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Global DNA methylation in a population with aflatoxin B_1 exposure

Hui-Chen Wu[1], Qiao Wang[1], Hwai-I Yang[2,3], Wei-Yann Tsai[4,5], Chien-Jen Chen[6,7], and Regina M Santella[1,8,*]

[1]Department of Environmental Health Sciences; Mailman School of Public Health of Columbia University; New York, NY USA; [2]Graduate Institute of Clinical Medical Science; China Medical University; Taichung, Taiwan; [3]Molecular and Genomic Epidemiology Center; China Medical University Hospital; Taichung, Taiwan; [4]Department of Biostatistics; Mailman School of Public Health of Columbia University; New York, NY USA; [5]Department of Statistics; National Chen Kung University; Tainan City, Taiwan; [6]Graduate Institute of Epidemiology and Preventive Medicine; College of Public Health; National Taiwan University; Taipei, Taiwan; [7]Genomics Research Center; Academia Sinica; Taipei, Taiwan; [8]Herbert Irving Comprehensive Cancer Center; Columbia University Medical Center; New York, NY USA

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Abbreviations: 8-oxodG, 8-oxodeoxyguanosine; AFB_1, aflatoxin B_1; Anti-HCV, antibodies to hepatitis C virus; BMI, body mass index; CSP, cancer screening program; ELISA, enzyme-linked immunosorbent assay; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; WBC, white blood cell

We previously reported that global DNA hypomethylation, measured as Sat2 methylation in white blood cells (WBC), and aflatoxin B_1 (AFB_1) exposure were associated with increased hepatocellular carcinoma risk. In this study, we assessed the association between AFB_1 exposure and global DNA methylation. We measured LINE-1 and Sat2 methylation in WBC DNA samples from 1140 cancer free participants of the Cancer Screening Program (CSP) cohort. Blood and urine samples were used to determine the level of AFB_1-albumin (AFB_1-Alb) adducts and urinary AFB_1 metabolites. In continuous models, we found reverse associations of urinary AFB_1 with LINE-1 and Sat2 methylation. The odds ratio (OR) per 1 unit decrease were 1.12 (95% CI = 1.03–1.22) for LINE-1 and 1.48 (95% CI = 1.10–2.00) for Sat2 methylation. When compared with subjects in the highest quartile of LINE-1, we found that individuals in the 2nd and 3rd quartiles were less likely to have detectable AFB_1-Alb adducts, with ORs (95% CI) of 0.61 (0.40–0.93), 0.61 (0.40–0.94), and 1.09 (0.69–1.72), respectively. The OR for detectable AFB_1-Alb was 1.81 (95% CI = 1.15–2.85) for subjects in the lowest quartile of Sat2 methylation. The OR for detection of urinary AFB_1 for those with LINE-1 methylation in the lowest quartile compared with those in the highest quartile was 1.87 (95% CI = 1.15–3.04). The corresponding OR was 1.75 (95% CI = 1.08–2.82) for subjects in the lowest quartile of Sat2 methylation. The association between AFB_1 exposure and global DNA methylation may have implications for the epigenetic effect of AFB_1 on hepatocellular carcinoma development and also suggests that changes in DNA methylation may represent an epigenetic biomarker of dietary AFB_1 exposure.

Introduction

Aflatoxins are naturally occurring mycotoxins produced by only a few *Aspergillus* species of which *A. flavus* and *A. parasiticus* are the most important; they live in hot and humid conditions. Aflatoxins commonly contaminate foods such as peanuts, grain and corn. Aflatoxin B_1 (AFB_1), the most potent hepatocarcinogen, can induce principally G→T mutations, including a *p53* codon 249 hotspot mutation.[1-3] Once ingested, AFB_1 is metabolized by the cytochrome P-450 system at the 8,9-vinyl bond to produce an unstable reactive intermediate, AFB_1-8,9-epoxide.[4] This intermediate can bind covalently to DNA,[5] forming AFB_1-guanine adducts, and to protein, forming AFB_1-albumin and other protein adducts.[6,7] Much epidemiological evidence suggests that dietary exposure to AFB_1 is an important contributor to the high incidence of hepatocellular carcinoma (HCC) in Eastern and South-Eastern Asia, including Taiwan (reviewed in ref. 8). We previously applied two biological markers of AFB_1 exposure, AFB_1-albumin (AFB_1-Alb) adducts and urinary AFB_1 metabolites, to understand the role of AFB_1 exposure on risk of HCC.[9-12] Using data on the analyses of urine and albumin samples banked up to 12 y before diagnosis in the Cancer Screening Program (CSP) in Taiwan, our results suggested that high dietary exposed to AFB_1 was associated with 1.5–1.8 times the risk of HCC compared with low exposure.[12] Other prospective studies also showed strong associations between biological markers of AFB_1 exposure in serum or urine and risk of subsequent HCC.[13-16]

DNA methylation may play an important role in causing disease by silencing genes through hypermethylation or activating genes through hypomethylation.[17] In addition to gene- specific DNA methylation, lower genome-wide aberrant DNA methylation (also referred to as global methylation) in regions that are normally methylated, such as repeats or transposable elements, can lead to genomic instability and altered gene transcription,

*Correspondence to: Regina M Santella; Email: rps1@columbia.edu
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Table 1. Mean levels of LINE-1 and Sat2 by subjects' characteristics

		% Methylation (Mean ± SD)	
Variable	**N**	**LINE1**	**Log(Sat2)**
Age			
Below Mean (≤51)	508	75.5 ± 2.1	4.6 ± 0.6
Above Mean (>51)	632	75.5 ± 2.1	4.6 ± 0.6
P value		0.86	0.15
BMI (kg/m²)			
Below Mean (≤24.1)	591	75.4 ± 2.1	4.6 ± 0.6
Above Mean (>24.1)	549	75.6 ± 2.1	4.6 ± 0.6
P value		0.20	0.31
Gender			
Female	129	75.8 ± 1.8	**4.8 ± 0.5**
Male	1011	75.4 ± 2.2	**4.6 ± 0.6**
P value		0.06	**0.0002**
Smoking			
No	603	75.6 ± 2.0	4.6 ± 0.6
Yes	535	75.5 ± 2.2	4.6 ± 0.6
P value		0.54	0.11
Alcohol			
No	925	75.6 ± 2.0	4.6 ± 0.6
Yes	212	75.3 ± 2.4	4.6 ± 0.7
P value		0.28	0.19
HBsAg			
Negative	686	75.4 ± 2.1	**4.6 ± 0.6**
Positive	454	75.7 ± 2.1	**4.7 ± 0.6**
P value		0.12	**0.001**
Anti-HCV			
Negative	983	75.5 ± 2.1	4.6 ± 0.6
Positive	123	75.4 ± 2.0	4.6 ± 0.6
P value		0.57	0.45

Smoking data missing on 2 subjects. Alcohol data missing on 3 subjects. Anti-HCV data missing on 34 subjects. One-way ANOVA test of the differences in the methylation markers among groups.

impacting normal growth and development.[18] DNA methylation typically occurs in CpG dinucleotides and up to 80% of CpG dinucleotides occurs in repetitive sequences. There are different types of repetitive sequences scattered throughout the genome (e.g., satellite repeats, SINE [short interspersed nuclear element], and LINES [long interspersed nuclear elements]).[19] Changes in DNA methylation patterns are one of the most common molecular alterations in HCC tumor tissues.[20-27] Global hypomethylation is associated with genomic instability and an increased number of mutational events (reviewed in ref. 28). We recently conducted a prospective case-control study nested within the CSP cohort where there is a high incidence of HCC and high AFB_1 exposure, and found that global hypomethylation in baseline white blood cell (WBC) DNA was significantly associated with increased HCC risk later in life.[29] Whether dietary exposure to AFB_1 is associated with WBC DNA methylation change has not been evaluated. Using biospecimens and data from 1140 cancer free participants in the CSP cohort, we tested the hypothesis that AFB_1 exposure is associated with a global decrease in methylation level in WBC DNA.

Results

Table 1 shows the levels of LINE-1 and Sat2 methylation by subjects' characteristics. Males had statistically significant lower Sat2 methylation compared with females (4.6 ± 0.6 vs. 4.8 ± 0.5 for logSat2; $P = 0.0002$). There was no difference in LINE-1 and Sat2 methylation in relation with smoking, alcohol or anti-HCV status. Individuals positive for HBsAg showed statistically higher Sat2 methylation than negative individuals (4.7 ± 0.6 vs. 4.6 ± 0.6 for logSat2, $P = 0.001$).

Table 2 shows the distributions of biomarkers of AFB_1 by subject's characteristics. Both older age and being positive for HBsAg were associated with high AFB_1-Alb adducts but not urinary AFB_1. Male gender and anti-HCV status were associated with both biomarkers of AFB_1. Level of urinary AFB_1 was associated with smoking and alcohol drinking.

The odds ratios (ORs) from logistic regression models of the association between DNA methylation and detection of AFB_1-Alb adducts are presented in **Table 3**. We did not find any association between detection of AFB_1-Alb adducts and LINE-1 or Sat2 methylation as continuous variables. When LINE-1 methylation levels were stratified into quartiles based on all values, we observed a U shaped association with AFB_1-Alb adducts; the adjusted ORs for detection of AFB_1-Alb adducts were 0.61 (95% CI = 0.40–0.93), 0.61 (95% CI = 0.40–0.94) and 1.09 (95% CI = 0.69–1.72) for subjects with adducts in the 3rd, 2nd, and 1st quartile of LINE-1, respectively, compared with those in the highest quartile. The ORs were 1.15 (95% CI = 0.76–1.76), 1.03 (95% CI = 0.68–1.56) and 1.81 (95% CI = 1.15–2.85) for subjects in the 3rd, 2nd, and lowest quartile of Sat2 methylation, respectively, compared with subjects in the highest.

The association of DNA methylation with detection of urinary AFB_1 is given in **Table 4**. We observed evidence of a reverse association between urinary AFB_1 and methylation in LINE-1 and Sat2. The association using a continuous measure was statistically significant (OR per 1 unit decrease in LINE-1 methylation = 1.12, 95% CI = 1.03–1.22). When LINE-1 methylation was modeled in quartiles, we found individuals with detectable urinary AFB_1 were more likely to have lower levels of LINE-1 methylation. The adjusted OR for those in the second and lowest quartile of LINE-1 methylation compared with those in the highest quartile were 1.70 (95% CI = 1.07–2.69) and 1.87 (95% CI = 1.15–3.04), respectively. We also found an association between Sat2 methylation and detection of urinary AFB_1 as a continuous or categorical variable; the OR per 1 unit decrease in natural log Sat2 methylation was 1.48 (95% CI = 1.10–2.00). The ORs were 1.33 (0.85–2.07), 1.26 (0.80–1.97) and 1.75 (1.08–2.82) for those in the 3rd, 2nd, and lowest quartiles, compared with subjects in the highest quartile.

Table 2. Distribution of subject's characteristics by levels of biomarkers of AFB_1 exposure

Variable	AFB_1-albumin				
	Q1(n = 284)	Q2(n = 289)	Q3(n = 284)	Q4(n = 282)	*P*
Age, y (Mean ± SD)	50.0 ± 9.8	50.2 ± 9.9	52.1 ± 8.8	52.0 ± 8.0	0.004
BMI, kg/m² (Mean ± SD)	24.2 ± 2.9	24.0 ± 3.4	24.3 ± 3.4	23.9 ± 3.4	0.45
Gender					
Female/Male	24/261	42/247	41/243	22/260	0.009
Smoking					
No/Yes	155/130	158/129	154/130	136/146	0.33
Alcohol					
No/Yes	229/54	247/42	225/59	224/57	0.20
HBsAg					
Negative/Positive	217/68	127/162	168/116	174/108	0.0001
Anti-HCV					
Negative/Positive	259/25	230/46	240/27	254/25	0.01
	Urinary AFB_1				
	Q1(n = 284)	Q2(n = 283)	Q3(n = 289)	Q4(n = 284)	
Age, y (Mean ± SD)	50.9 ± 9.6	51.2 ± 9.3	50.8 ± 8.6	51.3 ± 9.3	0.92
BMI, kg/m² (Mean ± SD)	23.9 ± 3.1	24.2 ± 3.3	24.4 ± 3.3	23.8 ± 0.4	0.10
Gender					
Female/Male	62/222	25/258	24/265	18/266	<0.0001
Smoking					
No/Yes	176/106	170/113	143/146	114/170	< 0.0001
Alcohol					
No/Yes	241/42	236/46	233/56	215/68	0.03
HBsAg					
Negative/Positive	163/121	183/100	172/117	168/116	0.32
Anti-HCV					
Negative/Positive	228/42	249/26	259/22	247/33	0.03

One-way ANOVA test of the differences in the markers of AFB_1 among groups.

Discussion

We previously reported that global DNA hypomethylation in WBC DNA was significantly associated with increased HCC risk later in life.[29] In this study, we found that decreased LINE-1 or Sat2 methylation was associated with dietary exposed to AFB_1. Those in the higher quartiles of AFB_1-Alb adducts had lower LINE-1 and Sat2 methylation than those in the first quartile. Compared with individuals with undetectable AFB_1 biomarkers, those with detectable AFB_1-Alb adducts or urinary AFB_1 metabolites were more likely to be in the lowest quartile of LINE-1 or Sat2 methylation. Our results provide data in humans supporting the hypothesis that exposure to AFB_1 contributes to decreased global DNA methylation and that AFB_1 may play a role in HCC by altering global DNA methylation status.

Dietary exposure to AFB_1 is one of the major risk factors for HCC in regions of high incidence. Despite the fact that most prospective studies, including ours, report strong associations between biological markers of AFB_1 exposure in serum or urine and risk of subsequent HCC (reviewed in ref. 8), the underlying mechanisms leading to development of HCC are not fully understood. The biotransformation of AFB_1 to the highly reactive exo-8,9-epoxide is the most well-known toxic and carcinogenic pathway.[30] In addition to the formation of adducts, it is believed that AFB_1 acts as a carcinogen by mechanisms that include the formation of reactive oxygen species leading to increased hepatic oxidative damage.[31,32] In our previous studies, we found that AFB_1 exposure was positively associated with level of oxidative DNA damage in humans as measured by urinary 8-oxo-7,8-dihydro-guanine (8-oxoG)[33] as well as urinary 15-F_{2t}-isoprostanes, a marker of lipid peroxidation.[34] Oxidative DNA damage such as 8-oxoG can interfere with the ability of methyltransferases to interact with DNA,[35,36] thus resulting in global decreased DNA methylation. Long-term exposure to oxidative stress has been shown to increase oxidative damage to methylated cytosine residues and depletion in the level of 5-methylcytosine in repeated elements.[36,37]

The effects of changes in epigenetic marks can be as profound as DNA sequence mutations and may increase susceptibility to disease including cancer.[38] Previous studies indicated

Table 3. The association of global DNA methylation with detection of AFB_1-albumin adducts

		AFB_1-Alb adducts			
	% Methylation	Undetectable	Detectable	OR(95%CI)	AdjOR(95%CI)
LINE-1	1 unit decrease	241	899	1.00(0.93–1.07)	1.01(0.94–1.01)
	Above mean (>75.5)	119	459	1.0	1.0
	Below mean (≤75.5)	122	440	1.00(0.70–1.24)	1.04(0.77–1.40)
	Quartile				
	Q4 (>76.79)	50	242	1.0	1.0
	Q3 (75.50–76.79)	69	217	**0.65(0.43–0.98)**	**0.61(0.40–0.93)**
	Q2 (74.20–75.49)	74	217	**0.61(0.41–0.91)**	**0.61(0.40–0.94)**
	Q1 (<74.20)	48	223	0.96(0.62–1.48)	1.09(0.69–1.72)
				P trend = 0.71	*P* trend = 0.75
logSat2	1 unit decrease	237	880	1.08(0.84–1.37)	1.26(0.96–1.66)
	Above mean (>4.6)	128	472	1.0	1.0
	Below mean (≤4.6)	113	427	1.03(0.77–1.36)	1.17(0.87–1.58)
	Quartile				
	Q4 (>5.0)	62	219	1.0	1.0
	Q3 (4.7–5.0)	62	225	1.03(0.69–1.53)	1.15(0.76–1.76)
	Q2 (4.3–4.6)	69	209	0.86(0.58–1.27)	1.03(0.68–1.56)
	Q1 (<4.3)	44	227	1.46(0.95–2.24)	**1.81(1.15–2.85)**
				P trend = 0.20	***P* trend = 0.03**

OR adjusted for age, sex, smoking, alcohol, HBsAg status, and anti-HCV status.

that environmental exposures may not cause immediate effects, but lead to changes in the epigenome that "prime" an individual for later pathogenesis,[39] i.e., increased individual susceptibility. Thus, epigenomic alteration provides a molecular mechanism for the long-term effects of carcinogenic exposures on cancer risks. Animal and a few human studies have demonstrated that dietary factors are one of the most important factors modifying DNA methylation resulting in phenotype changes.[40-43] This evidence, including ours, supports the hypothesis that certain dietary factors may contribute directly to "methylation equilibrium status" by preventing or encouraging either promoter hyper or global hypomethylation and thus have long-term effects on cancer development.[44,45]

Significant geographic variation in the methylation status of various CpG islands was observed when the methylation status of 12 CpG islands in 85 HCC tumors from various geographic locations was investigated,[46] indicating environmental factors may induce DNA methylation aberrations in HCC tumors. We previously measured promoter methylation in candidate genes in 83 HCC tumors and examined its relationship to AFB_1 exposure.[21-24] We found hypermethylation in promoter regions of tumor suppressor genes including *p16*^{INK4A} and *RASSF1A,* correlated with AFB_1-DNA adducts in liver tissues and AFB_1-Alb adducts.[21-24] The association of *RASSF1A* methylation and AFB_1-DNA adducts was also reported in another study.[47] These data also suggest that exposure to dietary AFB_1 may alter methylation status of key genes involved in development of HCC.

In agreement with our previous study,[29] we found individuals positive for HBsAg had statistically significant higher Sat2 methylation, compared with those negative for HBsAg and individuals negative for HBsAg and with detectable AFB_1 exposure had the lowest Sat2 methylation compared with other groups. An in vitro study demonstrated that exposure to HBV leads to upregulation of host DNA methyltransferase genes, resulting in increased methylation in host and viral DNA.[48] To better understand the role of HBV infection and the combined effect of HBV with AFB_1 exposure on HCC risk, it is important to characterize genome-wide DNA methylation among individuals with HBV infection alone and individuals with both HBV infection and AFB_1 exposure.

Men have a higher incidence of HCC than women (reviewed in ref. 49). Many studies have found that LINE-1 methylation was higher in males than in females (reviewed in ref. 50). We found males had lower Sat2 methylation than females. The conflicting data might be due to differences in the distribution of other risk factors including diet that differ by gender in the Taiwanese population. In our present study population, males had higher value of urinary AFB_1 metabolites (60.5 ± 44.9 fmol/mL for males verses 38.9 ± 43.1 for females, $P < 0.0001$). In general, AFB_1-Alb adducts have been recognized as long-term markers of AFB_1 exposure. Because albumin adducts are as long lived as albumin, which has a half-life of 21 d in humans, they provide information on accumulated exposure over a period of 2 to 3 mo.[7,51] The long-term stability of AFB_1-Alb adducts has been

Table 4. The association of global DNA methylation with of detection of urinary AFB_1 metabolites

		Urinary AFB_1			
	% Methylation	**Undetectable**	**Detectable**	**OR(95%CI)**	**AdjOR(95%CI)**
LINE-1	1 unit decrease	197	943	**1.09(1.03–1.20)**	**1.12(1.03–1.22)**
	Above mean (>75.5)	123	455	1.0	1.0
	Below mean (≤75.5)	74	488	**1.78(1.30–2.44)**	**1.75(1.25–2.44)**
	Quartile				
	Q4 (>76.79)	57	235	1.0	1.0
	Q3 (75.50–76.79)	66	220	0.81(0.54–1.21)	1.03(0.67–1.56)
	Q2 (74.20–75.49)	41	250	1.48(0.95–2.29)	**1.70(1.07–2.69)**
	Q1 (<74.20)	33	238	**1.75(1.10–2.79)**	**1.87(1.15–3.04)**
				***P* trend = 0.002**	***P* trend = 0.004**
logSat2	1 unit decrease	195	922	**1.67(1.26–2.21)**	**1.48(1.10–2.00)**
	Above mean (>4.6)	120	480	1.0	1.0
	Below mean (≤4.6)	77	463	**1.50(1.10–2.06)**	1.36(0.98–1.90)
	Quartile				
	Q4 (>5.0)	62	219	1.0	1.0
	Q3 (4.7–5.0)	51	236	1.31(0.87–1.98)	1.33(0.85–2.07)
	Q2 (4.3–4.6)	47	231	1.39(0.91–2.12)	1.26(0.80–1.97)
	Q1 (<4.3)	35	236	**1.91(1.21–3.00)**	**1.75(1.08–2.82)**
				P trend = 0.006	*P* trend = 0.03

OR adjusted for age, sex, smoking, alcohol, HBsAg status, and anti-HCV status.

confirmed,[52] as well as their significant correlation with AFB_1 intake.[53] Measurement of the adduct levels in urine, however, provides a noninvasive means of estimating the levels of AFB_1 exposure, with a correlation of 0.65 found between total dietary AFB_1 intake and urinary AFB_1 extraction.[54] We previously reported that there is a weak linear correlation between levels of AFB_1-Alb adducts and urinary AFB_1 metabolites.[12] Measuring AFB_1-Alb adducts in blood collected at 2 visits on average of 1.6 y apart, we previously found substantial intra-individual variability in AFB_1 exposure in Taiwan, which was likely due to dietary influences.[55]

Our finding of an association of biomarkers of AFB_1 with global DNA hypomethylation in the WBC DNA must be interpreted with caution. First, the putative causal role of AFB_1 exposure in global decreased DNA methylation could not be verified. Biomarkers of AFB_1 and of global DNA methylation were only measured at baseline, making temporal separation of cause and effect difficult. In addition, we previously reported that changes in WBC DNA methylation over time are highly associated with baseline values of the assay and vary by assay type.[56] A longitudinal rather than a cross-sectional study should be conducted to ascertain the causal association between AFB_1 exposure and global DNA hypomethylation. Nevertheless, the association indicates the presence of AFB_1 biomarkers in persons with lower levels of DNA methylation. Further investigations, incorporating prospective and dietary intervention studies, are required to confirm AFB_1-related HCC via altering DNA methylation status.

Second, studies have shown that global DNA methylation differs by blood cell type.[57,58] However, we measured LINE-1 and Sat2 by MethyLight in 48 women with DNA available from four different sources including white blood cells, granulocytes, mononuclear cells and lymphoblastoid cell lines. We found methylation levels were not significantly different among four cell types.[59] It is unclear if the associations we found in the present study were due to the differences in the distributions of cell types among people with different AFB_1 exposure. Currently only one animal study showed percentage changes in T cells and B cells with aflatoxin dosing.[60] Studies are needed to show that AFB_1 exposure does not change blood cell type human populations. Finally, although we observed a significant association of global DNA hypomethylation with AFB_1 exposure, the magnitude of change in the level of DNA methylation is small and the biological significance is unclear.

Despite these limitations, this study has numerous strengths. First, we applied antibody-based methods for measurement of AFB_1 exposure including AFB_1-albumin and urinary AFB_1 metabolites, which provide more accurate assessment of AFB_1 exposure at the individual level than food questionnaires. Second, this study design allowed us to investigate the associations of methylation levels of different types of DNA repetitive elements with AFB_1 exposure in a population with high AFB_1 exposure and high prevalence of HCC, providing potential biological mechanisms for the process of hepatocarcinogenesis.

In summary, we found an inverse association between biomarkers of AFB_1 and biomarkers of global DNA methylation. These results strongly suggest that AFB_1 exposure may result in a decrease in DNA methylation globally. Our results provide information on the application of biomarkers in human populations at high-risk for cancer and that AFB_1-induced global DNA hypomethylation may, in addition to the formation of AFB_1-DNA adducts, have an important role in AFB_1 carcinogenicity.

Materials and Methods

Study cohort. Subjects are from the CSP cohort recruited in Taiwan. This study was approved by Columbia University's Institutional Review Board as well as the Research Ethics Committee of the College of Public Health, National Taiwan University. Written informed consent was obtained from all subjects and strict quality controls and safeguards were used to protect confidentiality. The cohort characteristics have been described in detail previously.[9,12,29,33,34] Briefly, this cohort was originally set up for the evaluation of cancer screening efficacy. Study subjects were voluntary participants in a free cancer screening program implemented in seven urban townships in Taiwan. Individuals who were between 30 and 65 y old were recruited between July 1990 and June 1992 with a total of 12 020 males and 11 923 females. All participants were administrated a structured questionnaire to obtain epidemiological information on socio-demographic characteristics, habits of alcohol intake and cigarette smoking, health history and family history of cancer. Habitual cigarette smoking was defined as having smoked >4 d/wk for at least six months. Information about duration and intensity was also obtained. Habitual alcohol intake was defined as drinking alcohol containing products >4 d/wk for at least six months. For each subject, 15 ml of blood and spot urine were collected, and transported on dry ice to a central laboratory at the National Taiwan University. WBC, plasma and red blood cells were separated and all biospecimens were stored at −70 °C. Hepatitis B virus surface antigen (HBsAg), was tested by radioimmunoassay (Abbott Laboratories); antibody against hepatitis C virus (anti-HCV) was tested by enzyme immunoassay using commercial kits (Abbott Laboratories).

Currently, there is passive follow up of the cohort. New cases are detected by computerized data linkage with profiles on the national cancer registry and death certification systems in Taiwan. The overall follow-up rate is 98%. Through June 2008, 305 cases were identified. We randomly selected controls from cohort subjects who were not affected with HCC through the follow-up period by matching to each case by age (± 5 y), gender, residential township and date of recruitment (± 3 mo). We applied a nested case-control study design to examine the etiology of AFB_1 exposure by measuring biomarker of AFB_1 on HCC risk.[12] Baseline WBCs and urine were shipped to Columbia University on dry ice for determination of biomarkers of AFB_1 exposure and global DNA methylation.

Study subjects. For this study, we used information from 1140 cancer free participants in the CSP cohort for whom we previously measured biomarkers of AFB_1 exposure including AFB_1-Alb adducts and AFB_1 metabolites[9,12] and biomarkers of global DNA methylation including LINE-1 and Sat2 in baseline samples.[29]

AFB_1-albumin adducts in blood and AFB_1 metabolites in urine. Plasma samples were assayed for albumin adducts by enzyme-linked immunosorbent assay (ELISA) as previously described.[9,12] Briefly, 50 μl of albumin extracts, equivalent to 200 μg albumin, were added to plates previously coated with 3 ng of AFB_1 epoxide-modified human serum albumin (HSA). Polyclonal antiserum #7 was used at $1:2 \times 10^5$ dilution and the secondary antiserum, goat anti-rabbit IgG-alkaline phosphatase conjugate, was used at 1:750 dilution. Concentrations of AFB_1-Alb adducts were determined using a standard curve of serially diluted AFB_1 epoxide-modified HSA that had been enzymatically digested. Samples with less than 20% inhibition were considered undetectable and assigned a value of 0.01 fmol/μg. Two controls samples were analyzed with each batch of test samples, a pooled sample of plasma from non-smoking US subjects and a positive control of serum from a rat treated with 1.5 mg AFB_1. The coefficient of variation (CV) of the mean of the two controls was 20% (n = 13).

Urines were assayed essentially as described previously.[9,12] Seppak urine extracts (50 μl) were added to plates previously coated with 3 ng of AFB_1 epoxide-modified bovine serum albumin. AF8E11 was used at 1:1500 dilution and the secondary antiserum, goat anti-mouse alkaline phosphatase was used at 1:1000 dilution. AF8E11 mainly reacts with AFB_1, but there is significant cross-reactivity with a number of aflatoxin derivatives, including AFB_2, AFM_1, AFG_1, and AFP_1.[61] Concentrations of urinary metabolites were determined using a standard curve of serially diluted AFB_1. Samples with less than 20% inhibition were considered undetectable and assigned a value of 1 fmol/ml. A pooled sample of urine from 5 controls was used as a quality control and analyzed with each batch of test samples. The CV was 10% (n = 5).

DNA extraction and bisulfite treatment. Genomic DNA was extracted from WBCs by a salting out procedure. Cells were lysed with SDS in a nuclei lysis buffer and treated with RNase A (final 133 μg/mL) and RNase T1 (final 20 units/mL) to remove RNA. Proteins were co-precipitated with NaCl (330 μL of saturated NaCl added per 1 mL solution) by centrifugation. Genomic DNA was recovered from the supernatant by precipitation with 100% ethanol, washed in 70% ethanol, and dissolved in the Tris-EDTA buffer.

Aliquots of DNA (500 ng) were bisulfite-treated with the EZ DNA methylation kit (Zymo Research). The DNA was resuspended in 20 μL of distilled water and stored at −20 °C until use.

MethyLight assay. We used the sequences of probe and forward and reverse primers of Sat2-M1 described in Weisenberger et al.[62] PCR was performed in a 10 μl reaction volume with 0.3 μM forward and reverse PCR primers, 0.1 μL probe, 3.5 μM $MgCl_2$, using the following PCR program: 95 °C for 10 min, then 55 cycles of 95 °C for 15 sec, followed by 60 °C for 1 min. Assays were run on an ABI Prism 7900 Sequence Detection System (LifeTechnologies). The MethyLight data were expressed as percent of methylated reference (PMR) values and are the mean of duplicates.

PMR = 100% * 2 exp – [Delta Ct (target gene in sample – control gene in sample) – Delta Ct (100% methylated target in reference sample – control gene in reference sample)] Ten percent of subjects were assayed in duplicate, and the inter assay CV for the Sat2 MethyLight assay was 1.0%.

Pyrosequencing assay. Pyrosequencing for LINE-1 methylation levels was performed using PCR and sequencing primers as previously described, with minor modifications to the original protocol.[29,63] Briefly, PCR was performed in a 25 μL reaction mix containing 50 ng bisulfite-converted DNA, 1× Pyromark PCR Master Mix (Qiagen), 1× Coral Load Concentrate (Qiagen), and 0.2 μM forward and reverse primers, using the following PCR program: 95 °C for 15 min, then 44 cycles of 95 °C for 30 sec followed by 56 °C for 30 sec and 72 C for 30 sec, with a final extension at 72 °C for 10 min. Following amplification, the biotinylated PCR products were purified and incubated with the sequencing primer designed to bind adjacent to the CpG sites of interest. Pyrosequencing was conducted using a PyroMark Q24 instrument (Qiagen), with subsequent quantitation of methylation levels determined with the PyroMark Q24 1.010 software. Percent methylation within a sample was subsequently determined by averaging across all three interrogated CpG sites in the analysis. Ten percent of subjects were assayed in duplicate, and the inter assay CV for the LINE-1 pyrosequencing assay was 0.5%.

The laboratory investigator who performed all assays was blinded to epidemiologic data.

Statistical methods. We used one-way ANOVA to test if there is any difference in methylation among groups and performed the Tukey Studentized Range comparisons with Bonferroni correction to determine which groups are different. Because Sat2 methylation was not normally distributed, all statistical analyses used data after natural log transformation. To estimate associations with AFB_1 exposure, we used logistic regression models to calculate odds ratios (ORs) and 95% confidence intervals (Cls). We modeled the associations adjusting for age (years, continuous), sex, HBsAg (Yes vs. No), Anti-HCV (Yes vs. No), smoking (Ever vs. Never), and alcohol consumption (Ever vs. Never) in models. Markers of global DNA methylation were assessed both as continuous measures and as quartiles, based on the distribution among all subjects to accommodate possible nonlinear associations. All analyses were performed with SAS software 9.0 (SAS Institute). All statistical tests were based on two-tailed probability.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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