Carcinogenesis vol.33 no.7 pp.1340–1345, 2012
doi:10.1093/carcin/bgs160
Advance Access Publication May 12, 2012

Global DNA methylation levels in white blood cells as a biomarker for hepatocellular carcinoma risk: a nested case–control study

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 10032, 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, 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 and [8]Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, NY, USA

*To whom correspondence should be addressed. Department of Environmental Health Sciences, Mailman School of Public Health of Columbia University, 630 West 168th St, New York, NY 10032, USA. Tel: +212 305-1996; Fax: +212 305-5328;
Email: rps1@columbia.edu

Global DNA hypomethylation is associated with genomic instability and human cancer and blood DNAs collected at the time of cancer diagnosis have been used to examine the relationship between global methylation and cancer risk. To test the hypothesis that global hypomethylation is associated with increased risk of hepatocellular carcinoma (HCC), we conducted a prospective case–control study nested within a community-based cohort with 16 years of follow-up. We measured methylation levels in Satellite 2 (Sat2) by MethyLight and LINE-1 by pyrosequencing using baseline white blood cell DNA from 305 HCC cases and 1254 matched controls. We found that Sat2 hypomethylation was associated with HCC risk [odds ratio (OR) per unit decrease in natural log Sat2 methylation = 1.77, 95% confidence interval (CI) = 1.06–2.95]. The association was significant among individuals diagnosed with HCC before age 62 (OR per unit decrease in natural log Sat2 methylation = 2.47, 95% CI = 1.06–5.73) but not after (OR = 1.67, 95% CI = 0.84–3.32). We did not observe an association of LINE-1 with HCC overall risk by age at diagnosis. Among carriers of hepatitis B virus surface antigen (HBsAg), with each 1 U decrease in natural log Sat2 methylation level, the OR for HCC increased by 2.19 (95% CI = 1.00–4.89). LINE-1 hypomethylation was associated with about a 2-fold increased risk of HCC, with ORs (95% CI) of 2.39 (1.06–5.39), 2.09 (0.91–4.77) and 2.28 (0.95–5.51, P_{trend} = 0.14) for HBsAg carriers in the third, second and lowest quartile of LINE-1 methylation, respectively compared with carriers in the fourth. These results suggest that global hypomethylation may be a useful biomarker of HCC susceptibility.

Introduction

Hepatocellular carcinoma (HCC), the predominant form of human liver cancer, is the sixth most common tumor worldwide and globally the third leading cause of cancer mortality (1). Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the major etiological risk factors for HCC (2–4). However, environmental exposures to alcohol, aflatoxins and cigarette smoke have also been implicated (2,5,6).

Abbreviations: AFP, α-fetoprotein; ALT, alanine transaminase; AntiHCV, antibodies against hepatitis C virus; AST, aspartate transaminase; CI, confidence interval; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; m5C, 5-methylcytosine; OR: odds ratio; Satellite 2, Sat2; WBC, white blood cell

Hepatocarcinogenesis is characterized by the accumulation of genetic and epigenetic alterations leading to the activation of oncogenes and inactivation or loss of tumor suppressor genes (7–9). DNA methylation is involved in cancer etiology by silencing tumor suppressor genes through hypermethylation or activating oncogenes through demethylation (10,11). Moreover, global decrease in 5-methylcytosine content might contribute to the reactivation of transposable elements and the generation of chromosomal instability (12). Global hypomethylation of liver tumor DNA was observed in a mouse model (13). Total 5-methylcytosine content of human HCC tissues was also about 20–40% less than that of normal liver (14). Till date, the majority of methylation studies have used DNA obtained from tumor and adjacent tissues to assess differences in methylation levels between HCC tumor and histologically normal tissues (14–16). Limited data also suggest that DNA methylation changes in plasma can serve as a marker of circulating liver tumor DNA (17).

Recently, globally decreased DNA methylation in peripheral blood DNA was found to be an independent risk factor for many cancers [reviewed in (18)]. In addition, levels of global DNA methylation in LINE-1 and Alu in white blood cell (WBC) change over time (19–22). Exposure to carcinogens such as benzene is associated with a significant reduction in methylation of particular repetitive elements including LINE-1 in WBC DNA (18,23). Other studies also suggested global methylation changes resulting from environmental exposures including persistent organic pollutants, air pollution and metal-rich particulates (24–26). The main challenge in making inferences from studies examining the association of DNA methylation in WBC with cancer risk and/or risk factors is that most of the studies were retrospective in nature. There is some evidence that decreased methylation of particular repetitive elements such as Alu or LINE-1 in blood collected before cancer diagnosis is associated with increased risk of cancer including gastric later in life (27,28). A prospective design is required to ensure that the biomarker is not influenced by the disease process.

The main aim of this study was to examine whether global hypomethylation, reflected in particular repetitive elements [LINE-1 and Satellite 2 (Sat2)], in peripheral WBC DNA is a predictor of HCC risk. We also examined the association of risk factors of HCC with WBC DNA methylation. We measured methylation levels in Sat2 by MethyLight and LINE-1 by pyrosequencing using baseline WBC DNA from 305 HCCs and 1254 matched controls who participated in a community-based cancer screening cohort (5,6,29).

Materials and methods

Study cohort

Subjects are from the community-based Cancer Screening Program 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 and methods of screening and follow-up have been described in detail previously (5,6,29). Briefly, individuals who were between 30 and 65 years and lived in seven townships in Taiwan 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 regarding epidemiological information and they donated a 20 ml fasting blood sample at recruitment. Biospecimens were transported on dry ice to a central laboratory at the National Taiwan University and stored at −70°C until transport to Columbia University for analysis. Epidemiological information included socio-demographic characteristics, habits of alcohol intake and cigarette smoking, health history and family history of cancers, including HCC. Habitual cigarette smoking was defined as having smoked >4 days/week for at least 6 months. Information about duration and intensity was also obtained. Habitual alcohol intake was defined as drinking alcohol-containing products >4 days/week for at least 6 months.

© The Author 2012. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Downloaded from http://carcin.oxfordjournals.org/ at China Medical University Library on August 7, 2012

At enrollment, blood samples were tested in Taiwan for serological markers, including alanine transaminase (ALT), aspartate transaminase (AST) and α-fetoprotein (AFP). Hepatitis B virus surface antigen (HBsAg) and AFP were tested by radioimmunoassay (Abbott Laboratories, North Chicago, IL); antibody against HCV (anti-HCV) was tested by enzyme immunoassay using commercial kits (Abbott Laboratories, North Chicago, IL). Both ALT and AST levels were determined with a serum chemistry autoanalyzer (Hitachi Model 736; Hitachi Co., Tokyo, Japan) using commercial reagents (Biomerieus, Mercy I'Etoile, France). 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 pathologic examination. The criteria for HCC diagnosis included a histopathological 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 for every 3 months, was carried out during 1999–2005 on those subjects 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. Currently, there is passive follow-up of the cohort. New cases are detected by computerized data linkage with profiles on the National Cancer Registry in Taiwan.

Study subjects. Between February 1991 and June 2008, a total of 305 cases were newly diagnosed with HCC. Cases were primarily identified through linkage to the National Cancer Registry and death certification systems. We randomly selected 1254 controls 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 to 6. Baseline WBCs were shipped to Columbia University on dry ice for the determination of global DNA methylation.

DNA extraction and bisulfite treatment. Genomic DNA was extracted from WBCs by a salting out procedure. Cells were lysed with sodium dodecyl sulfate in a nuclei lysis buffer and treated with RNase A (final 133 µg/ml) and RNase T1 (final 20 U/ml) to remove RNA. Proteins were coprecipitated with NaCl (330 µl of saturated NaCl added per milliliter solution) by centrifugation. Genomic DNA was recovered from the supernatant by precipitation with 100% ethanol, washed in 70% ethanol and dissolved in the Tris-ethylenediaminetetraacetic acid buffer.

Aliquots of DNA (500 ng) were bisulfite treated with the EZ DNA methylation kit (Zymo Research, Orange, California) to convert unmethylated cytosines to uracils while leaving methylated cytosines unmodified. The DNA was resuspended in 20 µl of distilled water and stored at −20°C until use. The laboratory investigator who performed the assays was blinded to epidemiologic data.

MethyLight assay

We used the sequences of probe and forward and reverse primers of Sat2-M1 described in Weisenberger *et al* (30). PCR was performed in a 10 µl reaction volume with 0.3 µM forward and reverse PCR primers, 0.1 µl probe and 3.5 µM $MgCl_2$ using the following PCR program: 95°C for 10 min, then 55 cycles of 95°C for 15 s followed by 60°C for 1 min. Assays were run on an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA).

Universal methylated DNA served as a methylated reference, and an Alu-based control reaction (*AluC4*) was used to measure the levels of input DNA to normalize the signal for each reaction. MethyLight data specific for the repetitive elements were expressed as percent of methylated reference (PMR) values (31).

$$\text{PMR} = 100\% * 2\ \exp -[\text{Delta Ct(target gene in sample} - \text{control gene in sample)} - \text{Delta Ct(100\% methylated target in reference sample} - \text{control gene in reference sample)}]$$

Each MethyLight reaction was performed in duplicate, and the PMR values represent the mean. The assay for Sat2 failed on 17 samples (4 cases and 13 controls). The interassay coefficient of variation for the Sat2 MethyLight assay was 1.0%.

Pyrosequencing assay

Pyrosequencing for LINE-1 methylation levels was carried out using PCR and sequencing primers as described previously, with minor modifications to the original protocol (32). Briefly, PCR was carried out in a 25 µl reaction mix containing 50 ng bisulfite-converted DNA, 1X Pyromark PCR Master Mix (Qiagen, Valencia, CA), 1X 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 s, followed by 56°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. Each set of amplifications included bisulfite-converted CpGenome universal methylated (EMD Millipore, MA), unmethylated and non-template controls.

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. Relative peak height differences were used to calculate the percentage of methylated cytosines at each given site. Percent methylation within a sample was subsequently determined by averaging across all three interrogated CpG sites in the analysis. Non-CpG cytosine residues were used as internal controls to verify efficient sodium bisulfite DNA conversion. The assay for LINE-1 failed on seven samples (three cases and four controls). The interassay coefficient of variation for the LINE-1 pyrosequencing assay was 0.5%.

Statistical methods

We used the χ^2 test for categorical variables and ANOVA for continuous variables to assess the difference in selected characteristics between cases and controls. Because Sat2 methylation was not normally distributed, all statistical analysis used data after natural log transformation. To estimate associations with HCC risk, we used conditional logistic regression models stratified on the matching factors to calculate odds ratios (ORs) and 95% confidence intervals (CIs). We modeled the associations adjusting for age (years, continuous), HBsAg (yes versus no), smoking (ever versus never) and alcohol consumption (ever versus never) in all final models. Markers were assessed both as continuous measures and as quartiles based on the distribution among controls to accommodate possible non-linear associations. 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 respective quartiles. We also stratified cases by HBsAg status, gender and age at HCC diagnosis (≤62 versus >62) in conditional logistic regression models adjusted for age, smoking and alcohol consumption. We used multivariable linear regression models to examine the associations between methylation markers and demographic factors adjusting for case–control status and separately by case–control status. Age, body mass index (BMI), AST, ALT and ATP were assessed as continuous variables and relationships were expressed using standardized β-coefficients. All analyses were performed with SAS software 9.2 (SAS Institute, Cary, NC). All statistical tests were based on two-tailed probability.

Results

Table I presents the distributions of selected characteristics and methylation markers for cases and matched controls. Overall, the distributions of matching factors were similar with no differences in mean ages and BMIs. There were about 66.6 and 68.7% males in cases and controls, respectively. The distributions of habitual smoking and alcohol consumption were also similar. As expected, the percent positive for HBsAg and/or anti-HCV was higher in cases than in matched controls (61.6 versus 25.8% for HBsAg and 21.3 versus 7.9% for anti-HCV). Overall, mean levels of LINE-1 and Sat2 methylation were not statistically significantly different between cases and matched controls.

The ORs from conditional logistic regression models of the association between DNA methylation and HCC overall risk and those ORs stratified by age at HCC diagnosis are presented in Table II. We did not find any association between HCC risk and LINE-1 methylation as a continuous or categorical variable. However, we observed evidence of a reverse association between Sat2 methylation and HCC risk; the association using a continuous measure was statistically significant (OR per unit decrease in natural log Sat2 methylation = 1.77, 95% CI = 1.06–2.95). Thus, the OR for 1% decrease in Sat2 methylation was 5.9. When Sat2 methylation was modeled in quartiles, we found a dose-response relationship (higher HCC risk with lower levels of Sat2 methylation). The adjusted OR for those subjects in the lowest quartile of Sat2 methylation compared with those in the highest quartile was 1.49 (95% CI = 1.00–2.22, P_{trend} = 0.03). The age at diagnosis ranged from 36 to 78, with a mean of 62.0 (SD = 8.6). We did not

Downloaded from http://carcin.oxfordjournals.org/ at China Medical University Library on August 7, 2012

Table I. Socio-demographic characteristics of HCC cases and matched controls

Variable	Cases		Controls		*p*
	n = 305	%	*n* = 1254	%	
Age, years (mean, SD)	53.4 (7.8)		53.1 (7.8)		0.54
BMI, kg/m^2 (mean, SD)	24.6 (3.7)		24.4 (3.6)		0.56
Gender					
Female	101	33.1	392	31.3	0.53
Male	204	66.9	862	68.7	
HBsAg					
Negative	113	37.1	924	73.7	<0.0001
Positive	188	61.6	324	25.8	
Missing	4	1.3	6	0.5	
Anti-HCV					
Negative	205	67.2	993	79.2	<0.0001
Positive	65	21.3	99	7.9	
Missing	35	11.5	162	12.9	
Smoking					
Never	184	60.3	769	61.3	0.84
Ever	121	39.7	484	38.6	
Missing	0	0.0	1	0.1	
Alcohol					
Never	248	81.3	1068	85.1	0.11
Ever	56	18.4	184	14.7	
Missing	1	0.3	2	0.2	
LINE-1, %					
Continuous (mean, SD)	76.2 (2.2)		76.2 (2.1)		0.88
Q1 (<74.6)	58	19.0	295	23.5	0.22
Q2 (74.6–76.4)	89	29.2	312	24.9	
Q3 (76.4–77.8)	83	27.2	327	26.1	
Q4 (>77.8)	72	23.6	316	25.2	
Missing	3	1.0	4	0.3	
logSat2, %					
Continuous (mean, SD)	1.97 (0.28)		1.99 (0.26)		0.17
Q1 (<4.3)	95	31.1	308	24.6	0.09
Q2 (4.4–4.6)	69	22.6	311	24.8	
Q3 (4.6–4.9)	63	20.7	311	24.8	
Q4 (>4.9)	74	24.3	311	24.8	
Missing	4	1.3	13	1.0	

BMI: missing data in two controls and one case.

find any association between HCC risk and LINE-1 methylation by age at diagnosis as a continuous or categorical variable. For Sat2, the association was only significant among individuals diagnosed with HCC before age 62 (OR per unit decrease in natural log Sat2 methylation = 2.47, 95% CI = 1.06–5.73) but not after (OR = 1.67, 95% CI = 0.84–3.32). Thus, the OR for a 1% decrease in Sat2 methylation was 11.8 and 5.3 among individuals diagnosed with HCC before and after age 62, respectively. The ORs were 1.05 (0.53–2.09), 1.36 (0.67–2.76) and 2.37 (1.15–4.88) for those in the third, second and lowest quartiles compared with subjects in the highest quartile (P_{trend} = 0.01). The corresponding ORs were 1.18 (0.68–2.07), 1.15 (0.65–2.04) and 1.46 (0.84–2.52) for those diagnosed with HCC after 62 years.

Table III presents the association of DNA methylation with HCC by HBsAg status. There were 188 HCC cases and 199 matched controls positive for HBsAg and 113 cases and 342 matched controls negative for HBsAg. Among those positive for HBsAg, LINE-1 hypomethylation was associated with about a 2-fold increased risk of HCC. However, the association was statistically significant only in the third quartile (OR = 2.39, 95% CI = 1.06–5.39). In the continuous model, there was a borderline statistically significant association between HCC risk and natural log Sat2 methylation, with an adjusted OR of 2.19 (95% CI = 1.00–4.89). In the categorical model, there was also a slight dose-response relationship between Sat2 methylation and HCC risk. The ORs were 1.23 (0.61–2.46), 1.82 (0.90–3.66) and 1.91 (0.92–3.96) for those in the third, second and lowest quartiles compared with subjects in the highest quartile (P_{trend} = 0.05). There was no statistical association between DNA methylation and HCC risk among those negative for HBsAg.

We also examined the association of DNA methylation with HCC risk by gender. There was no statistically significant association between LINE-1 methylation and HCC risk among either males or females (data not shown). The effect of decreased Sat2 methylation on HCC risk was restricted to males. With 1 U decrease in methylation, the OR for males was 1.87 (95% CI = 1.01–3.47). The ORs were 1.22 (0.73–2.05), 1.43 (0.85–2.40) and 1.73 (1.05–2.86) for those in the third, second and lowest quartiles compared with males in the highest quartile (P_{trend} = 0.03).

Table IV presents the association of DNA methylation with the characteristics of the study subjects. Among controls, males had lower LINE-1 methylation than females. Both ALT and alcohol consumption were inversely associated with LINE-1 methylation. Controls positive for HBsAg had higher Sat2 methylation than those negative for HBsAg. Among HCC cases but not controls, increased BMI was

Table II. Association of DNA methylation with HCC overall risk by age at HCC diagnosis

DNA methylation	Total		Age at diagnosis with HCC	
	Cases/controls		≤62 years	>62 years
	305/1254		126/447	167/582
	OR (95% CI)	AdjOR[a] (95% CI)	AdjOR[a] (95% CI)	AdjOR[a] (95% CI)
LINE-1				
1 U decrease	1.01 (0.93–1.08)	1.02 (0.94–1.10)	1.01 (0.86–1.15)	0.98 (0.87–1.09)
Quartile				
Q4 (>77.8)	1.0	1.0	1.0	1.0
Q3 (76.4–77.8)	1.12 (0.77–1.63)	1.11 (0.74–1.66)	1.47 (0.73–2.97)	0.97 (0.57–1.62)
Q2 (74.6–76.4)	1.28 (0.84–1.95)	1.26 (0.81–1.97)	1.03 (0.47–2.25)	1.37 (0.76–2.47)
Q1[a] (<74.6)	0.88 (0.54–1.43)	0.92 (0.55–1.54)	0.89 (0.39–2.04)	0.80 (0.37–1.74)
	P_{trend} = 0.82	P_{trend} = 0.96	P_{trend} = 0.60	P_{trend} = 0.93
logSat2				
1 U decrease	1.47 (0.90–2.40)	**1.77(1.06–2.95)**	**2.47 (1.06–5.73)**	1.67 (0.84–3.32)
Quartile				
Q4 (>4.9)	1.0	1.0	1.0	1.0
Q3 (4.6–4.9)	0.86 (0.59–1.26)	0.99 (0.66–1.50)	1.05 (0.53–2.09)	1.18 (0.68–2.07)
Q2 (4.3–4.6)	0.96 (0.66–1.41)	1.18 (0.78–1.78)	1.36 (0.67–2.76)	1.15 (0.65–2.04)
Q1[b] (<4.3)	1.35 (0.94–1.95)	**1.49 (1.00–2.22)**	**2.37 (1.15–4.88)**	1.46 (0.84–2.52)
	P_{trend} = 0.07	P_{trend} = 0.03	P_{trend} = 0.01	P_{trend} = 0.20

[a]AdjOR, ORs adjusted for age, smoking, alcohol consumption and HBsAg status.
[b]Hypomethylation.
Bold value indicts the association is statistically significant at the p value of 0.05

Downloaded from http://carcin.oxfordjournals.org/ at China Medical University Library on August 7, 2012

Table III. Association of DNA methylation with HCC by hepatitis B virus infection status

DNA methylation	HBV+		HBV−	
	Cases/controls		Cases/controls	
	(188/199)	AdjOR[a] (95% CI)	(113/342)	AdjOR[a] (95% CI)
LINE-1				
1 U decrease	185/198	1.11 (0.98–1.26)	113/341	0.95 (0.81–1.12)
Quartile				
Q4 (>77.8)	38/47	1.0	33/91	1.0
Q3 (76.4–77.8)	50/48	**2.39 (1.06–5.39)**	32/113	0.77 (0.40–1.49)
Q2 (74.6–76.4)	50/55	2.09 (0.91–4.77)	37/83	1.01 (0.50–2.07)
Q1[b] (74.6)	47/48	2.28 (0.95–5.51)	11/54	0.51 (0.19–1.36)
		P_{trend} = 0.14		P_{trend} = 0.45
log Sat2				
1 U decrease	186/196	**2.19 (1.00–4.89)**	111/338	1.81 (0.68-4.82)
Quartile				
Q4 (>4.9)	47/60	1.0	25/92	1.0
Q3 (4.6–4.9)	35/47	1.23 (0.61–2.46)	27/75	1.29 (0.66–2.54)
Q2 (4.3–4.6)	43/42	1.82 (0.90–3.66)	26/92	1.15 (0.58–2.28)
Q1[b] (<4.3)	61/47	1.91 (0.92–3.96)	33/79	1.75 (0.89–3.42)
		P_{trend} = 0.05		P_{trend} = 0.15

[a]AdjOR, ORs adjusted for age, smoking and alcohol consumption.
[b]Hypomethylation.
Bold value indicts the association is statistically significant at the p value of 0.05

associated with decreased LINE-1 methylation. HCC cases who were ever smokers had higher Sat2 methylation than never smokers.

Discussion

In this study, we demonstrated that decreased Sat2 methylation of WBC DNA was significantly associated with increased HCC risk later in life. The association was significant only among cases diagnosed at younger age. Using an *APC*$^{Min/+}$ mice model, Yamada *et al*. (33) demonstrated that global DNA hypomethylation promotes early-stage tumor formation in liver by tumorigenesis through the mechanisms of loss of heterozygosity and/or chromosomal instability (33). Comparing HCC tumor and non-neoplastic liver tissues, hypomethylation occurred earlier in *Sat2* than in *LINE-1* or *Alu* (34).

Chronic infection with HBV is the major risk factor among the Taiwanese population (2). Integration of HBV DNA and resulting induction of genomic instability is one of the proposed mechanisms suggested for HBV-related liver carcinogenesis (35). The insertion of viral DNA also results in activation of proto-oncogenes (35). HBV DNA integration occurs in human satellite DNA sequences (36); genomic repetitive sequences are suspected to be hot spots for HBV DNA integration (37). Increasing evidence also suggests that viral infection plays a role in regulating DNA methylation in the host genome during hepatocarcinogenesis. An *in vivo* study demonstrated that HBV X protein (HBx) represses E-cadherin expression via activation of DNA methyltranferase 1 (38) and induces global hypomethylation of Sat2 repeat sequences by downregulating DNA (cytosine-5-)-methyltransferase 3 beta (39). Comparing methylation levels at five selected candidate genes in tumor and adjacent liver tissues, higher frequencies of methylation in the promoter of glutathione *S*-transferase pi and E-cadherin were observed in tumor tissues than in normal tissue from patients positive for HBsAg (40). Observation of aberrant DNA methylation in HBV-related HCC tumor tissues was reported in other studies (15,41). These results suggest a mechanism for epigenetic tumorigenesis during HBV-mediated hepatocarcinogenesis. We observed that the significant association of Sat2 hypomethylation with HCC risk was mainly among HBsAg-positive individuals. Among those positive for HBsAg, hypomethylation in LINE-1 was associated with an about 2-fold increased risk of HCC, although the association was statistically significant only in those in the third quartile. Those negative for HBsAg who had Sat2 hypomethylation, had a non-significant increased risk of HCC. This suggests that other risk factors of HCC such as alcohol consumption might decrease Sat2 methylation. However, in our study population, there were only 17 HCC cases negative for HBsAg who consumed alcohol. Thus, we did not have sufficient power to test the association of global DNA hypomethylation and alcohol-related HCC.

A number of risk factors have been associated with global WBC methylation and methylation at specific repetitive elements such as LINE-1 and Alu but there is no prior study of Sat2 [reviewed in (18)]. We found no association for either LINE-1 or Sat2 methylation and age. Age differences in WBC DNA methylation levels are inconsistent. It is believed that the overall impact of age on global DNA methylation accounts for only a small proportion of interindividual variability (21). The fact that environmental factors might affect patterns of methylation was supported by a study of monozygotic twins who are epigenetically indistinguishable during the early years of life, whereas older monozygotic twins exhibit remarkable differences in overall content and genomic distribution of 5-methylcytosine (20). We observed some risk factors for HCC other than age to be associated with LINE-1 methylation. It is well known that men have a higher incidence of HCC than women (42). In contrast with other studies [reviewed in (18)], we found males had lower LINE-1 methylation than females. The conflicting data might be due to differences in the distribution of other risk factors by gender in the Taiwanese population. Decreased LINE-1 methylation associated with higher BMI was only observed in patients with HCC but not in the normal controls in our study population. BMI was associated with decreased LINE-1 methylation in one study, but not others [reviewed in (18)]. Obesity-related methylation changes in WBC DNA were observed using a genome-wide methylation profile (43). Epidemiological evidence has suggested that obesity is associated with HCC (44). More studies are needed to understand if epigenetic change such as LINE-1 hypomethylation is involved in obesity-related hepatocarcinogenesis.

We observed a decrease in LINE-1 methylation among controls who consumed alcohol (Table IV). In an animal model, chronic alcohol consumption induced genomic DNA demethylation in rat colon (45). However, in prior studies, alcohol consumption was associated with decrease in WBC Alu but not LINE-1 methylation [reviewed in (18)]. Alcohol can disrupt folate-mediated one-carbon metabolism by inhibiting the methionine synthase reaction, causing methionine levels to decrease and homocysteine levels to increase (46). The effect of alcohol consumption on LINE-1 methylation found in the current study requires confirmation. A prospective cohort study conducted in China found that blood folate level was inversely associated with serum ALT level (47). Along with our finding of a reverse association of ALT and LINE-1 methylation, these results suggest that hypomethylation due to decreased folate level may be associated with the development of liver damage and HCC.

We found smoking was associated with increased Sat2 not LINE-1 methylation among patients with HCC. Most current studies have not found an effect of smoking on methylation globally or of specific repetitive elements such as LINE-1 and Alu among healthy individuals [reviewed in (18)]. However, a recent study comparing genome-wide WBC DNA methylation profiles among individuals with different smoking status found significantly lower methylation in smokers compared with former and non-smokers (48). Among patients with head and neck squamous cell carcinoma but not healthy controls, each one pack-year increase in lifetime pack-years of smoking was significantly associated with an ~0.02% increase in the LRE1 methylation (49).

It has been hypothesized that DNA methylation evolved as a defense mechanism against DNA pathogens as a way to silence foreign DNA sequence (50,51). In a study of the methylation status of HBV DNA in infected liver tissues, hypomethylation of HBV DNA correlated with HBV expression (52). In our study, increase in Sat2 methylation was found in controls with chronic HBV infection. In another study, among controls, antibody response to human papillomavirus 16 was

Downloaded from http://carcin.oxfordjournals.org/ at China Medical University Library on August 7, 2012

Table IV. Association of DNA methylation with the characteristics of the study subjects

Characteristics	All subjects		Cases		Controls	
	β	P	β	p	β	p
LINE-1						
HCC (yes versus no)	0.05	0.17				
Age (years)	0.02	0.45	0.04	0.57	0.02	0.59
BMI (kg/m^2)	–0.03	0.35	**–0.13**	**0.04**	–0.003	0.94
Gender (M versus F)	**–0.09**	**0.005**	–0.05	0.54	**–0.10**	**0.006**
HBsAg (yes versus no)	0.02	0.62	–0.03	0.76	0.02	0.55
Anti-HCV(yes versus no)	0.01	0.73	–0.01	0.88	0.01	0.74
Smoking (ever versus never)	0.01	0.68	–0.09	0.22	0.04	0.28
Alcohol (ever versus never)	–0.05	0.08	0.02	0.79	**–0.06**	**0.04**
Family history of HCC (yes versus no)	–0.01	0.76	0.05	0.45	–0.03	0.35
AST (IU/ml)	0.09	0.08	0.09	0.35	0.09	0.13
ALT (IU/ml)	**–0.12**	**0.01**	–0.11	0.24	**–0.13**	**0.03**
AFP (ng/ml)	–0.55	0.05	–0.10	0.14	–0.05	0.09
Cirrhosis (yes versus no)	–0.07	0.05	–0.14	0.09	–0.001	0.97
logSat2						
HCC (yes versus no)	–0.02	0.50				
Age (years)	0.04	0.15	0.05	0.41	0.04	0.18
BMI (kg/m^2)	0.02	0.39	–0.003	0.96	0.03	0.38
Gender (M versus F)	–0.02	0.52	–0.07	0.37	–0.01	0.79
HBsAg (yes versus no)	**0.07**	**0.02**	–0.03	0.76	**0.08**	**0.01**
Anti-HCV (yes versus no)	–0.01	0.84	–0.09	0.20	0.03	0.40
Smoking (ever versus never)	0.04	0.27	**0.16**	**0.03**	0.006	0.86
Alcohol (ever versus never)	0.003	0.92	0.002	0.98	0.002	0.94
Family history of HCC (yes versus no)	–0.03	0.36	–0.05	0.40	–0.01	0.78
AST (IU/ml)	–0.04	0.47	–0.03	0.77	–0.04	0.46
ALT (IU/ml)	0.04	0.48	–0.005	0.96	0.06	0.35
AFP (ng/ml)	–0.03	0.23	–0.07	0.31	–0.03	0.40
Cirrhosis (yes versus no)	–0.01	0.69	0.07	0.39	–0.05	0.10

Bold value indicts the association is statistically significant at the p value of 0.05

associated with increased global methylation level (49). Higher methylation may be related to the ability of those individuals to mount a protective immune response to the virus.

Caution should be exercised when interpreting our findings. Although we found Sat2 hypomethylation is a predictor of HCC risk, we cannot exclude the possibility that this association was due to chance because most of the effects are of borderline significance. LINE-1 and Sat2, the most prevalent repetitive sequences, have been used as surrogate markers for genomic methylation levels (32). In the current study, we only observed an association with HCC risk for Sat2 not LINE-1 hypomethylation. It is still unclear if global hypomethylation or hypomethylation in specific repetitive element is involved in the development of HCC. In addition, we were limited to a single measure of DNA methylation. Because WBC progenitor cells are rapidly dividing, they may respond more quickly to factors that influence DNA methylation than cells that turn over more slowly. For this reason, WBC DNA methylation may not identify DNA methylation errors directly indicative of cancer, a process that occurs over decades, but rather may serve as an early biomarker for biological processes that systemically influence DNA methylation.

Despite these limitations, this study has numerous strengths. First, the prospective study design provides causal evidence for the role of global DNA methylation in WBC as a biomarker for cancer susceptibility (18). Second, this study design allowed us to investigate the associations of methylation levels of different types of DNA repetitive elements with HCC risk. Third, we observed that several HCC risk factors are associated with DNA methylation, providing the potential biological mechanisms for the process of hepatocarcinogenesis.

In summary, this study provides evidence that global hypomethylation in WBC DNA is associated with increased HCC risk, with associations being particularly strong among individuals with chronic HBV infection. These findings provide greater understanding of the factors that may modify HCC risk and could lead to the development of a simple non-invasive blood measure of DNA hypomethylation to identify people at high risk of HCC. This study adds to the growing literature, suggesting the potential of using selected markers of global methylation in WBC as biomarkers of cancer risk.

Funding

This work was supported by National Institutes of Health grants (RO1ES005116, P30ES009089, and P30CA013696).

Conflict of Interest Statement: None declared.

References

1. Ferlay, J. *et al*. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer*, **127,** 2893–2917.
2. Chen, C.-J. *et al*. (1991) Effects of hepatitis B virus, alcohol drinking, cigarette smoking and familial tendency on hepatocellular carcinoma. *Hepatology*, **13,** 398–406.

Downloaded from http://carcin.oxfordjournals.org/ at China Medical University Library on August 7, 2012

3. Yang, H.-I. *et al*. (2002) Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N. Engl. J. Med.,* **347,** 168–174.
4. Thein, H.H. *et al*. (2011) Trends in incidence of hepatocellular carcinoma after diagnosis of hepatitis B or C infection: a population-based cohort study, 1992–2007. *J. Viral. Hepat.,* **18,** e232–e241.
5. Wu, H.-C. *et al*. (2009) Aflatoxin B1 exposure, hepatitis B virus infection, and hepatocellular carcinoma in Taiwan. *Cancer Epidemiol. Biomarkers Prev.,* **18,** 846–853.
6. Wang, L.-Y. *et al*. (1996) Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan. *Int. J. Cancer,* **67,** 620–625.
7. Lee, S. *et al*. (2003) Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *Am. J. Pathol.,* **163,** 1371–1378.
8. Herath, N.I. *et al*. (2006) Review of genetic and epigenetic alterations in hepatocarcinogenesis. *J. Gastroenterol. Hepatol.,* **21,** 15–21.
9. Shen, L. *et al*. (2002) DNA methylation and environmental exposures in human hepatocellular carcinoma. *J. Natl Cancer Inst.,* **94,** 755–761.
10. Jones, P.A. *et al*. (2002) The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.,* **3,** 415–428.
11. Tycko, B. (2003) Genetic and epigenetic mosaicism in cancer precursor tissues. *Ann. N. Y. Acad. Sci.,* **983,** 43–54.
12. Ehrlich, M. (2002) DNA methylation in cancer: too much, but also too little. *Oncogene*, **21,** 5400–5413.
13. Ushijima, T. *et al*. (1997) Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. *Proc. Natl Acad. Sci. USA,* **94,** 2284–2289.
14. Lapeyre, J.-N. *et al*. (1979) 5-Methylcytosine content of nuclear DNA during chemical hepatocarcinogenesis and in carcinomas which result. *Biochem. Biophys. Res. Commun.,* **87,** 698–705.
15. Hernandez-Vargas, H. *et al*. (2010) Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. *PLoS One,* **5,** e9749.
16. Christine, P. (2011) Methylation profile as a new tool for classification of hepatocellular carcinoma. *J. Hepatol.,* **54,** 602–603.
17. Zhang, Y.-J. *et al*. (2007) Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. *Clin. Cancer Res.,* **13,** 2378–2384.
18. Wu, H.C, *et al*. (2011) Global DNA methylation levels in girls with and without a family history of breast cancer. *Epigenetics*, **6,** 29–33.
19. Bjornsson, H.T. *et al*. (2008) Intra-individual change over time in DNA methylation with familial clustering. *JAMA,* **299,** 2877–2883.
20. Fraga, M.F. *et al*. (2005) Epigenetic differences arise during the lifetime of monozygotic twins. *Proc. Natl Acad. Sci. USA,* **102,** 10604–10609.
21. Bollati, V. *et al*. (2009) Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech. Ageing Dev.,* **130,** 234–239.
22. Zhu, Z.-Z. *et al*. (2012) Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. *Int. J. Epidemiol.,* **41,** 126–39.
23. Bollati, V. *et al*. (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res.,* **67,** 876–880.
24. Rusiecki, J.A. *et al*. (2008) Global DNA hypomethylation is associated with high serum-persistent organic pollutants in greenlandic inuit. *Environ. Health Perspect.,* **116,** 1547–1552.
25. Baccarelli, A. *et al*. (2009) Rapid DNA methylation changes after exposure to traffic particles. *Am. J. Respir. Crit. Care Med.,* **179,** 572–578.
26. Tarantini, L. *et al*. (2008) Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. *Environ. Health Perspect.,* **117,** 217–222.
27. Gao, Y. *et al*. (2012) Blood leukocyte Alu and LINE-1 methylation and gastric cancer risk in the Shanghai Women’s Health Study. *Br. J. Cancer,* **106,** 585–591.
28. Zhu, Z. *et al*. (2011) Repetitive element hypomethylation in blood leukocyte DNA and cancer incidence, prevalence, and mortality in elderly individuals: the Normative Aging Study. *Cancer Causes Control*, **22,** 437–47.
29. Sun, C.-A. *et al*. (2001) Genetic polymorphisms of glutathione S-transferases M1 and T1 associated with susceptibility to aflatoxin-related hepatocarcinogenesis among chronic hepatitis B carriers: a nested case–control study in Taiwan. *Carcinogenesis,* **22,** 1289–1294.
30. Weisenberger, D.J. *et al*. (2005) Analysis of repetitive element DNA methylation by MethyLight. *Nucl. Acids Res.,* **33,** 6823–6836.
31. Wu, H.-C., *et al*. (2011) Global methylation profiles in DNA from different blood cell types. *Epigenetics,* **6,** 76–85.
32. Yang, A.S., *et al*. (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res.,* **32,** e38.
33. Yamada, Y. *et al*. (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc. Natl Acad. Sci. USA,* **102,** 13580–13585.
34. Lee, H.S. *et al*. (2009) Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clin. Cancer Res.,* **15,** 812–820.
35. Kremsdorf, D. *et al*. (2006) Hepatitis B virus-related hepatocellular carcinoma: paradigms for viral-related human carcinogenesis. *Oncogene,* **25,** 3823–3833.
36. Shaul, Y. *et al*. (1986) Integration of hepatitis B virus DNA in chromosome-specific satellite sequences. *J. Virol*., **59,** 731–734.
37. Berger, I. *et al*. (1987) Integration of hepatitis B virus: analysis of unoccupied sites. *J. Virol.*, **61,** 1180–1186.
38. Lee, J.-O. *et al*. (2005) Hepatitis B virus X protein represses E-cadherin expression via activation of DNA methyltransferase 1. *Oncogene,* **24,** 6617–6625.
39. Park, I. *et al*. (2007) Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein. *Gastroenterology*, **132,** 1476–94.
40. Su, P.-F., *et al*. (2007) Differential DNA methylation associated with hepatitis B virus infection in hepatocellular carcinoma. *Intl. J. Cancer,* **121,** 1257–1264.
41. Lambert, M.-P., *et al*. (2011) Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. *J. Hepatol.,* **54,** 705–715.
42. Lee, C.-M., *et al*. (1999) Age, gender, and local geographic variations of viral etiology of hepatocellular carcinoma in a hyperendemic area for hepatitis B virus infection. *Cancer,* **86,** 143–1150.
43. Wang, X. *et al*. (2010) Obesity related methylation changes in DNA of peripheral blood leukocytes. *BMC Med.,* **8,** 87.
44. Caldwell, S. *et al*. (2004) Obesity and hepatocellular carcinoma. *Gastroenterology*, **127,** S97–S103.
45. Choi, S.-W. *et al*. (1999) Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. *J. Nutr.,* **129,** 1945–1950.
46. Mason, J. *et al*. (2005) Effects of alcohol on folate metabolism: implications for carcinogenesis. *Alcohol*, **35,** 235–241.
47. Welzel, T.M. *et al*. (2007) Blood folate levels and risk of liver damage and hepatocellular carcinoma in a prospective high-risk cohort. *Cancer Epidemiol. Biomarkers Prev.,* **16,** 1279–1282.
48. Breitling, L. *et al*. (2011) Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. *Am. J. Hum. Genet*., **88,** 450–457.
49. Hsiung, D.T. *et al*. (2007) Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiol. Biomarkers Prev.,* **16,** 108–114.
50. Bird, A.P. (1993) Functions for DNA methylation in vertebrates. *Cold Spring Harb. Symp. Quant. Biol.,* **58,** 281–285.
51. Yoder, J.A. *et al*. (1997) Cytosine methylation and the ecology of intragenomic parasites. *Trends Genetics,* **13,** 335–340.
52. Bowyer, S. *et al*. (1987) Expression of the hepatitis B virus genome in chronic hepatitis B carriers and patients with hepatocellular carcinoma. *Proc. Natl Acad. Sci. USA,* **84,** 847–850.

Received March 19, 2012; revised April 20, 2012; accepted April 24, 2012

Downloaded from http://carcin.oxfordjournals.org/ at China Medical University Library on August 7, 2012