and control groups. Thus, overwhelming dietary exposure to AF may result in no significant difference of AFB₁-lysine adduct levels between cases and controls in this study.

In summary, this study confirmed the association of BCP mutations with HCC development; based on data from this study and available evidence, the accumulation of 1762^T/1764^A double mutations, 1753^V mutations, and 1752^V mutations may occur at different stages of hepatocarcinogenesis. Limitations of this study include the nature of the case-control design and the limited sample size. Further evaluation and confirmation will be valuable in cohort studies with a larger sample size.

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Abbreviations

HBV	hepatitis B virus
AFB₁	aflatoxin B ₁

HCC	hepatocellular carcinoma
BCP	basic core promoter
OR	odds ratio
CI	confidence interval
AF	aflatoxin; hepatitis C virus (HCV)
IARC	International Agency for Research on Cancer
HBsAg	HBV surface antigen
HPLC	high performance liquid chromatography
nPCR	nested polymerase chain reaction

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Fig. 1. Serum AFB₁-lysine adducts level in cases and controls

The median and interquartile range of percentages in cases and controls were 4.84 (1.88–7.65) pg/mg albumin and 3.77 (2.62–6.78) pg/mg albumin, respectively. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively.

Table 1

Demographic characteristics of HCC cases and controls.

Factor	Cases	Controls	<i>p</i> value
Age (years) (mean $\pm$ SD [*])	41 $\pm$ 9	42 $\pm$ 7	0.300
Gender [n (%)]			
Male	54(90.0)	108(90.0)	
Female	6(10.0)	12(10.0)	1.000
Current cigarette smoker [n (%)]			
Yes	26(43.3)	63(52.5)	
No	34(56.7)	57(47.5)	0.246
Total pack-years of smoking (mean $\pm$ SD)	16.6 $\pm$ 9.0	23.2 $\pm$ 14.1	0.059
Alcohol drinker [n (%)]			
Yes	20(33.3)	36(30.0)	
No	40(66.7)	84(70.0)	0.649
First-degree family history of cancer [n (%)]			
Yes	14(23.3)	13(10.8)	
No	46(76.7)	107(89.2) ^{**}	0.027

^{*} SD, standard deviation

^{**} *p* <0.05 from chi-square test

Table 2

HBV genotypes in HCC cases and controls.

Groups	N	HBV genotypes [n (%)]			
		A	B	C	D
HCC Cases	60	13(21.7)	45(75.0)	0(0.0)	2(3.3)
Controls	120	17(14.2)	101(84.2)	2(1.7)	0(0.0)

Table 3

HBV BCP mutations in HCC cases and controls.

BCP Mutations	Controls (%)	Cases (%)	OR (95% CI) *	Adjusted OR (95%CI)
1762 ^T /1764 ^A mutations	No (1762 ^A /1764 ^G)	5 (8.3)	1.00	1.00
	Yes (1762 ^T /1764 ^G or 1762 ^T /1764 ^A) **	89(74.1)	3.83(1.41–10.41)	3.89(1.40–10.77) #
1753 ^V mutations	No (1753 ^T)	30 (50.0)	1.00	1.00
	Yes (1753 ^{CAG})	30(50.0) ***	2.87 (1.50–5.50)	2.78(1.43–5.40) ##
1752 ^V mutations	No (1752 ^A)	116 (96.7)	1.00	1.00
	Yes (1752 ^{TCC})	4 (3.3)	5.80(1.74–19.37) ***	5.96(1.75–20.25) #

* OR, odds ratio; CI, confidence interval

** 1 single mutation (1762^T/1764^G) was found in control group, all others were 1762^T/1764^A double mutations.

*** $p < 0.05$ from unconditional logistic regression in comparing the distribution of mutations in cases and controls

adjusted for age, gender, first-degree family history of cancer;

adjusted for age, gender, alcohol drinking status, first-degree family history of cancer.

Table 4

HCC risks in relation to HBV mutations and AFB₁ exposure in cases and controls.

BCP Mutations	AFB ₁ -lysine adduct	Controls (%)	Cases (%)	OR (95% CI)	Adjusted OR (95%CI)*
1762 ^T /1764 ^A double mutations or 1762 ^T single mutations					
Concentration (pg/mg alb.)					
No	<6.46	22(18.3)	3 (5.0)	1.00	1.00
	6.46	9 (7.5)	2 (3.3)	1.63 (0.23–11.45)	2.01 (0.24–14.49)
Yes	<6.46	66 (55.0)	36 (60.0)**	4.00(1.12–14.27)	4.26 (1.16–15.38)
	6.46	23(19.2)	19(31.7)**	6.06(1.57–23.37)	6.94 (1.68–27.78)
1752 ^V mutations					
Concentration (pg/mg alb.)					
No	<6.46	65 (54.2)	21(35.0)	1.00	1.00
	6.46	24 (20.0)	9 (15.0)	1.16(0.47–2.89)	1.20 (0.47–3.08)
Yes	<6.46	23(19.2)	18(30.0)**	2.42(1.10–5.33)	2.28(1.01–5.31)
	6.46	8 (6.7)	12(20.0)**	4.65(1.67–12.89)	5.13 (1.79–14.71)

* adjusted for age, gender, first-degree family history of cancer

** $p < 0.05$ from unconditional logistic regression in comparison with baseline group (no mutation and lower than average AFB₁-lysine adduct)