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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

LINE-1 Methylation Level and Patient Prognosis in a Database of 208 Hepatocellular Carcinomas

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ABSTRACT

Background. The level of long interspersed nucleotide element-1 (LINE-1) methylation has become regarded as a surrogate marker of global DNA methylation. Previously, we demonstrated that LINE-1 hypomethylation might contribute to the acquisition of aggressive tumor behavior through genomic gains of oncogenes such as cyclin-dependent kinase 6 (*CDK6*) in esophageal squamous cell carcinoma. However, the relationship between LINE-1 hypomethylation and clinical outcome in hepatocellular carcinoma (HCC) remains unclear.

Methods. LINE-1 methylation level in 208 samples of curatively resected HCCs was measured by pyrosequencing assay, and the prognostic value of LINE-1 methylation level in HCC was examined.

Results. LINE-1 methylation levels in the 208 HCC patients investigated were distributed as follows: mean 64.7; median 64.6; standard deviation (SD) 13.6; range 21.5–99.1; interquartile range 62.9–66.6. Univariate Cox regression analysis revealed a significantly higher cancer recurrence rate in the low-methylation-level group than in the high-methylation-level group (hazard ratio 1.58; 95 % CI 1.05–2.47; $p = 0.028$). Interestingly, the influence of LINE-1 hypomethylation on patient outcome was modified by hepatitis virus infection (p of interaction $= 0.023$); LINE-1 hypomethylation was associated with a higher cancer recurrence rate in patients without hepatitis virus infection (log-rank $p = 0.0047$). CDK6 messenger RNA expression levels were inversely associated with LINE-1 methylation levels ($p = 0.0075$; $R = -0.37$).

Conclusions. Genome-wide DNA hypomethylation, as measured by LINE-1 levels, might be associated with poor disease-free survival in HCC patients, suggesting a potential role for LINE-1 methylation level as a biomarker for identifying patients who will experience an unfavorable clinical outcome.

Liver cancer is the fifth most commonly diagnosed cancer and the third most frequent cause of cancer mortality.[1] Hepatocellular carcinoma (HCC), accounting for approximately 70–85 % of liver cancers, is the major histological subtype worldwide.[2] The apparent prevailing risk factors of HCC are chronic viral hepatitis B and C infections, alcohol exposure, and non-alcoholic fatty liver disease.[3] Several molecular alterations have been identified in HCC, some of which are potential biomarkers and therapeutic targets.[4,5] Recently, HCC has been linked to genetic and epigenetic changes occurring during tumor development.[6,7] Therefore, the contribution of epigenetic alterations to HCC development and progression has attracted increasing attention in recent years.[8,9]

DNA methylation alterations associated with human cancers include global DNA hypomethylation and site-specific CpG island promoter hypermethylation.[10] Promoter hypermethylation can silence tumor suppressor genes, whereas global DNA hypomethylation appears to play an important role in genomic instability, leading to cancer development.[11,12] Because long interspersed nucleotide element-1 (LINE-1) retrotransposons constitute

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a substantial portion (approximately 17 %) of the human genome, the level of LINE-1 methylation has become regarded as a surrogate marker of global DNA methylation.[13] Pyrosequencing has emerged as a cost-effective and high-throughput method for assessing the global DNA methylation status of LINE-1.[14–16] LINE-1 methylation is highly variable, and LINE-1 hypomethylation is strongly associated with a poor prognosis in several types of human neoplasms, including colon, esophageal, gastric, and ovarian cancers.[17–20] In addition, we demonstrated that LINE-1-hypomethylated tumors presented highly frequent genomic gains at various loci containing candidate oncogenes, including cyclin-dependent kinase 6 (*CDK6*) in esophageal squamous cell carcinoma (ESCC), leading to the acquisition of aggressive tumor behavior.[21] However, the relationship between LINE-1 hypomethylation and clinical outcome in HCC had not been clarified by the date of this study.

In the present study, we quantified the LINE-1 methylation levels in 208 samples of curatively resected HCC by pyrosequencing assay, and examined the prognostic value of LINE-1 hypomethylation in HCC. In addition, we evaluated the relationship between LINE-1 methylation level and *CDK6* messenger RNA (mRNA) expression level in HCC. Our data suggest a potential role for LINE-1 hypomethylation as a prognostic biomarker.

METHODS

Study Subjects

All subjects were randomly selected from 344 HCC patients who had undergone surgical resection as their first therapy at Kumamoto University Hospital (Kumamoto, Japan) between January 2000 and December 2010. Among the 283 subjects, 75 were excluded because their tissue samples were inadequate or their results inconclusive. Thus, 208 patients were finally included in the study. The clinical, epidemiological, and pathological variables and prognosis of the study group (208 patients) were similar to those of the excluded group (136 patients) [electronic supplementary Fig. 1 and electronic supplementary Table 1]. Patients were observed at 1- to 3-month intervals until death or until 30 December 2013, whichever came first. Disease-free survival (DFS) was defined as the duration between surgical cancer treatment and the next sign of cancer recurrence. Overall survival (OS) was defined as the duration between the operation date and the date of death. Tumor staging was conducted by the American Joint Committee on Cancer's Cancer Staging Manual (7th edition).[22] Written informed consent was obtained from each subject, and the study procedures were approved by the Institutional Review Board. Throughout this article, the term 'prognostic marker' conforms to the REMARK guidelines.[23]

DNA Extraction

Prepared slides of the tumors were stained with hematoxylin and eosin, and the areas of tumor and normal liver parenchyma were delineated. In each case, hematoxylin and eosin-stained tissue sections were scraped off the slides for DNA extraction. Section area depended on the sizes of the tissue and tumor (average section = 10 μm × 1 μm for large tumors). DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen).

Sodium Bisulfite Treatment and Pyrosequencing of Long Interspersed Nucleotide Element-1 (LINE-1)

Sodium bisulfite treatment of genomic DNA was performed as previously described using an EpiTect Bisulfite Kit (Qiagen).[17–19,24,25] Polymerase chain reaction (PCR) and subsequent pyrosequencing of LINE-1 were performed as previously described using the PyroMark kit (Qiagen).[17–19,24,25] This assay amplifies a region of LINE-1 containing four CpG sites (base positions 305–331 in Accession No. X58075). In each tumor sample, the overall LINE-1 methylation level was the average relative amount of C in the four CpG sites (electronic supplementary Fig. 2).

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was obtained from the frozen tissue samples by using a mirVana™ microRNA (miRNA) isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.[26] Complementary DNA (cDNA) synthesis and quantitative reverse transcription PCR were carried out as previously described.[21]

Statistical Methods

The JMP program (version 9, SAS Institute, Cary, NC, USA) was used for statistical analyses. All *p*-values were two-sided. We confirmed, by using the Shapiro–Wilk test, that the continuous variables were within normal distribution, and confirmed the homoscedasticity between specific groups by using the *F*-test and Bartlett's test. We then compared the means by applying the Student's *t* test and analysis of variance method. The survival time distribution in the survival analysis was assessed by the Kaplan–Meier method using a log-rank test. We constructed a

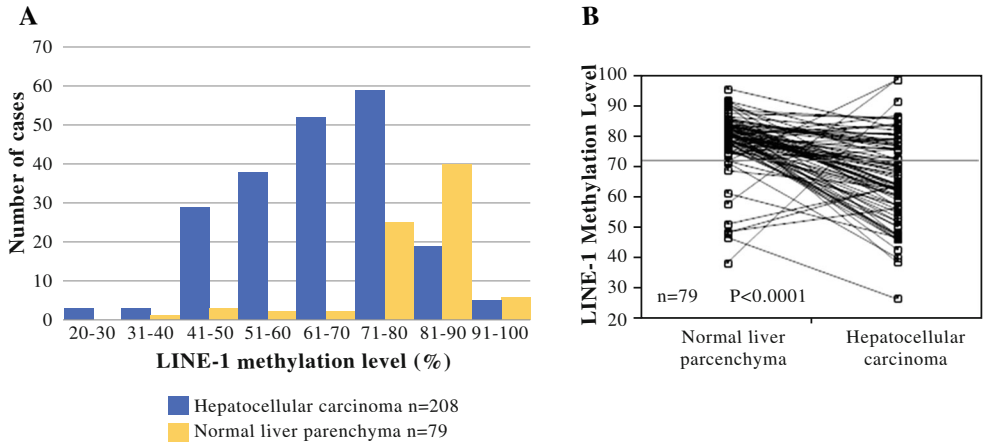


FIG. 1 The distribution of LINE-1 methylation levels in tumors and normal liver parenchymata. **a** Distributions are shown for 208 hepatocellular carcinomas and 79 normal liver parenchymata. **b** LINE-1 methylation levels in 79 hepatocellular carcinomas and matched normal liver parenchymata. The methylation levels are significantly lower in the cancer tissues than in the matched normal parenchymata (paired *t*-test; $p < 0.0001$)

multivariate model to compute the hazard ratio (HR) from the LINE-1 methylation level status, accounting for sex (male vs. female), age at surgery (<66 vs. ≥66 years), hepatitis virus infection (absence or presence of hepatitis B virus [HBV] or hepatitis C virus [HCV] infection), indocyanine green retention at 15 min (ICG-R15) [<14 vs. ≥14 %], portal vein invasion (present vs. absent), hepatic vein invasion (present vs. absent), tumor differentiation (well-defined vs. moderate–poor), stage of fibrosis,[27] (F0–2 vs. F3–4), cancer type (simple nodular vs. other types), Union for International Cancer Control (UICC) stage (I vs. II, IIIa or IIIb), α-fetoprotein (AFP) level (<20 vs. ≥20 ng/ml), and resection method (major resection vs. minor resection, and anatomic resection vs. non-anatomic resection). To assess interaction between the variables, the LINE-1 methylation level was cross-correlated with another variable of interest in the univariate Cox model, and interaction was evaluated by the Wald test.

RESULTS

LINE-1 Methylation Level in Hepatocellular Carcinoma and Normal Liver Parenchyma

We examined LINE-1 methylation levels in 208 HCC tissues and 79 normal liver parenchymata. The LINE-1 methylation levels in the HCC tissues (vs. normal liver parenchymata) were distributed as follows: mean 64.7 (75.8); median 64.6 (80.5); standard deviation (SD) 13.6 (13.4); range 21.5–99.1 (9.4–97.3); interquartile range 62.9–66.6 (72.4–79.2) [Fig. 1a]. LINE-1 methylation level was within normal distribution (Shapiro–Wilk test; $p = 0.21$). The LINE-1 methylation level in HCC tissues was significantly lower than in matched normal liver parenchymata ($p < 0.001$ by the paired *t* test) [Fig. 1b].

Association Between LINE-1 Methylation Level and Clinical, Epidemiological, and Pathological Variables

We then examined the relationship between the LINE-1 methylation level in HCC and various clinical, epidemiological, and pathological variables. Weak correlations between LINE-1 methylation level in HCC and age at operation ($p = 0.11$) and between LINE-1 methylation and tumor differentiation ($p = 0.11$) were not statistically significant (Table 1). Importantly, none of the clinical HCC features was significantly correlated with LINE-1 methylation level.

LINE-1 Methylation Level and Patient Survival

A follow-up study of the 208 patients revealed 127 cancer recurrences and 69 deaths. The median follow-up time for censored patients was 4.7 years. Using the methylation level as a quartile categorical variable (i.e. first quartile cases [Q1; ≥74.5 %], second quartile cases [Q2; 64.6–74.5 %], third quartile cases [Q3; 55.0–64.6 %], and fourth quartile cases [Q4; ≤55.0 %]), we conducted a univariate Cox regression analysis. The cancer recurrence rate was higher in Q2, Q3, and Q4 than in Q1 (electronic supplementary Table 2). Thus, we adopted a dichotomous LINE-1 methylation level, defining Q1 as the 'high-methylation-level group' and combining Q2, Q3, and Q4 into the 'low-methylation-level-group'. The 'low-methylation-level group' (Q2–4) experienced significantly shorter DFS

TABLE 1 Status of LINE-1 methylation in hepatocellular carcinoma tissues, and their relations to clinical and tumor features

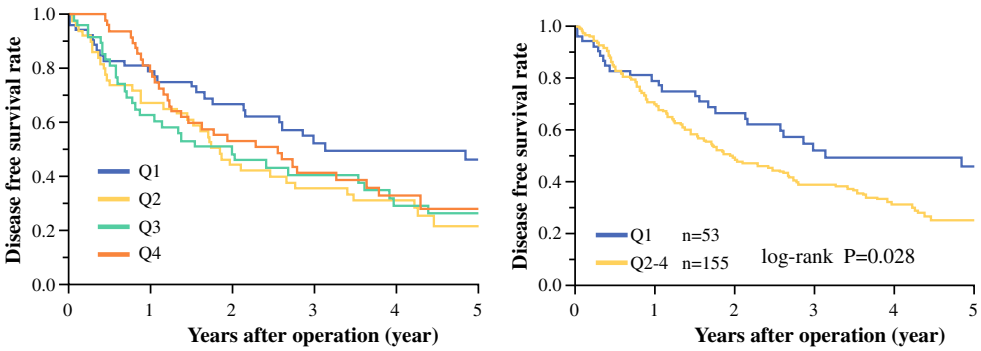
Clinical or pathological feature	Total *N*	LINE-1 methylation level (%) [mean ± SE]	*p*-Value
All cases	208	64.74 ± 0.95	
Age (years)			0.11
<66	97	66.37 ± 1.38	
≥66	111	63.31 ± 1.29	
Sex			0.71
Male	174	64.90 ± 1.03	
Female	34	63.94 ± 2.34	
Child–Pugh classification			0.19
A	185	65.18 ± 1.00	
B	23	61.24 ± 2.84	
C	0	0	
Fibrosis stage			0.71
F0–2	101	64.87 ± 1.37	
F3–4	99	64.15 ± 1.39	
Hepatitis virus infection (HBV or HCV)			0.42
Present	157	64.34 ± 1.09	
Absent	50	66.13 ± 1.93	
HBV infection			0.18
Present	61	66.65 ± 1.74	
Absent	147	63.87 ± 1.13	
HCV infection			0.085
Present	103	63.05 ± 1.34	
Absent	105	66.31 ± 1.33	
ICG-R15 (%)			0.48
<14	91	65.48 ± 1.46	
≥14	91	64.02 ± 1.46	
AFP (ng/ml)			0.97
<20	118	64.71 ± 1.44	
≥20	118	64.78 ± 1.26	
Tumor type			0.17
Simple nodular	131	63.76 ± 1.19	
Others	77	66.41 ± 1.55	
Stage			0.88
I	89	64.20 ± 1.45	
II	105	65.19 ± 1.34	
III	14	64.80 ± 3.66	
Hepatic vein invasion			0.33
Present	31	63.61 ± 1.51	
Absent	177	65.48 ± 1.21	
Portal vein invasion			0.94
Present	82	64.07 ± 1.13	
Absent	126	66.41 ± 1.75	
Tumor number			0.26
Solitary	145	64.07 ± 1.13	
Multiple	61	66.41 ± 1.75	
Differentiation			0.11
Well-defined	62	67.04 ± 1.73	
Moderate–Poor	144	63.69 ± 1.14	
Operative method			0.11
Minor	164	67.04 ± 1.73	
Major	44	63.69 ± 1.14	
Anatomic resection			0.81
Anatomic	135	64.57 ± 1.18	
Non-anatomic	73	65.1 ± 1.60	

SE standard error, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG-R15* indocyanine green retention at 15 min, *AFP* α-fetoprotein

than the 'high-methylation-level group' (Q1) [Kaplan–Meier analysis; log-rank $p = 0.028$] (Fig. 2). The low-methylation-level group also experienced a significantly higher cancer recurrence rate (univariate Cox analysis; HR 1.58; 95 % CI 1.05–2.47; $p = 0.0279$) [electronic supplementary Table 2]. When adjusted for clinical and pathological features, the multivariate Cox model revealed no significant association between reduced methylation level and increased cancer recurrence rate (multivariate HR 1.37; 95 % CI 0.83–2.25; $p = 0.22$). The prognostic influence of LINE-1 hypomethylation was essentially diminished by adjusting for age at surgery and ICG-R15 in the multivariate analysis; adjusting for these factors, the HRs for DFS rates were 1.44 (95 % CI 0.95–2.27) and 1.53 (95 % CI 0.99–2.48), respectively.

Next, we analyzed the relationship between OS and LINE-1 methylation. Low methylation levels were not significantly associated with poor OS, in either univariate Cox regression analysis (HR 1.32; 95 % CI 0.77–2.39; $p = 0.31$) or multivariate analysis (HR 0.84; 95 % CI 0.44–1.67; $p = 0.84$), possibly because the sample size was small (only 69 deaths had occurred since the initial surgery).

Additionally, we examined whether the influence of low LINE-1 hypomethylation on cancer recurrence was modified by any of the clinical and pathological variables, including sex, age, hepatitis virus infection (HBV or HCV), Child–Pugh classification, ICG-R15, stage of fibrosis, tumor size, number of tumors, stage, tumor type, and differentiation. The relationship between LINE-1 methylation level and DFS rate was significantly modified by hepatic virus infection (p of interaction = 0.023; Fig. 3a), although multiple hypothesis testing permits a chance emergence of this trend. In the absence of hepatitis infection, low methylation was associated with a significantly



Number at risk

Years	0	1	2	3	4	5
Q1	53	40	30	20	16	14
Q2	52	32	21	16	12	3
Q3	51	28	19	15	11	5
Q4	52	37	24	16	10	5

FIG. 2 Kaplan–Meier curves of disease-free survival for different quartiles (Q1–4) of LINE-1 methylation level in 208 hepatocellular carcinomas. In the *right panel*, Q1 represents the 'hypermethylation group' and Q2, Q3, and Q4 collectively represent the 'hypomethylation group'. *Qx* quartile *x*

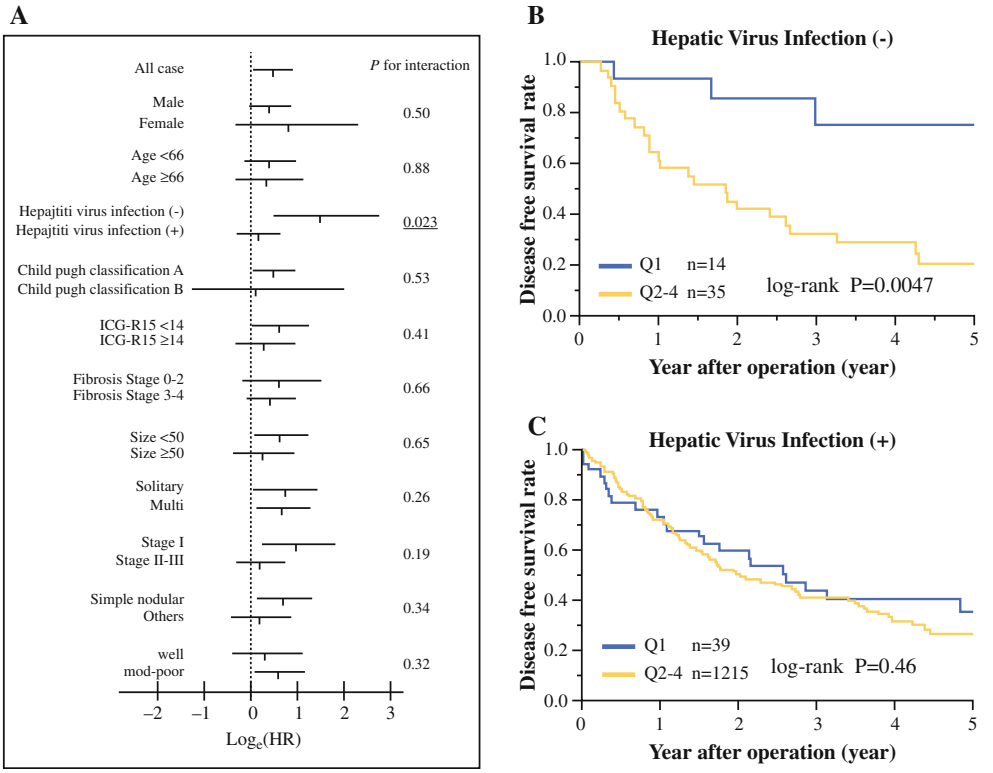


FIG. 3 LINE-1 methylation level and disease-free survival in various strata. **a** Log_e (adjusted HRs) plots of disease-free survival rate in the hypomethylation (Q2–4) and hypermethylation (Q1) groups. The 95 % CI is also indicated. **b** Kaplan–Meier curves of disease-free survival among patients without hepatic virus infection. **c** Kaplan–Meier curves of disease-free survival among patients with hepatic virus infection. Q1 represents the 'hypermethylation group' and Q2, Q3, and Q4 collectively represent the 'hypomethylation group'. *HRs* hazard ratios, *ICG-R15* indocyanine green retention at 15 min, *mod* moderate, *Qx* quartile *x*

poorer outcome (log-rank $p < 0.0047$; Fig. 3b, c). In contrast, the LINE-1 methylation level was uncorrelated with DFS in hepatitis patients (log-rank $p = 0.46$; Fig. 3b, c). Other tested variables did not significantly interact in the relationship between LINE-1 methylation level and DFS (p of all interactions > 0.05; Fig. 3a).

Correlation Between LINE-1 Methylation Level and Cyclin-Dependent Kinase 6 (CDK6) mRNA Expression in Hepatocellular Carcinoma

Given that we previously showed that LINE-1 hypomethylation activates *CDK6* expression through DNA

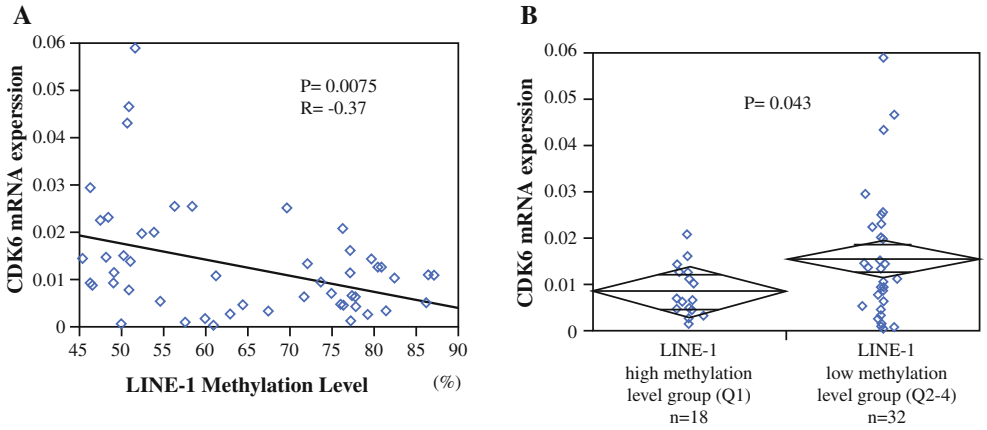


FIG. 4 Correlation between LINE-1 methylation level and CDK6 mRNA expression in HCC. **a** CDK6 mRNA expression levels were inversely associated with LINE-1 methylation levels ($p = 0.0075$, $R = -0.37$). **b** CDK6 mRNA expression levels were significantly higher in the low-methylation-level group ($p = 0.043$). *CDK6* cyclin-dependent kinase 6, *mRNA* messenger RNA, *HCC* hepatocellular carcinoma, *Qx* quartile *x*

copy number aberrance in ESCC, we theorized a correlation between *CDK6* expression and LINE-1 methylation levels in HCC. *CDK6* mRNA expression levels were inversely associated with LINE-1 methylation levels in 50 HCCs, which were randomly selected from 97 HCCs with available data of LINE-1 and RNA ($p = 0.0075$; $R = -0.37$; Fig. 4a). Moreover, CDK6 mRNA expression levels were significantly higher in Q2–4 (low methylation level group) than Q1 (high methylation level group) [$p = 0.043$; Fig. 4b].

DISCUSSION

In the current study, we examined the prognostic impact of LINE-1 methylation level in 208 patients with HCC. Since LINE-1 constitutes a substantial portion of the human genome, its methylation status is considered to reflect the global DNA methylation level.[13] This study implicated LINE-1 hypomethylation in higher HCC recurrence, suggesting a potential role for LINE-1 methylation level as a biomarker for identifying patients who will experience an unfavorable clinical outcome. Moreover, LINE-1 methylation levels correlated with *CDK6* expression.

The relationship between LINE-1 methylation level and prognosis has been examined in several types of human neoplasms.[17–20,28–32] Global DNA hypomethylation has been linked to poor survival in colon cancer, ESCC, gastric cancer glioma, and ovarian cancer. Our current findings in HCC patients are consistent with these results. Conversely, this relationship was absent in a study of cutaneous melanoma, possibly because this tumor is histologically different from the internal tumors. Nonetheless, our data certainly support a potential role for LINE-1 hypomethylation as a prognostic HCC biomarker.

Many human cancers are characterized by hypermethylation patterns of the CpG islands in the promoter regions of tumor suppressor genes.[33] Importantly, hypermethylation inactivates certain gene types in HCC.[9] Recently, the relationship between CpG hypermethylation and prognosis has been elucidated. In HCC, poor prognosis is associated with epigenetic silencing (by CpG hypermethylation) of genes whose products inhibit the Ras pathway.[8] Conversely, the relationship between global DNA hypomethylation and prognosis in HCC has not been clarified. Although LINE-1 hypomethylation in HCC should be associated with higher cancer recurrence, this relationship was not significant in the multivariate analysis of the present study.

HCC is characterized by global DNA hypomethylation and gradually decreasing methylation levels as the tumor progresses.[8,9] The mechanism by which global DNA hypomethylation may confer a poor prognosis remains partially explored. First, genome-wide DNA hypomethylation is associated with genomic instability.[11,34–37] The hypomethylation of retrotransposons, which reside in repetitive elements, inhibits heterochromatin formation and promotes recombination and genomic instability.[38–40] Furthermore, by utilizing a 'copy and paste' mechanism, retrotransposon is inserted into new genomic loci and may thus alter gene structure and expression, leading to facilitate oncogenic pathways in HCC.[41] For instance, a LINE-1 element that is inserted into a *c-MET* gene drives the transcription of *c-MET*, which is termed *L1-MET*.[42] Zhu et al. have shown that LINE-1 hypomethylation in HCC promotes *c-MET* expression, resulting in a poor prognosis.[43] Second, hypomethylation of gene regulatory regions or loss of genetic imprinting release the expression of oncogenes.[44–46] Third, genomic DNA hypomethylation is associated with inflammatory mediators and oxidative

stress, which facilitate malignant angiogenesis in HCC.[47,48] The results of the present study support the established relationship between DNA hypomethylation and increased malignancy. However, our findings require validation by further study.

CDK6, a member of the serine–threonine kinases family, and cyclin D1 jointly inactivate the retinoblastoma protein, causing a promotion of cellular proliferation by regulating G1–S-phase progression.[49] Additionally, CDK6 is a critical player in carcinogenesis via controlling cell-cycle progression in HCC.[50] Recently, we demonstrated that LINE-1 hypomethylation might be an important influential factor for DNA copy number variations in ESCC, leading to acquire aggressive tumor behavior by promoting the expression of oncogenes such as *CDK6*.[21] In our present study, HCC with LINE-1 hypomethylation showed higher *CDK6* expression than HCC with LINE-1 hypermethylation, supporting the possibility that LINE-1 hypomethylation might contribute to an increased *CDK6* expression. However, the mechanism by which LINE-1 hypomethylation affects tumor behavior could not be sufficiently analyzed in our study and must therefore be an objective of future study projects.

In the present study, LINE-1 hypomethylation correlates with higher recurrence rates of HCC, but exhibits no adverse effect on mortality rate. This result contradicts previous reports that linked LINE-1 hypomethylation level to lower OS of HCC patients.[43,51] Several factors may explain this discrepancy. First, OS depends on post-recurrence therapies such as re-resection, ablation therapy, molecular target treatment, and best supportive care, which vary among treatment facilities.[52,53] Second, overall post-resection survival is associated with hepatic viral status and liver function.[54,55] The present study included more cases of HCV-infected HCC patients than previous studies.

Interestingly, LINE-1 hypomethylation significantly affected the prognosis of HCC, but this effect was negated by hepatitis virus infection. This result may be explained as follows. Hepatitis virus infection has a high probability of multicentric recurrence. In addition, HCC tumor tissue is histologically altered by hepatitis virus infection. Therefore, we suggest that hepatitis virus infection attenuates the effect of LINE-1 hypomethylation on tumor recurrence.

CONCLUSIONS

The present study suggests that genome-wide DNA hypomethylation, as measured by LINE-1 methylation levels, reduces the DFS of HCC patients. Future studies are needed to confirm this association and to elucidate the mechanism by which genome-wide DNA hypomethylation affects tumor behavior.

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CONFLICT OF INTEREST No conflicts of interest exist.

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