

Role of Serum LINE-1 Hypomethylation in Patients with Hepatocellular Carcinoma

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ABSTRACT

Purpose: Global hypomethylation is one of the most consistent epigenetic changes in hepatocellular carcinoma (HCC), and may play crucial roles in multistage hepatocarcinogenesis. However, the clinical implications of this epigenetic alteration in sera of patients with HCC have not been investigated.

Experimental Designs: The combined bisulfite restriction analysis PCR was applied to assess the methylation status of LINE-1 repetitive sequences in genomic DNA derived from serum samples of 85 patients with HCC, 73 patients with cirrhosis, and 20 healthy carriers of hepatitis B.

Results: Serum LINE-1 hypomethylation was found to be significantly elevated in patients with HCC (mean $53.17 \pm 7.74\%$) compared to those of healthy carriers ($48.07 \pm 6.20\%$), and cirrhosis ($48.78 \pm 8.01\%$) ($P = 0.003$ and $P = 0.001$, respectively). There was no significant difference in serum LINE-1 hypomethylation level between non-HCC individuals. The levels of serum LINE-1 hypomethylation at initial presentation correlated significantly with presence of HBsAg, a large tumor size, and advanced tumor stages classified by the CLIP score.

Conclusions: Significant correlations of serum LINE-1 hypomethylation levels with tumor size and tumor stage suggest that global hypomethylation may play important roles in promoting tumor progression and invasiveness. These findings indicate that serum LINE-1 hypomethylation may be served as a molecular marker for predicting the prognosis of patients with HCC.

Key words : LINE-1, global hypomethylation, hepatocellular carcinoma, COBRA LINE-1

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, accounting for more than 500,000 new cases diagnosed annually⁽¹⁾. The prevalence of HCC is geographically variable, with the highest frequencies observed in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic. Despite remarkable improvements in surgical and ablative therapies, the overall prognosis of patients with HCC remains unsatisfactory because of its aggressiveness, and high recurrence rates⁽²⁾. As a result, a reliable serum marker is important for monitoring of tumor progression, treatment responsiveness, and predicting the prognosis. Although several molecular biological factors for HCC have been studied in recent years, a marker for this cancer in routine clinical practice is not yet available.

Recent advances in molecular biology have shown that accumulated genetic and epigenetic alterations through the repeating destruction and regeneration of hepatocytes are responsible for multistage hepatocarcinogenesis⁽³⁾. Among these changes, widespread global DNA hypomethylation accompanied by region-specific hypermethylation are common features detected in HCC^(4,5). Global hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the whole genomes both in noncoding repetitive sequences and genes^(6,7). Global losses of methylation in cancer may lead to alterations in the expression of proto-oncogenes critical to carcinogenesis and may facilitate chromosomal instability^(8,9). Previous studies have described the hypomethylation of genomic repetitive sequences, a marker of the global genomic hypomethylation, in several malignancies including carcinoma of urinary bladder, prostate, colon, and liver⁽¹⁰⁻¹⁵⁾. In addition, the extent of global hypomethylation appears to correlate with tumor progression and invasiveness in several cancer types, including HCC⁽¹⁶⁻²¹⁾, indicative of its role in tumor development and progression.

Recently, we have developed an improved quantitative combined bisulfite restriction analysis (COBRA) PCR protocol that efficiently evaluates the methylation status of LINE-1 repetitive sequences in genomic DNA derived from microdissected tissue samples⁽²²⁾. We demonstrated that most cancers exhibited significantly increased levels of hypomethylation compared with their normal tissue counterparts,

which was independent of patients' age and gender. Furthermore, DNA derived from sera of patients with carcinoma displayed significantly higher LINE-1 hypomethylation levels than those of age- and sex-matched controls. These findings suggest the potential value of COBRA LINE-1 as a serum marker of various cancer types, including HCC. However, whether serum level of LINE-1 hypomethylation can be served as a molecular marker for HCC has not been investigated. To address this issue, we examined the methylation status of LINE-1 in serum samples of patients with HCC, and compared to those of patients with benign liver diseases and healthy individuals. We also determined the clinicopathological correlations of this serum marker in patients with HCC.

MATERIALS AND METHODS

Patients and Blood Samples

Eighty-five patients who were diagnosed of HCC in King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between April 1999 and December 2003 were recruited into the study. HCC was diagnosed based on liver tumor characteristics detected by imaging studies (ultrasonography or computed tomography), serum alpha-fetoprotein (AFP) above 400 ng/ml, and/or histopathology. The clinicopathological data of the patients were collected, which included sex, age, liver function test, Child-Pugh stage, serum AFP level, tumor size, number of tumors, tumor cell differentiation, venous invasion, extrahepatic metastasis, and HCC staging classified by the CLIP score.

The patients with HCC consisted of 69 males and 16 females, with the mean age of 53.6 ± 12.7 years. All patients had cirrhosis as underlying liver disease. In regard to predisposing etiologic factors, 58 patients (68.2%) were positive for serum hepatitis B surface antigen (HBsAg), 8 patients (9.5%) were positive for hepatitis C virus antibody (anti-HCV), and 12 patients (14.1%) were associated with alcohol-dependence. For the remaining 7 patients (8.2%), the etiology could not be determined. According to the CLIP score at initial presentation, there were 6 patients (7.1%) in score 0, 14 patients (16.5%) in score 1, 21 patients (24.7%) in score 2, 19 (22.4%) in score 3, 19 (22.4%) in score 4, and 6 (7.1%) in score 5 subgroup. Thirty patients (35.3%) had venous invasion, while extrahepatic metastasis was found in 8 patients (9.4%).

The control groups comprised 20 healthy carri-

ers of hepatitis B (14 males, mean age 31.5 ± 10.3 years), and 73 patients with cirrhosis (55 males, mean age 50.3 ± 11.7 years). A healthy carrier was individual who was positive for HBsAg and had persistent normal serum alanine aminotransferase (ALT) level for at least 12 months. Cirrhosis was diagnosed based on histological examinations and/or imaging studies. With the patients' written consent, all serum samples were collected at the time of the diagnosis and stored at -70°C until they were assayed.

Measurement of Serum LINE-1 Hypomethylation

COBRA LINE-1 in the sera was performed as described previously⁽²²⁾. We used 50UTR of LINE-1.2 sequence from NCBI Accession Number M80343. DNA was isolated and extracted from sera, and treated with bisulfite. Bisulfited DNA was subjected to 35 cycles of PCR with two primers, 5'-CCGTAAGGGGTTAGGGAGTTTTT-3' and 5'-RTAAAACCC TCCRAAC CAAATATAAAA-3', with an annealing temperature of 50°C . The amplicons were digested in 10 (1 reaction volume with 2U of TaqI or 8U of TasI in 1xTaqI buffer (MBI fermentas) at 65°C overnight and were then electrophoresed in 12% nondenaturing polyacrylamide gel. Intensities of DNA fragments were measured by PhosphorImager using Image Quant software (Molecular Dynamics). LINE-1 methylation level was calculated as a percentage of intensity of TaqI divided by the sum of TaqI-and TasI-positive amplicons.

Statistical Analysis

All data are expressed as mean values \pm standard deviation, and percentage. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann-Whitney test or Student's t test when appropriate for quantitative variables. P values <0.05 for a two-tailed test were considered statistically significant. All statistical analyses were performed using the SPSS software for windows 10.0 (SPSS Inc., Chicago, IL).

RESULTS

Serum LINE-1 Hypomethylation in Patients with HCC

Serum LINE-1 hypomethylation of 85 patients with HCC obtained at the time of diagnosis ranged from

32.73% to 70.42%, with a mean of $53.17 \pm 7.74\%$. The average level of serum LINE-1 hypomethylation in patients with HCC was significantly higher than those of controls ($48.07 \pm 6.20\%$), and patients with cirrhosis ($48.78 \pm 8.01\%$) ($P = 0.003$ and $P = 0.001$, respectively). There was no significant difference in the mean level of serum LINE-1 hypomethylation between controls and patients with cirrhosis ($P = 0.674$).

Serum LINE-1 Hypomethylation and Clinicopathological Features

To evaluate the association between serum LINE-1 hypomethylation levels and clinicopathological features, the patients with HCC were divided into two groups based on the mean value (53.17%) of the whole HCC group. Accordingly, there were 36 patients with serum LINE-1 hypomethylation levels of $<53.17\%$ and 49 patients with serum levels of $\geq 53.17\%$. The correlation between these two groups and various clinicopathological parameters listed in Table 1 were further analysed. There was no significant correlation between serum LINE-1 hypomethylation level and patients' age ($P = 0.488$), gender ($P = 0.900$), serum AFP level ($P = 0.508$), tumor cell differentiation ($P = 0.199$), number of tumor ($P = 0.342$), the presence of venous invasion ($P = 0.648$), and extrahepatic metastasis ($P = 0.130$). However, high serum LINE-1 hypomethylation levels were significantly associated with HBsAg positive ($P = 0.011$), large tumor size >5 cm ($P < 0.001$), and high CLIP score ($P = 0.017$).

Serum LINE-1 hypomethylation levels exhibited a positive correlation with tumor size ($P < 0.001$; Pearson $r = 0.439$), and the CLIP score ($P = 0.005$; Pearson $r = 0.304$) as shown in Figure 1 and 2, respectively. In addition, serum LINE-1 hypomethylation levels in patients with the CLIP score 3-6 subgroups ($55.56 \pm 6.83\%$) were significantly higher than those of patients with the score 0-2 subgroups ($50.61 \pm 7.92\%$), and patients with cirrhosis ($48.78 \pm 8.01\%$) ($P = 0.003$ and $P < 0.001$, respectively). However, serum LINE-1 hypomethylation levels in patients with the score 0-2 subgroups were not significantly different from those of patients with cirrhosis ($P = 0.241$).

DISCUSSION

An increasing number of studies have described the critical roles of epigenetic alterations in hepato-

Table 1 Relationship between serum LINE-1 hypomethylation levels and Clinicopathological features

Variables	Serum LINE-1 hypomethylation		P
	<53.17%	≥53.17%	
Age (yrs)			
≥60 (n = 27)	13	14	0.488
<60 (n = 58)	23	35	
Gender			
Male (n = 69)	29	40	0.900
Female (n = 16)	7	9	
Etiology of liver disease			
HBV positive (n = 58)	19	39	0.011
HBV negative (n = 27)	17	10	
Serum AFP level (ng/ml)			
≥400 (n = 47)	18	29	0.508
<400 (n = 38)	18	20	
Tumor cell differentiation			
Well (n = 13)	9	4	0.199
Moderately and poorly (n = 39)	17	22	
Tumor number			
Solitary (n = 53)	24	29	0.342
Multiple (n = 31)	11	20	
Tumor size			
≤5 cm (n = 15)	13	2	<0.001
>5 cm (n = 70)	23	47	
Venous invasion			
Presence (n = 30)	14	16	0.648
Absence (n = 55)	22	33	
Extrahepatic metastasis			
Presence (n = 8)	1	7	0.130
Absence (n = 77)	35	42	
CLIP score			
Score 0-2 (n = 41)	23	18	0.017
Score 3-5 (n = 44)	13	31	

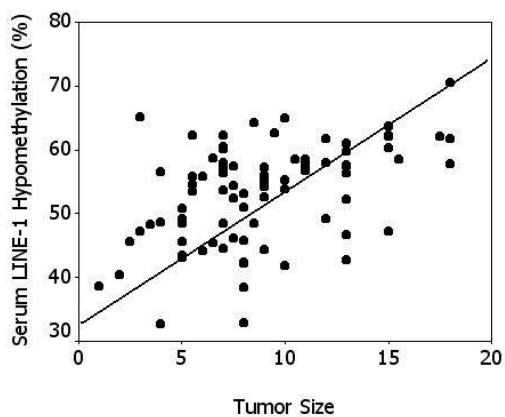


Figure 1 Serum LINE-1 hypomethylation levels in patients with HCC exhibited a positive correlation with tumor size

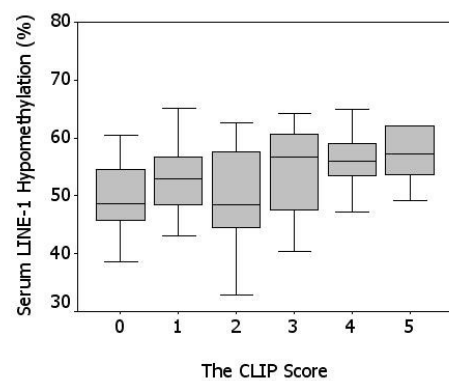


Figure 2 Association between serum LINE-1 hypomethylation levels and HCC staging classified by the CLIP score. The horizontal line indicated the mean levels of the patients' subgroups

carcinogenesis, though most reports have focused on the function of DNA hypermethylation in silencing tumor suppressor genes^(4,5). Previous data have shown that region-specific hypermethylation are already detectable in potentially precancerous lesions including cirrhosis and dysplastic nodules⁽²³⁾, indicative of its contribution in early stages of hepatocarcinogenesis. Recently, the presence of free-circulating DNA hypermethylation has been described in serum samples of patients with various types of cancers^(24,25), including HCC^(26,27). Although the clinical significance of this epigenetic alteration in sera of patients with HCC remains to be elucidated, these data suggest the potential use of this molecular approach in cancer diagnosis and monitoring. Conversely, limited information is available on the status and clinical implications of global hypomethylation in sera of patients with HCC, as well as other cancer types.

In this study, we first demonstrated that serum LINE-1 hypomethylation levels were significantly higher in patients with HCC than those of healthy individuals, as well as patients with cirrhosis. These results were consistent with recent data demonstrating that global hypomethylation is significantly increased in tumor tissues compared with in cirrhotic and non-cirrhotic tissues^(15,21). Though the paired cancer tissues of the patients were not examined in this study, we have previously shown a highly concordant of hypomethylation levels between the primary cancer and their paired serum specimens⁽²²⁾. Thus, the increase in serum LINE-1 hypomethylation level was likely reflected the global hypomethylation status in the tumor tissues. Notably, there was no significant difference of serum LINE-1 hypomethylation levels among non-HCC samples, suggesting that there was no sequential increase of global hypomethylation from normal controls and cirrhosis. These results confirmed previous data that the level of hypomethylation in normal liver tissues is comparable with that detected in chronic liver diseases.

While global hypomethylation is one signature of most cancer types, it often displays considerably specificity with regard to tumor type, tumor stage, and sequences affected⁽²⁸⁾. For examples, in the processes of colorectal and gastric carcinogenesis, global hypomethylation seems to be an early event that already occurs during premalignant stages⁽²⁹⁾. By contrast, global hypomethylation is present in the latter stages of cervical carcinogenesis, i.e., high-grade squa-

mous intraepithelial lesions and invasive squamous carcinoma⁽³⁰⁾. With respect to hepatocarcinogenesis, experimental studies have revealed conflicting results whether this epigenetic change is in the initiation or in the progression of the cancer^(31,32). In human HCC, DNA hypomethylation on certain loci on 16q and 17p, which may reflect overall hypomethylation in cancers, are detected in HCC but not precancerous liver tissues. In addition, global hypomethylation seems to be a later event during hepatocarcinogenesis compared with regional DNA hypermethylation^(33,34). By contrast, more recent data have demonstrated that DNA hypomethylation on pericentromeric satellite regions may contribute to hepatocarcinogenesis from early stages even in precancerous lesions⁽³⁵⁾. Together, these data indicate an essential role of DNA hypomethylation but could not clarify whether this aberrant methylation is more relevant in early or late stage events of hepatocarcinogenesis.

In this study, analysis of serum LINE-1 hypomethylation in relation to clinicopathological features showed that a high level of LINE-1 hypomethylation was significantly correlated with a more aggressive tumor behavior in patients with HCC. Specifically, a high-serum LINE-1 hypomethylation level was observed more frequently in patients with tumors >5 cm in diameter, and advanced disease stages (CLIP score 3-6 subgroups). This is in accordance with previous reports demonstrating that there is a positive correlation between the expression levels of LINE-1 hypomethylation in liver tissues and advanced disease stages^(20,21). Similar findings of global hypomethylation in tissue specimens association with tumor progression are observed in a variety of cancers, including carcinoma of breast, cervix, ovary, and prostate gland⁽¹⁶⁻¹⁹⁾. These data suggest an important and active role of global hypomethylation in the progression of HCC, as well as other tumor types. Unlike tissue-based techniques, measurement of COBRA LINE-1 in the serum has several advantages of its rapidity, reproducibility, and noninvasiveness. Therefore, measurement of serum LINE-1 hypomethylation levels may be more useful and feasible in clinical setting to predict tumor progression than the detection of this epigenetic alteration in tumor specimens.

Recent data have shown that inhibition of global hypomethylation may be a potential therapeutic approach to control disease progression of cancers. For instance, antisense inhibition of demethylase activity,

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which is able to reverse the status of DNA hypomethylation, could reduce tumorigenesis⁽³⁶⁾. In accordance with this observation, it has been shown that pharmacological administration of S-adenosyl-methionine (AdoMet), which acts through enhancing DNA methyltransferase activity and inhibiting active demethylation⁽³⁷⁾, prevents the development and progression of HCC in animal experiments⁽³⁸⁾. More recently, it has been reported that reversal of the hypomethylation status of urokinase promoter by AdoMet treatment blocks growth and metastasis of breast cancer⁽³⁹⁾. Together, these data suggest that targeting global hypomethylation may be particularly useful in improving the prognosis of HCC, particularly in subgroup of patients with high serum LINE-1 hypomethylation levels.

In conclusion, this is the first study demonstrating the clinical implications of serum LINE-1 hypomethylation in HCC. Our results showed that a high level of LINE-1 hypomethylation was significantly associated with tumor progression and invasiveness. Apart from these data, it will be interesting to determine in future studies whether serum LINE-1 hypomethylation could be useful for tumor prognostication and monitoring tumor progression or treatment response in patients with HCC.

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