

## Combination of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation in serum DNA is a non-invasion prognostic biomarker for early recurrence of hepatocellular carcinoma after curative resection

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Hepatocarcinogenesis, a multistep process, involves not only genetic mutations but also epigenetic alterations. Widespread of global DNA hypomethylation is accompanied with specific regional hypermethylation especially at tumor suppressor genes' promoters. The aim of this study is to determine the efficacy of combined DNA methylation analysis of a global DNA methylation marker – *LINE-1* and a tumor suppressor gene highly associated with the malignancy of HCC- *RASSF1A* in serum as a novel prognostic marker for diagnosis of early recurrence after curative resection.

*LINE-1* was hypomethylated in 66.7% (70/105) and *RASSF1A* promoter was hypermethylated in 73.3% (77/105) of HCC serum DNA samples by methylation specific PCR, but in none of the healthy controls: *LINE-1* hypomethylation (0/50) and *RASSF1A* hypermethylation (0/50). A significant association was found between *LINE-1* hypomethylation and clinical pathologic features including HBsAg positivity ( $p=0.009$ ), tumor size ( $p=0.001$ ) and AFP levels ( $p<0.001$ ). Besides, significant correlation was detected between *RASSF1A* promoter hypermethylation and lymph nodes metastasis ( $p=0.045$ ).

The results of Kaplan-Meier estimates of survival suggested that *LINE-1* hypomethylation was highly associated with poor survival of patients (disease-free survival  $p=0.002$ , overall survival  $p=0.0123$ ). More importantly, co-evaluation of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation was found to be significantly correlated to early recurrence and poor prognosis (disease-free survival  $p=0.0001$ , overall survival  $p=0.05$ ) in patients after curative resection.

In conclusion, our study showed that the combined examination of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation was effective in predicting early recurrence of HCC after curative resection. Patients with dual positivity of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation should be supplied with more intensive care and close follow-up after they undergo tumor resection.

*Key words: hepatocellular carcinoma, RASSF1A hypermethylation, LINE-1 hypomethylation, prognosis*

Hepatocellular carcinoma (HCC), the most common primary cancer of the liver, is the sixth of most common malignant tumor type and the second leading cause of cancer-related mortality around the world [1]. For HCC patients, curative hepatic resection is the standard treatment, but their long-term prognosis remains poor [2]. Intrahepatic recurrences, especially within 2 year after surgical resection, are common and have been considered the main cause of poor prognosis

in HCC patients. Inadequate resection, tumor invasiveness, tumor size, infiltration of the fibrous capsule, portal vein invasion and intrahepatic metastases have been reported as the main reasons for tumor recurrence [3]. But most of these factors can be detected only after tumor resection. Thus, it would be necessary to have a noninvasive, preoperative marker to identify patients who are at high risk of developing early recurrent tumors so that they may receive intensive therapy.

Recent advance molecular biology studies suggest that genetic mutations and epigenetic alterations accumulated in repeated destruction and regeneration of hepatocytes are responsible for multistage hepatocarcinogenesis [4]. A widespread global DNA hypomethylation is accompanied by specific region hypermethylation including tumor suppressor genes' promoters in HCC [5]. Global loss of methylation in cancer may lead to chromosomal instability and alter expression of proto-oncogenes which is critical to carcinogenesis [6]. Hypermethylated status of CpG islands in the promoter region of the tumor suppressor genes which involve in cell cycle regulation, DNA repair, tumor metabolism, cell interactions, cell apoptosis and angiogenesis are able to cause their inappropriate silencing negatively affecting the initiation and progression of tumors [7]. Additionally, the technical advantage of DNA methylation is chemically stable and can be detected with a very high sensitivity up to 1:1000 molecular. And many cancer specific methylated DNA can be found in body fluids including serum which is the most easily treated sample [8]. Thus, detection of the methylation status of certain genes in serum DNA can be a useful non-invasive marker [9].

Long interspersed nucleotide element-1 retrotransposons – *LINE-1* constitutes a substantial portion (about 17%) of the human genome. And the level of *LINE-1* methylation has been considered as a marker of global DNA methylation [10]. *LINE-1* methylation status is highly variable, and *LINE-1* hypomethylation can affect genomic instability, alter gene expression and closely associated with a poor prognosis in several types of human tumors, including colon, esophageal, gastric, and ovarian cancers [11, 12]. Pattamadilok J showed that *LINE-1* hypomethylation status in HCC tissue could be a biomarker for identifying poor prognosis [13]. Duo to *LINE-1* hypomethylation could be detected in the white blood cells of cancer patients [14, 15]. The methylation status of *LINE-1* in serum DNA may not be a specific biomarker alone for early recurrence of HCC patients. To enhance the specificity and accuracy, we combined with the hypermethylation status of a suppressor gene. The RAS association domain family 1A gene – *RASSF1A*, located in the

3p21.3 region, is a significant member of the RAS signaling pathway. It plays an important role in apoptosis, cell cycle, microtubule stability, cell adhesion and migration [16]. The promoter region hypermethylation of *RASSF1A* is suspected as a major silence mechanism in human malignant tumors, including HCC [17, 18]. And a meta-analysis on *RASSF1A* methylation in HCC body fluids showed that *RASSF1A* hypermethylation could be a reliable biomarker for HCC discrimination and prognosis [19].

To determine whether combination of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation could be a noninvasive prognostic marker for postoperative HCC patients, we detected *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation status in serum DNA, analyzed the association between genes methylation status and clinical pathologic features, and examined the correlation with disease free survival and overall survival after HCC patients underwent curative hepatic resection.

## Patients and methods

**Clinical samples.** The study material consisted of 105 blood samples from HCC patients, and 75 among them underwent curative hepatic resection from West China Hospital, Sichuan University, between March and November in 2014. The diagnoses of HCC were verified by senior pathologist. None of the patients had received prior radiotherapy and chemotherapy treatment when the blood samples were collected. Complete pathological and clinical data were all collected and described in Table 2. All patients were informed consent. In addition, 50 blood samples were used as control group in NO.4 West China Teaching Hospital, Sichuan University. Besides, 1 normal liver tissue from healthy person who died of sudden accident was used as control to test the methylation status of *LINE1* and *RASSF1A*.

**Follow-up.** Patients were followed up once a month after operation during the first half year, and then every 3 months afterwards. Serum AFP level and abdominal ultrasonography were performed each time during the routine review. Computed tomography (CT) was conducted every 3 to 6 months. Survival status of all patients was obtained from follow-up telephone calls by the endpoint of May 2016. The date of death for each patient was verified by local civil affairs department. Overall survival was calculated from the date of operation to the date of death or the last follow-up. Recurrence was confirmed on the basis of positron emission tomography–CT (PET-CT), or the combination of evidences from CT, ultrasonography, magnetic resonance imaging (MRI) and increased serum AFP levels, according to the latest guidelines released.

**Cell line preparation.** Cell line of Huh-7 which has been confirmed to contain CpG islands hypermethylation of *RASSF1A* promoter was chosen as positive hypermethylated control [20]. Cell line of K562 was chosen as hypomethylation control of *LINE-1* [21].

**Table 1. Primer sequence used in MSP analyses.**

Gene	Direction	Sequence	bp
Unmethylated			
<i>RASSF1A</i>	Forward	5'-GGGGTTTTGTGAGAGTGTGTTTAG-3'	175
	Reverse	5'-TAAACACTAACAAAACACAAACCAAAC-3'	
<i>LINE1</i>	Forward	5'-TGTGTGTGAGTTGAAGTAGGGT-3'	116
	Reverse	5'-ACCCAATTTCCAAATACAACCATCA-3'	
Methylated			
<i>RASSF1A</i>	Forward	5'-GGGTTTTGCGAGAGCGCG-3'	169
	Reverse	5'-GCTAACAAACGCGAACCG-3'	
<i>LINE1</i>	Forward	5'-CGCGAGTCGAAGTAGGGC-3'	111
	Reverse	5'-ACCCGATTTCCAAATACGACCG-3'	

**Table 2. Association of RASSF1A methylation status with demographic and clinicopathological features of the patients**

Patient's characteristics	n	<i>LINE1</i> hypomethylation	<i>p</i> value	<i>RASSF1A</i> hypermethylation	<i>p</i> value
Gender			0.349		0.380
Females	17	13 (76.47)		11 (64.7)	
Males	88	57 (56.82)		66 (75)	
Age,years			0.435		0.218
>45	77	53 (68.83)		54 (70.13)	
≤45	28	17 (60.71)		23 (82.14)	
HBsAg			<b>0.009</b>		0.12
Positive+	97	68 (70.1)		73 (75.26)	
Negative-	8	2 (25)		4 (50)	
Tumor size,cm			<b>0.001</b>		0.124
≥5	23	22 (95.65)		14 (60.87)	
<5	26	14 (53.85)		21 (80.77)	
AFP leve, ng/L			<b>&lt;0.001</b>		0.092
>400	45	39 (86.67)		36 (80)	
≤400	60	31 (51.67)		39 (65)	
Cirrhosis			1		0.372
With	60	40 (66.67)		46 (76.7)	
Without	45	30 (75)		31 (68.89)	
Porta vein tumor embolus			0.851		0.129
With	17	11 (64.7)		15 (88.24)	
Without	88	59 (67)		62 (70.45)	
Degree of cell differentiation			0.280		0.501
Moderately	39	30 (76.92)		27 (69.23)	
Poorly	10	6 (60)		8 (80)	
Lymph nodes metastasis			0.442		<b>0.045</b>
With	16	12 (75)		15 (93.75)	
Without	89	58 (65.17)		62 (69.66)	

Statistically significant values are highlighted in bold

Huh-7 was cultured in DMEN (Gibco), while K562 was cultured in PRMI1640 (Gibco). Both media contained 10% fetal bovine serum (BI), 100 IU/ml penicillin and streptomycin (Gibco). Well-grown passages were collected for genomic DNA extraction.

**DNA extraction.** According to the manufacture's instruction, DNA was extracted from tissues and cell lines with the TIANamp kit (TIANGEN) and dissolved in 150µL TE. Likewise, plasma DNA was isolated from 400µL sample using the Body Fluid Viral DNA/RNA Miniprep Kit (Axygen) and then dissolved in 40µL TE.

**Bisulfite conversion of DNA and MSP.** In order to convert all unmethylated-cytosines to uracil, extracted DNA was modified, using sodium bisulfite (SB) from the EZ DNA Methylation (ZYMO Research Co.). Based on the manufacturer's instructions, extracted DNA was added to each conversion system up to 500ng. then modified DNA was stored at -80°C until used.

The methylated status of *LINE-1* and *RASSF1A* in serum DNA samples was tested by Methylation Specific PCR (MSP) using specific primer pairs for the methylated and unmethylated sequences. The primer sequences and length of products

are shown in Table 1. According to the manufacture's instruction, two µL of sodium bisulfite modified DNA was added into a 23µL reaction mixture that contained 12.5µL 2xTaq Master Mix (Novoprotein Scientific Inc.) and 1.5µL of corresponding forward and reverse primers (10µmol/L); then dH<sub>2</sub>O was added to a final volume of 25µL. And sodium bisulfite treated DNA was amplified with PCR instrument. Thermo-cycling condition was used as follows: (i) *LINE-1*, one cycle at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 58°C for 45 s and 72°C for 30 s, with a final extension cycle of 72°C for 10 min [22]. (ii) *RASSF1A*, one cycle at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 63°C for 30 s and 72°C for 30 s, with a final extension cycle of 72°C for 10 min [23].

MSP products were fractionated on 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Our criterion for methylation status of *LINE-1* was follow: samples with band in both the methylation and unmethylation specific reaction were regarded as hypomethylated. For *RASSF1A*, samples only with band in the methylation or unmethylation specific reaction were considered as hypermethylated or unmethylated, respectively.

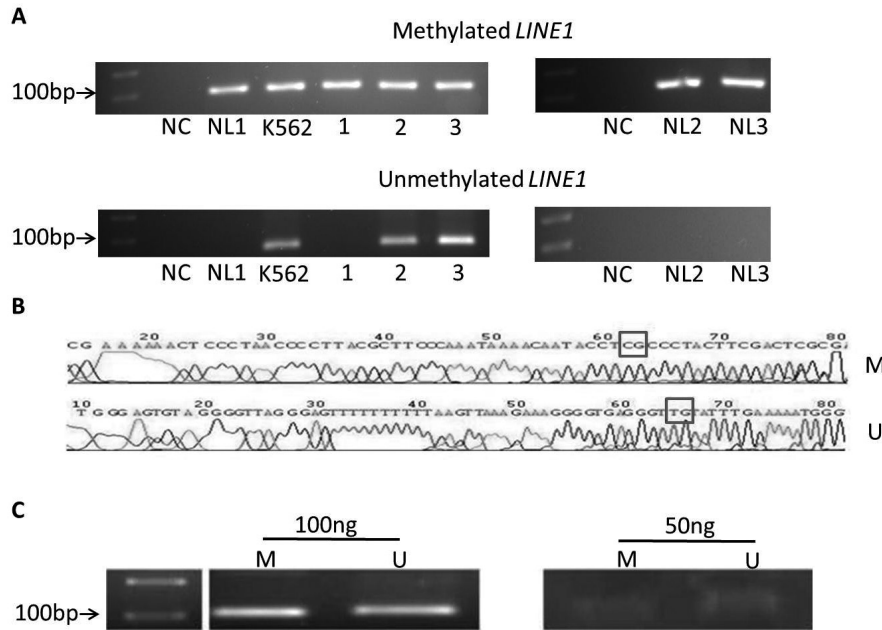


Figure 1. Methylation analysis of *LINE-1* by methylation specific PCR in serum DNA from HCC. (A) methylated *LINE-1* (NC= negative control, no DNA. NL=normal liver tissue DNA, 100% methylated. 1-3=patient's samples) and unmethylated *LINE-1* (NC= negative control, no DNA. cell line DNA of K562 used as hypomethylated control. 1-3=patient's samples). (B) The specificity was proved by the sequence of MSP product (M= the methylated *LINE-1*, U= the unmethylated *LINE-1*, black frame represented the methylated CpG site). The sequences were consistent with the purpose fragments and CpG island in methylated was replaced by TpG in unmethylated fragment. (C) The sensitivity was detected using normal plasma DNA added K562 DNA with 100ng-50ng. And *LINE-1* unmethylation status could be tested with strong signals, when K562 DNA concentration reached or exceeded 100ng. Or weak signals could be detected if the reaction system contained DNA greater than or equal to 50ng.

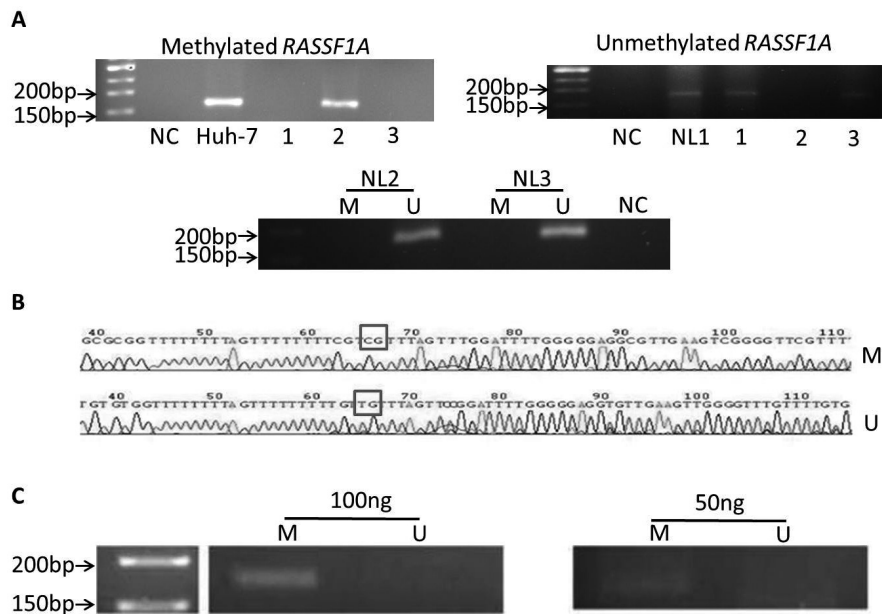


Figure 2. Methylation analysis of *RASSF1A* by methylation specific PCR in serum DNA from HCC. (A) methylated *RASSF1A* (NC= negative control, no DNA. Cell line DNA of Huh-7 used as positive control, 100% hypermethylated. 1-3=patient's samples) and unmethylated *RASSF1A* (NC= negative control, no DNA. NL=normal liver tissue DNA, totally unmethylated. 1-3=patient's samples). (B) The specificity was proved by the sequence of MSP product (M= the methylated *RASSF1A*, U= the unmethylated *RASSF1A*, Black frame represented the methylated CpG site). The sequences were consistent with purpose fragments and CpG island in methylated was replaced by TpG in unmethylated fragment. (C) The sensitivity was detected using normal plasma DNA added Huh-7 DNA with 100ng-50ng. And *RASSF1A* methylation status could be tested with strong signals, when Huh-7 DNA concentration reached or exceeded 100ng. Or weak signals could be detected if the reaction system contained DNA greater than or equal to 50ng.

After electrophoresis detection, the methylated and unmethylated products of MSP in HCC samples were randomly collected with DNA Purification Kit (TIANGEN) and sequenced (Sangon Biotech). The methylated and unmethylated fragments of *LINE-1* and *RASSF1A* were compared with DNAMAN, separately.

To test the sensitivity of MSP system, K562 and Huh-7 cell lines was chose as positive controls for unmethylated *LINE-1* and methylated *RASSF1A*, separately. And DNA from cell lines (50ng-100ng) was respectively added in 400 $\mu$ L normal plasma. Then, plasma DNA isolated, bisulfite conversion and MSP were performed.

**Statistical analyses.** The data was analysed by using the Social Sciences (SPSS), version 20.0 (IBM). The methylation status of *LINE-1* and *RASSF1A* and all other qualitative variables were represented as frequencies and percentages (%). The chi-square test was used to assess any potential association of *LINE-1* and *RASSF1A* methylation status with patient's demographic and clinical pathological characteristics. Survival rates were calculated by using Kaplan-Meier method with log-rank test and Breslow test. Multivariate Cox proportional hazards regression analysis was applied to investigate the independent effect of *LINE-1* and *RASSF1A* methylation status on survival. Patient's gender, age, tumor size, AFP level and HBsAg condition were also involved in the multivariate model as potential confounding factors. All tests were two tailed and p values <0.05 was considered as statistical significance.

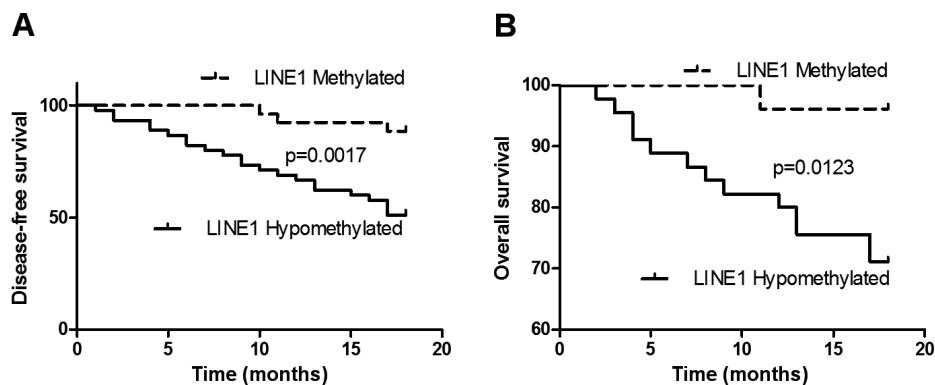
## Results

**The methylation status of *LINE-1* and *RASSF1A*** was evaluated in serum DNA samples from 105 patients diagnosed with HCC in double-blinded experiments. Patient's clinical pathological characteristics (Table 2) and clinical data become available after tumor resection for the statistical correlations. *LINE-1* hypomethylated in 70 (66.7%) and *RASSF1A* promoter hypermethylated in 77 (73.3%). But none of them was found in control sample.

**Association between *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation status and tumor parameters in serum DNA with HCC patients.** Chi-square analysis showed a significant association between hypomethylated *LINE-1* status and HBsAg positive condition (70.1% vs 25%,  $p=0.009$ ), bigger tumor size (95.65% vs 53.85%,  $p=0.001$ ) as well as high AFP levels (86.67% vs 51.67%,  $p<0.001$ ). And only a significant relation between hypermethylated *RASSF1A* promoter status with lymph nodes metastasis (93.75% vs 69.66%,  $p=0.045$ ) was detected. No other significant associations between *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation status and other tumor parameters detected were found. The associations of demographic and clinical pathological characteristics with *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation status are given in Table 2. Co-expression of hypomethylated *LINE-1* and hypermethylated *RASSF1A* promoter were found 54 (51.43%) of HCC samples and its presence was associated with high AFP levels (82.22% vs 28.33%,  $p=0.02$ ) and lymph nodes metastasis (81.25% vs 46.07%,  $p=0.01$ ).

**Association between *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation status and survival in serum DNA with HCC patients.** Following up 75 cases of HCC patients underwent curative hepatic resection for 18 months, 14 (19.72%) patients have died due to a consequence of disease progression. *LINE-1* hypomethylation and *RASSF1A* hypermethylation was detected in 13 (92.86%) and 10 (71.43%) of these patients, respectively. The incidence of death was significantly higher in patients with hypomethylated *LINE-1* than methylated *LINE-1* (28.9% vs 3.8%,  $p=0.015$ ), but it was independent of the *RASSF1A* promoter hypermethylation status (14.28% in unmethylated vs 20% in hypermethylated,  $p=0.766$ ).

**The Kaplan-Meier estimates of survival rates,** significantly benefited patients with a methylated *LINE-1* status (Fig. 3A disease-free survival rates, Log Rank test:  $p=0.0017$ , Breslow test:  $p=0.0015$ ; Fig. 3B overall survival rate, Log Rank



**Figure 3.** Kaplan Meier estimate of disease-free survival (A) and overall survival (B) for HCC with *LINE-1* methylation or hypomethylation. Survival time was defined as the time from curative resection to death or last time know follow up. The difference between groups was tested by log rank test.

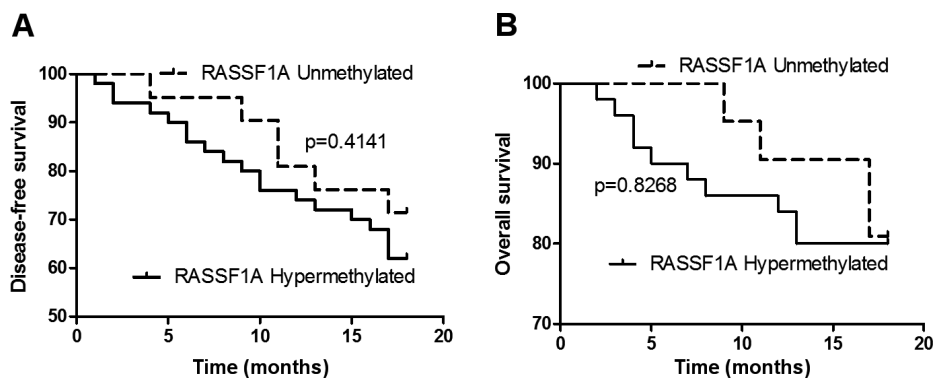


Figure 4. Kaplan Meier estimate of disease-free survival (A) and overall survival (B) for HCC with *RASSF1A* promoter hypermethylation or unmethylation. Survival time was defined as the time from curative resection to death or last time know follow up. The difference between groups was tested by log rank test.

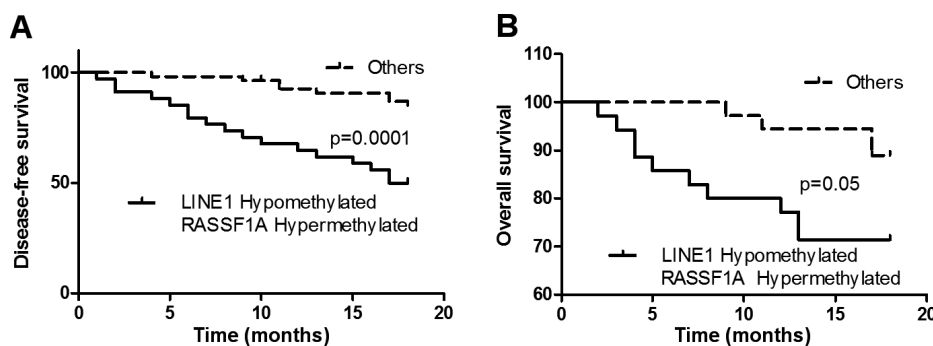


Figure 5. Kaplan Meier estimate of disease-free survival (A) and overall survival (B) for HCC categorized according to co-expression of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation. The difference between groups was tested by log rank test.

test:  $p=0.0123$ , Breslow test:  $p=0.0122$ ). Patients' survival was not significantly correlated with *RASSF1A* promoter hypermethylation status (Fig. 4A disease-free survival rates, Log Rank test:  $p=0.41$ , Breslow test:  $p=0.38$ ; Fig. 4B overall survival rate, Log Rank test:  $p=0.827$ , Breslow test:  $p=0.734$ ). The association of overall survival with the co-expression of hypomethylated *LINE-1* and hypermethylated *RASSF1A* promoters was marginal statistical significance (Fig. 5B Log Rank test:  $p=0.05$ , Breslow test:  $p=0.0393$ ). Remarkably, the correlation with disease-free survival was statistical significance (Fig. 5A Log Rank test:  $p=0.0001$ , Breslow test:  $p<0.0001$ ).

Further investigation with multivariate Cox proportional hazards regression analysis revealed that only hypomethylated *LINE-1* status ( $p=0.045$ ) remained the main statistically significant independent determinants for poor overall survival. Other parameters, such as gender ( $p=0.543$ ), age ( $p=0.545$ ), HBsAg status (0.564), tumor size ( $p=0.289$ ), AFP level (0.987), cirrhosis (0.661), lymph nodes metastasis ( $p=0.153$ ) and *RASSF1A* hypermethylated status ( $p=0.685$ ) were not significantly associated with overall survival.

## Discussion

Epigenetic alterations play important role in human carcinogenesis, which contribute to the initiation of cancer and also trigger genetic events leading to tumor development [24]. To date, DNA methylation is the most commonly studied epigenetic mechanism, and is involved in the tumorigenesis of nearly all cancer types, including HCC [25]. Widespread global DNA hypomethylation accompanied by specific region hypermethylation including tumor suppressor genes is a common feature found in HCC [9]. It is one of the major molecular alterations in the process of HCC carcinogenesis and also a potential diagnostic and prognostic bio-marker [26].

In this study, as a marker of global DNA methylation, *LINE-1* hypomethylation was detected in 70 (66.7%) out of the 105 examined cases, which indicated an important role in HCC. A significant correlation between *LINE-1* hypomethylation status and HBsAg positive, bigger tumor size was also observed. The result is in keeping with the study reported by Pisit Tangkijvanich [27]. In addition, significant correlation between *LINE-1* hypomethylation status and

high AFP levels was found. This finding is in accordance with Iman I. Ramzy [28]. In general, *LINE-1* hypomethylation was possibly an efficacy epigenetic biomarker in HBV induced and high AFP level patients alone, but not for all HCC patients.

Regarding *RASSF1A* promoter, it was found to be hypermethylated in 77 (73.3%) from our cases, which is a relative high methylation frequency. And it may be indicative of its crucial role in HCC carcinogenesis. Besides, the protein encoded by *RASSF1A* which was reported to share a similar conformation with RAS effector proteins is thus highly possible to act as a tumor suppressor in normal hepatocytes to prohibit the development and progress of HCC. Additionally, we detected that hypermethylation of *RASSF1A* was only significantly correlated with metastasis of regional lymph nodes ( $p=0.045$ ). And there was no significant relation with HBsAg positive ( $p=0.12$ ) and high AFP level ( $p=0.092$ ).

patient's disease-free survival and overall survival were analyzed with combination of *LINE-1* hypomethylated and *RASSF1A* promoter hypermethylated status or respectively. There is no significant relation with the *RASSF1A* promoter hypermethylation and prognosis. Thus, *RASSF1A* promoter hypermethylation may not be a useful biomarker alone for HCC agreeing with Xu B [29]. Interestingly, A more significant correlation was detected in disease-free survival rate (Log Rank test,  $p<0.001$ ; Breslow test,  $p<0.001$ ) than *LINE-1* hypomethylation alone (Log Rank test:  $p=0.0017$ , Breslow test:  $p=0.0015$ ). HCC patients co-expressing *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation have short disease free time and more easily relapse after curative hepatic resection. The mechanism is not very clear. Maybe HCC both with *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation possessed more potential deteriorative and aggressive ability. Besides, due to inadequate observed time, there is only marginal significant correlation was detected in overall survival rate (Log Rank test,  $p=0.05$ ; Breslow test,  $p=0.0393$ ). In additional study, a longer follow-up time need to be further explored.

In conclusion, we found that *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation are frequent epigenetic event in HCC patients. We also observed a significant correlation between *LINE-1* hypomethylated status and survival as well as between a hypermethylated *RASSF1A* promoter and lymph nodes metastasis. Notably, patients with co-expression of *LINE-1* hypomethylation and *RASSF1A* promoter hypermethylation status have shorter disease free time and more easily recur after tumor resection. Therefore, HCC patients with both hypomethylated *LINE-1* and hypermethylated *RASSF1A* promoter in serum DNA should require more intensive therapy and need to be closely observed after curative resection.

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