Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Association of hypomethylation of LINE-1 repetitive element in blood leukocyte DNA with an increased risk of hepatocellular carcinoma

Jian-zhong DI[§1], Xiao-dong HAN[§1], Wen-ye GU[2], Yu WANG[1], Qi ZHENG[1], Pin ZHANG[†‡1], Hui-min WU[3], Zhong-zheng ZHU[4]

([1]*Department of General Surgery, the Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China*)

([2]*Department of Gastroenterology, Shanghai Guanghua Hospital, Shanghai 200052, China*)

([3]*Department of General Surgery, Tongji Hospital, Tongji University, Shanghai 200065, China*)

([4]*Department of Oncology, No. 113 Hospital of People's Liberation Army, Ningbo 315040, China*)

[†]E-mail: zhangpindjz@163.com

Received Dec. 5, 2010; Revision accepted July 27, 2011; Crosschecked Sept. 12, 2011

Abstract: Global DNA hypomethylation has been associated with increased risk for cancers of the colorectum, bladder, breast, head and neck, and testicular germ cells. The aim of this study was to examine whether global hypomethylation in blood leukocyte DNA is associated with the risk of hepatocellular carcinoma (HCC). A total of 315 HCC cases and 356 age-, sex- and HBsAg status-matched controls were included. Global methylation in blood leukocyte DNA was estimated by analyzing long interspersed element-1 (LINE-1) repeats using bisulfite-polymerase chain reaction (PCR) and pyrosequencing. We observed that the median methylation level in HCC cases (percentage of 5-methylcytosine (5mC)=77.7%) was significantly lower than that in controls (79.5% 5mC) (P=0.004, Wilcoxon rank-sum test). The odds ratios (ORs) of HCC for individuals in the third, second, and first (lowest) quartiles of LINE-1 methylation were 1.1 (95% confidence interval (CI) 0.7–1.8), 1.4 (95% CI 0.8–2.2), and 2.6 (95% CI 1.7–4.1) (P for trend <0.001), respectively, compared to individuals in the fourth (highest) quartile. A 1.9-fold (95% CI 1.4–2.6) increased risk of HCC was observed among individuals with LINE-1 methylation below the median compared to individuals with higher (>median) LINE-1 methylation. Our results demonstrate for the first time that individuals with global hypomethylation measured in LINE-1 repeats in blood leukocyte DNA have an increased risk for HCC. Our data provide the evidence that global hypomethylation detected in the easily obtainable DNA source of blood leukocytes may help identify individuals at risk of HCC.

Key words: Cancer risk, Epigenetics, Global hypomethylation, Hepatocellular carcinoma, LINE-1 repetitive element
doi:10.1631/jzus.B1000422 **Document code:** A **CLC number:** R735.7

1 Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, while ranks the third most common cause of cancer-related death (Parkin *et al.*, 2005; Gomaa *et al.*, 2008). Its overall incidence remains high especially in the developing world and is steadily rising across most of the developed world (Shariff *et al.*, 2009). Chronic infection with hepatitis B or C virus (HBV or HCV), aflatoxin exposure, liver cirrhosis, chronical alcohol consumption, and genetic factors have been characterized to play a major role in the HCC etiology (Morgan *et al.*, 2004; Kim and Lee, 2005; Parkin *et al.*, 2005; Okuda, 2007; Gomaa *et al.*, 2008; Shariff *et al.*, 2009).

‡ Corresponding author
§ The two authors contributed equally to this work

Epigenetic changes in methylation patterns are increasingly implicated in cancer development (Feinberg and Tycko, 2004). Global DNA hypomethylation seems to occur in early neoplasia and has been regarded as an important component of cancer development (Das and Singal, 2004; Zhu *et al.*, 2011). Compared with normal tissue counterparts, lower global DNA methylation was observed in tumor tissues in a broad panel of cancers including HCC (Das and Singal, 2004; Formeister *et al.*, 2010). Animal studies have shown that experimentally-induced hypomethylation can lead to cancer development at multiple sites (Wilson *et al.*, 1984; Thomas and Williams, 1992; Gaudet *et al.*, 2003), indicating the causal role of global DNA hypomethylation in cancer development. In addition, lower levels of global methylation were reported in leukocyte DNA from subjects with colorectal adenoma, the precursor of colorectal cancer (Pufulete *et al.*, 2003; Lim *et al.*, 2008). Further, in healthy populations, global hypomethylation in blood leukocyte DNA has been associated with exposure to environmental risk factors for carcinogenesis, such as benzene, tobacco smoke, persistent organic pollutants, and perfluorooctane sulfonate (Bollati *et al.*, 2007; Rusiecki *et al.*, 2008; Breton *et al.*, 2009; Liu *et al.*, 2010; Wan *et al.*, 2010). Several case-control studies have shown that global hypomethylation measured in blood DNA was associated with an increased risk for cancers of the colorectum, bladder, breast, head and neck, and testicular germ cells (Pufulete *et al.*, 2003; Hsiung *et al.*, 2007; Moore *et al.*, 2008; Choi *et al.*, 2009; Cho *et al.*, 2010; Mirabello *et al.*, 2010), suggesting that global DNA hypomethylation is a potential biomarker of cancer susceptibility.

Global DNA methylation level derives from the overall 5-methylcytosine (5mC) dinucleotide CpG sites in the human genome, about 55% of which consists of repetitive elements (Lander *et al.*, 2001), including approximately 500000 long interspersed element-1 (LINE-1) repeats which represent approximately 17% of the human genome (Cordaux and Batzer, 2009). Because of heavy methylation in normal tissue, high representation throughout the genome, and close correlation with genomic DNA methylation content, LINE-1 methylation status has been used as a surrogate marker for estimating the genomic DNA methylation level (Yang *et al.*, 2004; Weisenberger *et al.*, 2005). In the present case-control study based on 315 HCC cases and 356 controls from a Chinese population, we sought to determine the association between LINE-1 hypomethylation and the risk of HCC.

2 Materials and methods

2.1 Subjects

This is a hospital-based case-control study. A total of 315 newly diagnosed HCC patients were recruited from the Sixth People's Hospital of Shanghai Jiao Tong University and Tongji Hospital of Tongji University, Shanghai, China from February 2003 to October 2009. All cases did not receive chemotherapy or radiation therapy before blood sample collection. Final diagnoses were pathologically confirmed from the specimens obtained by surgery. As a control, blood samples of 356 cancer-free subjects were randomly selected from the individuals who attended for the physical examination at the same hospitals during the period when the case patients were recruited. The controls were frequency-matched to the cases by age (±5 years), sex, and serum HBsAg status. For both case and control subjects, those with other cancers, diabetes, autoimmune disorders, or other major systemic diseases were excluded. Information on age, sex, habits of alcohol drinking and cigarette smoking, and HCC family history was obtained using structured questionnaire through in-person interviews. An ever-drinker was defined as a person who reported drinking alcoholic beverages at least once per week for at least six months. An ever-smoker was defined as a smoker of at least one cigarette per day for at least six months. Chronic infections of HBV and HCV were considered if the seropositivities of HBsAg and anti-HCV were detected, respectively. Tumor characteristics including tumor size, Edmondson-Steiner grade, and tumor stage (tumour-node-metastasis (TNM) staging, 6th Ed. (Lei *et al.*, 2006)) were made on the basis of medical records. Written informed consent was obtained from each participant. The study protocol was approved by the ethics review committee of the Institutional Review Board of the two participant hospitals.

2.2 DNA extraction and bisulfite modification

Genomic DNA of blood leukocytes was extracted using the QIAamp DNA Blood Mini kit (Qiagen, Shanghai, China). Extracted DNA was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo, CA, USA) according to the manufacturer's protocol.

2.3 LINE-1 polymerase chain reaction (PCR) and pyrosequencing

A modified method of PCR pyrosequencing originally described by Yang *et al.* (2004) was performed to quantitate methylation of the repetitive LINE-1 sequences. In brief, PCR was carried out in a 50-μl reaction volume containing 25 μl of GoTaq Green Master mix (Promega, WI, USA), 1 pmol of forward primer (TTT TGA GTT AGG TGT GGG ATA TA), 1 pmol of biotinylated reverse primer (biotin-AAA ATC AAA AAA TTC CCT TTC), and 50 ng of bisulphite-treated DNA. PCR conditions were 40 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. PCR product was bound to streptavidin sepharose beads (Amersham Biosciences, Uppsala, Sweden), and then was purified, washed, denatured, and washed again. Then, 0.3 μmol/L pyrosequencing primer (AGT TAG GTG TGG GAT ATA GT) was annealed to the purified PCR product. Pyrosequencing actions were performed in the PSQ HS 96 Pyrosequencing System. The non-CpG cytosine residues in LINE-1 repetitive sequences, which had been documented to be rarely methylated (Burden *et al.*, 2005), were used as built-in controls to verify bisulfite conversion, and complete conversion of cytosine at non-CpG sites ensured successful bisulfite conversion. The percentage of 5mC at each of three CpG dinucleotide positions in LINE-1 repetitive sequences, as described in detail by Tarantini *et al.* (2009), was measured. The degree of LINE-1 methylation was expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (percentage of 5mC). To validate PCR-pyrosequencing assay, each CpG dinucleotide position was assayed in duplicate and their averages were used in final analysis. The within-sample coefficient of variation was 0.68%, which suggests good reliability in the measurement.

2.4 Statistical analysis

Differences between HCC cases and controls in age, sex, alcohol drinking, cigarette smoking, HBsAg status, anti-HCV status, and HCC family history were evaluated using the χ^2-test. The nonparametric comparisons of median LINE-1 methylation levels between HCC cases and controls were evaluated by Wilcoxon rank-sum test. The association of LINE-1 methylation with HCC risk was estimated using the odds ratios (ORs) and 95% confidence intervals (CIs) from multivariate logistic regression analyses, with adjustment for age, sex, alcohol drinking, cigarette smoking, HBsAg status, anti-HCV status, and HCC family history. Statistical analyses were conducted using the Stata 10.1 (Stata Corp., College Station, TX). All tests were two-sided and a P value of ≤ 0.05 was considered statistically significant.

3 Results

The general characteristics of the HCC cases and controls are shown in Table 1. No significant differences between HCC cases and controls were found in the distribution of age, sex, alcohol drinking, cigarette smoking, or HBsAg status. Compared with controls, more cases were anti-HCV positive (P=0.01) and had HCC family history (P=0.04).

Fig. 1 depicts the distribution of the LINE-1 methylation levels in HCC cases and controls as box plots of the data. The median methylation level (77.7% 5mC) in cases was significantly lower than that (79.5% 5mC) in controls (P=0.004). Logistic regression analysis showed that the levels of LINE-1 methylation were inversely associated with the risk of HCC (Table 2). The ORs of HCC for individuals in the third, second and first (lowest) quartiles of LINE-1 methylation were 1.1 (95% CI 0.7–1.8), 1.4 (95% CI 0.8–2.2) and 2.6 (95% CI 1.7–4.1) (P for trend <0.001), respectively, compared to individuals in the fourth (highest) quartile. A 1.9-fold (95% CI 1.4–2.6) increased risk of HCC was observed among individuals with LINE-1 methylation below the median, compared to individuals with higher (>median) LINE-1 methylation.

Table 1 Characteristics of HCC and control subjects

Variable	Number of subjects[a]		P[b]
	HCC cases (n=315)	Controls (n=356)	
Age			
≤50 years	143 (45.4%)	167 (46.9%)	0.70
>50 years	172 (54.6%)	189 (53.1%)	
Sex			
Female	52 (16.5%)	59 (16.6%)	0.98
Male	263 (83.5%)	297 (83.4%)	
Alcohol drinking			
Never	195 (61.9%)	218 (61.2%)	0.86
Ever	120 (38.1%)	138 (38.8%)	
Cigarette smoking			
Never	171 (54.3%)	206 (57.9%)	0.35
Ever	144 (45.7%)	150 (42.1%)	
HBsAg			
Negative	42 (13.3%)	48 (13.5%)	0.96
Positive	273 (86.7%)	308 (86.5%)	
Anti-HCV			
Negative	298 (94.6%)	350 (98.3%)	0.01
Positive	17 (5.4%)	6 (1.7%)	
HCC family history[c]			
No	295 (93.7%)	345 (96.9%)	0.04
Yes	20 (6.3%)	11 (3.1%)	
Tumor size			
≤5 cm	155 (49.2%)		
>5 cm	160 (50.8%)		
Edmondson-Steiner grade			
I/II	64 (20.3%)		
III/IV	251 (79.7%)		
Tumor stage			
I/II	227 (72.1%)		
III/IV	88 (27.9%)		

[a] Data are expressed as n (%). [b] χ^2-test; [c] Family history of HCC in first-degree relatives

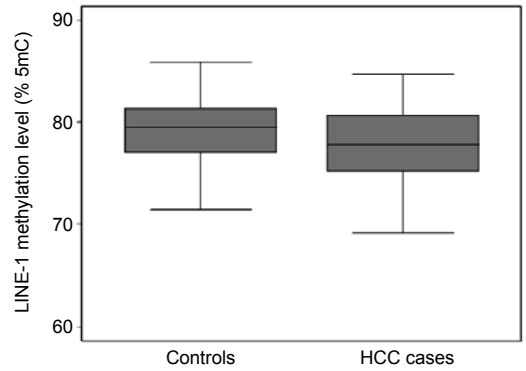

Fig. 1 Comparison of LINE-1 methylation levels measured in blood leukocyte DNA in HCC cases and controls (P=0.004)

Table 2 Association of LINE-1 methylation with risk of HCC

LINE-1 (% 5mC)	Number of subjects[a]		OR (95% CI)[b]	P[b]
	HCC cases	Controls		
Quartile[c]				
Q4	52 (16.5%)	88 (24.7%)	1.0[d]	
Q3	58 (18.4%)	89 (25.0%)	1.1 (0.7–1.8)	0.68
Q2	70 (22.2%)	90 (25.3%)	1.4 (0.8–2.2)	0.22
Q1	135 (42.9%)	89 (25.0%)	2.6 (1.7–4.1)	<0.001
Median[c]				
High	110 (34.9%)	177 (49.7%)	1.0[d]	
Low	205 (65.1%)	179 (50.3%)	1.9 (1.4–2.6)	<0.001

[a] Data are expressed as n (%). [b] Adjusted for age, sex, alcohol drinking, cigarette smoking, HBsAg status, anti-HCV status, and HCC family history; [c] The quartiles and the median of LINE-1 measures were based on values among control subjects; [d] Reference. P for trend <0.001 for the ORs of HCC from the fourth, third, second to first quartiles of LINE-1 methylation

Stratification by HCC risk factors including age (≤50 years, >50 years), sex (female, male), alcohol drinking (never, ever), cigarette smoking (never, ever), HBsAg status (negative, positive), anti-HCV status (negative, positive), and HCC family history (no, yes), produced comparable results, regardless of quartile or median LINE-1 methylation evaluated (data not shown). Additional analyses showed that the interactions between LINE-1 methylation and these HCC risk factors in relation to HCC risk were not statistically significant (data not shown).

No significant associations were observed of age, alcohol drinking, cigarette smoking, HBsAg status, anti-HCV status or HCC family history with LINE-1 methylation, based on control subjects. However, males (79.8% 5mC) had higher LINE-1 methylation than females (78.2% 5mC; P=0.02) among controls, consistent with previous findings (El-Maarri *et al*., 2007; Hsiung *et al*., 2007). No significant correlation was found between LINE-1 methylation and tumor size, Edmondson-Steiner grade, or tumor stage (data not shown).

4 Discussion

Global hypomethylation in blood leukocyte DNA has been reported to correlate with increased

risk for several types of cancer (Pufulete *et al.*, 2003; Hsiung *et al.*, 2007; Moore *et al.*, 2008; Choi *et al.*, 2009; Cho *et al.*, 2010; Mirabello *et al.*, 2010). In the present study, we demonstrated for the first time that individuals with global hypomethylation measured in LINE-1 repeats in leukocyte DNA had an increased HCC risk. Taken together, these findings support the idea that there exist common epigenetic basis for the pathogenesis of these different cancers. DNA methylation is a reversible epigenetic mechanism and DNA methylation patterns can be changed in response to exposure to exogenous and endogenous factors (Hsieh, 2000; Gluckman *et al.*, 2008). Global hypomethylation in leukocyte DNA has been reported to be associated with exposure to risk factors for carcinogenesis (Bollati *et al.*, 2007; Rusiecki *et al.*, 2008; Breton *et al.*, 2009; Liu *et al.*, 2010; Wan *et al.*, 2010), suggesting that leukocyte DNA hypomethylation may reflect cumulative effects from carcinogenic exposures. On the other hand, global methylation change has been indicated to be partly under genetic control (Bjornsson *et al.*, 2008; Hillemacher *et al.*, 2008), suggesting that DNA hypomethylation in leukocyte DNA may also reflect transgenerational risks for common human diseases including cancers (Bjornsson *et al.*, 2004). Thus, global methylation levels in leukocyte DNA could provide a useful biomarker of susceptibility to certain cancer types.

Global DNA hypomethylation has been proposed to contribute to activation of oncogenes and genomic instability in cancer tissues, thereby causing aberrant activation of a wide spectrum of genes, formation of abnormal chromosomal structures and increased mutation rates that convey various growth advantages (Gaudet *et al.*, 2003; Feinberg *et al.*, 2006). LINE-1 hypomethylation is often found in association with hypermethylation of specific genes in cancer tissues (Cho *et al.*, 2007; Ogino *et al.*, 2008). Thus, global DNA hypomethylation may also contribute to carcinogenesis by inactivating tumor suppression genes. Moreover, LINE-1 methylation status by itself is likely to have biological effects, which may contribute directly to carcinogenesis through new retrotransposon insertions in human genomes (Iskow *et al.*, 2010). Global DNA hypomethylation has been demonstrated to lead to the transcriptional activation of LINE-1 promoters, which can alter the transcriptome both in primary bladder tumors and in their premalignant urothelium counterparts (Wolff *et al.*, 2010). These findings suggested that hypomethylation of LINE-1 may play a role not only in cancer but also in cancer predisposition.

Our study had the advantages of being based on a relatively large sample size, careful matching of controls to HCC cases by age, sex and HBsAg status, and pyrosequencing-based quantitative analysis which produced individual measures of methylation at three CpG dinucleotide positions, thus more accurately reflecting DNA methylation in the region. Several limitations in the present study should be noted. Firstly, this was a hospital-based case-control study, which may result in selection bias of participants. Moreover, given that DNA methylation in cancer patients was measured after cancer diagnosis and DNA hypomethylation could be detected in sera as well as HCC cells in peripheral blood of HCC cases (Tangkijvanich *et al.*, 2007; Lee *et al.*, 2009), blood DNA hypomethylation in cancer patients we observed may partly represent the methylation level of DNA derived from HCC cells. As degree of hypomethylation in tumor cells and number of HCC cells in circulating peripheral blood progress according to increased tumor stage, further analyses of LINE-1 methylation in relation to HCC risk were repeated using cases only with early stage of tumor (Stage I/II), which showed no major differences in risk estimates from the results based on the total number of cases (data not shown). In this context, the potentially-confounding effect of contaminating DNA from HCC cells on the association between LINE-1 hypomethylation and HCC risk observed in the present study would be minimal. However, further prospective studies using HCC-free subjects are needed to verify the risk-effect of global hypomethylation in blood DNA on HCC development. Secondly, information on diet was not available here, which may also influence methylation levels in genomic DNA (Suzuki *et al.*, 2006; Schernhammer *et al.*, 2010). Lastly, although LINE-1 repetitive elements have been used as a surrogate for global DNA methylation content, its methylation level is not equivalent to global DNA methylation content (Yang *et al.*, 2004; Choi *et al.*, 2009). Moreover, PCR primers were designed towards a consensus LINE-1 sequence and allowed for the amplification of only a small pool of LINE-1 repetitive elements dispersed

throughout the genome (Yang *et al*., 2004).

In conclusion, global hypomethylation measured in LINE-1 repeats in blood leukocyte DNA shows an increased risk for HCC. Our data provide the evidence that global hypomethylation detected in the easily obtainable DNA source of blood leukocytes may help identify individuals at risk for HCC.

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