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Quantitative analysis of RASSF1A promoter methylation in hepatocellular carcinoma and its prognostic implications

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and is caused by the accumulation of genetic and epigenetic alterations in regulatory genes. In this study, we used methylight to detect the methylation status of the RASSF1A promoter in 87 paired HCC samples and analysed the relationship between methylation status and clinicopathological parameters, including prognosis after surgery. We found that the methylation level of the RASSF1A promoter in HCC tissues was significantly higher than that in the corresponding non-tumorous tissues ($p < 0.0001$). Furthermore, the methylation level of the RASSF1A gene promoter in HCC samples was higher in patients with a tumor size $\geqslant 6$ cm ($p = 0.0149$) and in patients younger than 50 years old ($p = 0.0175$). However, hypermethylation of the RASSF1A promoter in HCC tissues did not affect the overall survival of patients ($p = 0.611$). Thus, RASSF1A promoter hypermethylation may not be a useful biomarker for the prognosis of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and one of the main causes of cancer-related death worldwide. It is well documented that hepatitis B and C virus infections, aflatoxin B exposure, and heavy consumption of alcohol are important risk factors for HCC [1]. In addition, numerous genetic abnormalities associated with the development of HCC have been described.

In addition to genetic alterations, the DNA methylation-based mechanism has been demonstrated to contribute to the inactivation of tumor suppressor genes [2]. Aberrant methylated genes can be used as biomarkers for early detection, tumor classification, and response to treatments, such as target therapy, epigenetic agents, and traditional chemotherapy drugs. A number of studies have found that the methylation signal in the peripheral blood or tissue of cancer patients is a potential biomarker for early diagnosis or prediction of therapy response and prognosis [3].

RASSF1A, a major isoform of RASSF1 located at 3p21.3, is a cell cycle-related tumor suppressor protein that decreases colony formation, suppresses anchorage-independent growth, and dramatically reduces tumorgenicity in vivo. Many studies have shown that the inactivation of RASSF1A by promoter hypermethylation occurs frequently in HCC. However, to our knowledge, previous studies used different detection methods, and hence, the reported methylation frequencies of RASSF1A in hepatocellular carcinoma varied among these studies. Even when using the same method, repeated measurements can obtain differing methylation levels/frequencies [5–11]. Furthermore, in these studies, the methylation signal of the RASSF1A promoter was determined by qualitative or quantitative assay in no more than 50 paired samples.

In this study, to obtain a more exact quantitative assessment of the methylation status of the RASSF1A promoter, we used the Taqman probe-based quantitative methylation-specific PCR methylight method to detect the promoter of the RASSF1A gene in 87 paired HCC and paratumor non-malignant liver samples. We further analysed the correlation between the methylation status of the RASSF1A promoter and clinicopathologic features and evaluated whether RASSF1A can serve as a potential biomarker for HCC.

2. Material and methods

2.1. Human tissues

Human primary HCC and corresponding noncancerous liver samples (3 cm from the tumor) were collected from 87 patients who were diagnosed and treated at Guangxi Medical University, Guangxi Province, China from January 2003 to June 2005. This

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study protocol was approved by the Clinical Research Ethics Committee of Shanghai Cancer Institute, and informed consent was obtained from all patients. The tissue samples were snap frozen in liquid nitrogen immediately after surgical resection and then stored at −80 °C until they were analysed. Clinical information was collected from the patients' records and pathology reports. All patients were followed up from the date of surgery, and their death and survival statuses were recorded. Details are listed in Table 1

2.2. DNA preparation

Total genomic DNA was extracted from frozen tissue specimens (50–100 mg) according to the standard protocol with some modifications, which are briefly described below. The samples were frozen and pulverised, and the resulting powder was resuspended with 2 ml of warmed lysis buffer: 50 mM Tris–HCl pH 8.0, 50 mM EDTA, 1% SDS, 10 mM NaCl and 100 μg/ml boiling-treated RNase A (Sigma). Following one hour of incubation at 37 °C, Proteinase K (Roche, USA) was added to the cellular lysates for a final concentration of 100 μg/ml, and the digestion was carried out at 55 °C for 2 h. Organic extractions with a half volume of Phenol/Chloroform/Isoamyl alcohol (1:1:0.04) were repeatedly carried out until no visible interphase remained after centrifugation. DNA was precipitated from the aqueous phase in the presence of 0.3 M NaOAc pH 7.0 and two and a half volumes of ethanol. The DNA pellet was washed once with 70% ethanol, dissolved at 65 °C for 30 min with 0.2–0.4 ml of TE (10 mM Tris–HCl pH 7.4 and 1 mM EDTA) and then stored at 4 °C until further use. The DNA concentrations were calculated according to their OD readings at 260 nm [12].

2.3. Bisulfite treatment

Ten micrograms of DNA in 50 μl of TE was incubated with 5.5 μl of 3 M NaOH at 37 °C for 10 min, followed by a 16 h treatment at 50 °C with 30 μl of freshly prepared 10 mM hydroquinone and 520 μl of freshly prepared 3.6 M sodium-bisulfite at pH 5.0. The DNA was desalted using a home dialysis system with 1% agarose (detailed protocol available upon request). The DNA in the desalted sample (approximately 100 μl in volume) was denatured at 37 °C for 15 min with 5.5 μl of 3 M NaOH, followed by ethanol precipitation with 33 μl of 10 M NH4OAC and 300 μl of ethanol. After washing with 70% ethanol, the gently dried DNA pellet was dissolved with 30 μl of TE at 65 °C for 10 min. The DNA sample was finally stored at −20 °C until further use. Fifty nanograms of the DNA sample was reserved for PCR reaction [13].

2.4. SssI methylation assay

Peripheral blood leukocyte (PBL) DNA (Promega) was used as a substrate for M.SssI treatment. PBL DNA (0.05 μg/μl) was incubated with M.SssI at a concentration of 1 U/μg DNA (0.05 U/μl) and 0.16 mM AdoMet overnight at 37 °C. Then, extra AdoMet (to 0.20 mM) and M.SssI (to 0.065 U/μl) were added, followed by a second overnight incubation at 37 °C. The sample was stored at 4 °C, and 18 μl (0.9 μg DNA) aliquots were used for bisulfite conversion and recovery as described above [3].

2.5. Taqman probe-based quantitative methylation-specific PCR (Methylight)

PCR was performed using a 96-well optical tray with caps at a final reaction volume of 20 μl. Samples contained 8 μl of Real MasterMix (Taqman; Tiangen), 1 μl of bisulfite-treated DNA, 250 nM of the primers and 125 nM FAM-labelled probes. The modified DNA was amplified using the Methylight real-time PCR reaction using the TaqMan gene assay and the 7500/7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers and probes for RASSF1A and Alu-C4 have been previously described [3,5]. Each PCR program consisted of an initial denaturation step (95 °C, 10 min) and 45 cycles of denaturation (95 °C, 15 s) and annealing/extension (60 °C, 1 min). The methylation ratio was determined by absolute quantification of the

Table 1
Clinical information of 87 paired HCC samples and their RASSF1A promoter methylation level detected by Methylight assay. We listed the clinical information of 87 HCC and matched non-tumor liver tissues and their RASSF1A promoter methylation level detected by Methylight assay. The level of methylation was evaluated by percentage of methylation (PMR). The data were shown as means ± SEM. Differences among variables were assessed by *t*-test.

Parameters	Number of cases	PMR of NT (mean ± SEM)	PMR of HCC (mean ± SEM)	*P*-value
Total case number	87	15.37 ± 1.874	33.26 ± 4.236	<.0001*
Age (yr)				
>50	32	19.16 ± 3.933	25.32 ± 6.241	0.0175*
⩽50	55	13.17 ± 1.853	37.41 ± 5.617	
Gender				
Male	76	15.76 ± 2.086	31.13 ± 4.131	0.2588
Female	11	12.67 ± 3.538	45.65 ± 18.09	
Tumor size (cm)				
>6	48	16.13 ± 2.591	39.04 ± 6.588	0.0149*
⩽6	35	11.89 ± 1.872	20.45 ± 3.669	
Tumor Embolus				
Positive	29	16.00 ± 3.211	43.09 ± 8.818	0.0649
Negative	54	15.87 ± 458	29.02 ± 4.800	
Tumor Capsule				
Complete	23	15.76 ± 4.048	42.65 ± 10.64	0.8551
Incomplete	36	16.60 ± 2.594	26.43 ± 4.983	
TNM stage				
I,II	50	14.47 ± 2.149	32.32 ± 5.579	0.7445
III,IV	31	16.74 ± 3.519	35.36 ± 7.665	
Paracirrhosis				
Positive	49	15.04 ± 2.455	35.46 ± 5.945	0.6826
Negative	34	17.17 ± 3.188	31.75 ± 6.636	
AFP(ug/L)				
<20	22	17.87 ± 4.047	28.76 ± 5.831	0.5539
20–400	30	12.01 ± 2.623	28.97 ± 6.771	
>400	33	17.35 ± 3.352	38.64 ± 8.535	

real-time PCR. The quantity of amplified target genes in the test samples was normalised with that of Alu-C4 to measure the levels of input DNA, and DNA treated with M.SssI served as a methylated reference. The amount of methylated DNA (PMR, percentage of methylated reference) at a specific locus was calculated by dividing the GENE:Alu-C4 ratio of a sample by the GENE: Alu-C4 ratio of SssI-treated human genomic DNA (presumably fully methylated) and multiplying it by 100.

2.6. Statistical analysis

Data are expressed as the means ± SEM from at least three separate experiments. The data were analysed with either a two-tailed Student's *t*-test/$\chi 2$ or a one-way analysis of variance for the comparison of more than two groups, unless otherwise specified. The Kaplan–Meier method and the log-rank test were used to derive the overall survival function, and the log-rank test was used to compare the curves for two groups. For the Kaplan–Meier analysis, the median PMR level was used as a cut-off level. Therefore, the definition varied for each gene with the aim of obtaining equal sample sizes for each KM curve. This generates the highest statistical power for determining differences when there is a linear association. $P < 0.05$ was considered significant.

3. Results

3.1. Methylation level of RASSF1A promoter in HCC and corresponding noncancerous tissues

To determine the CpG island methylation level of the RASSF1A promoter, we used the methylight assay to detect the methylation level in 87 pairs of HCC and matched non-tumorous liver tissues. The data showed that the levels of methylation of the RASSF1A promoter were higher in 55 of 87 (63.22%) tumor tissues compared to their adjacent noncancerous liver tissues (Fig. 1A). It also revealed that the rate of DNA methylation ([(gene)sample/(Alu-C4)sample]/[(gene)SssI treated/(Alu-C4)SssI treated]*100) at the RASSF1A gene promoter was significantly higher in HCC tissues compared to their adjacent non-cancerous tissues. The median rate of methylation was 33.3% (range, 0.012–165.5) and 15.4% (0.13–89.2) in HCC tissues and non-cancerous liver tissues, respectively. The median level of DNA methylation was 2.16-fold up-regulated in HCC tissues compared to their corresponding non-cancerous tissues (paired *t*-test, $p = 0.0244$) (Fig. 1B). Occasionally, in cases when the SssI treatment of the standard DNA was not complete or in cases of aneuploidy of the gene locus of interest, PMR values may be >100%. Details are shown in Table 1. These data showed that the rate of DNA methylation at the RASSF1A locus is up-regulated in HCC tissues compare to their non-cancerous adjacent tissues.

3.2. Correlation between RASSF1A methylation status and clinicopathological data

To determine whether hypermethylation of RASSF1A is a characteristic biomarker of HCC, we also analysed the correlations between methylation status and multiple clinicopathological parameters. Of great interest, the CpG island methylation level of the RASSF1A promoter in HCC samples was higher in patients with larger tumors when the cutoff was set at 6 cm. Of the 48 patients with tumors $\geqslant$6 cm, the mean methylation level of the promoter of the tumor suppressor gene RASSF1A was 41.38%, while of the 35 patients with tumors <6 cm, the mean methylation level was 20.46% ($p = 0.0149$, Fig. 2).

Moreover, the methylation level of the RASSF1A promoter in HCC samples was higher in younger patients when the cutoff was set at 50 years old. Of the 55 patients who were younger than 50 years old, the mean methylation level was 37.41%, which is slightly higher than the mean methylation level of the other 32 patients (25.32%, $p = 0.0175$, Fig. 3A). By linear regression analysis, there was no apparent relationship between the methylation level of the RASSF1A promoter in the HCC samples and age in patients younger than 50 years old ($p = 0.998$, $R^2 < 0.001$, Fig. 3B), but for those older than 50 years old, the methylation level of the RASSF1A promoter in the HCC samples had a negative trend with age ($p = 0.1210$, $R^2 = 0.081$, Fig. 3C). For the other analysed clinical parameters, including gender, tumor embolus, tumor capsule, TNM stage, paracirrhosis and AFP level, there was no significant relationship to the methylation level of the RASSF1A promoter in HCC. Details are shown in Table 1.

3.3. Relationship between survival and methylation status of RASSF1A in HCC and adjacent non-tumorous tissues

We also explored the survival of HCC patients and evaluated whether the methylation status of the RASSF1A gene promoter is significantly associated with survival. Complete follow-up data

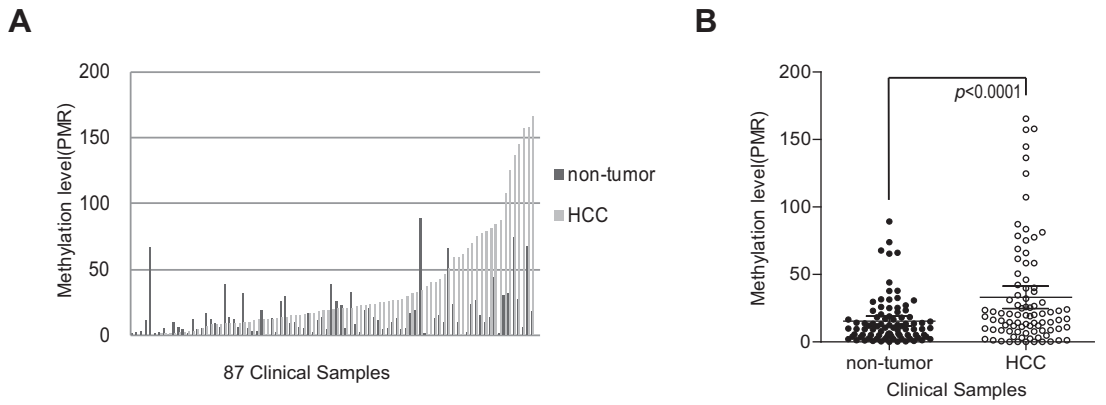

Fig. 1. Methylation level of RASSF1A gene promoter in HCC was higher than that in non-tumor liver tissues (A) We used Taqman probe-based quantitative methylation-specific PCR (methylight) to detect the promoter methylation level of RASSF1A in 87 HCC and matched non-tumor liver tissues. The level of methylation was evaluated by percentage of methylation (PMR). Comparing to matched adjacent noncancerous liver tissues, 55 of 87 (63.22%) tumor tissues showed a higher methylation level. (B) Methylation level of RASSF1A gene promoter in HCC was significantly higher than that in paired non-tumor liver tissues ($p < 0.001$). The median rate of methylation was 33.3% (range, 0.012–165.5) and 15.4% (0.13–89.2) in HCC tissues and non-cancerous liver tissues, respectively. The median DNA methylation level was 2.16-fold up-regulated in HCC tissues compared to their corresponding non-cancerous tissues.

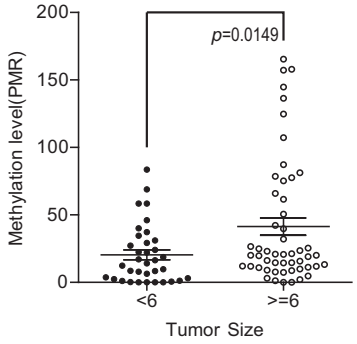

Fig. 2. Methylation level of RASSF1A promoter in HCC samples was related to tumor size The CpG island methylation level of the RASSF1A promoter in HCC samples was higher in patients with larger tumors when the cutoff was set at 6 cm. Of the 48 tumors ⩾6 cm, the mean methylation level of the promoter of the tumor suppressor gene RASSF1A was 41.38%, while of the 35 patients with tumors <6 cm, the mean methylation level was 20.46% ($p = 0.0149$).

were available in 80/87 patients (92.0%). Among those 80 patients, 57 (65.5%) died of disease (median, 51 months), and 23 (26.4%) were still alive (median, 21 months) at the termination of the study. Overall, the patients survived between 2 to 58 months, with a median survival of 29 months. The follow-up results did not reveal a significant difference between overall survival and the hypomethylation or hypermethylation status of the RASSF1A gene promoter ($p = 0.611$, Supplementary Fig. 1).

4. Discussion

Promoter hypermethylation of tumor suppressor or tumor related genes plays an important role in tumorgenesis. Hypermethylation of RASSF1A, a cell cycle-related tumor suppressor, has been detected frequently in lung, breast, ovarian, thyroid and nasopharyngeal carcinomas as well as in HCC tissues [14]. However, most previous studies used methylation-specific PCR, which has been considered an easy-to-use qualitative method for the detection of DNA methylation. Although methylation-specific PCR is a very sensitive assay to assess DNA hypermethylation, the results are dependent on the number of PCR cycles, the amount of input DNA, and the PCR reaction mixture conditions. Thus, the reported methylation frequencies of identical genes in hepatocellular carcinoma varied among previous studies [5–10]. Taqman probe-based quantitative methylation-specific PCR (MethyLight) can overcome issues related to PCR cycling and provide reliable quantitative information about the methylation status of the target CpG island loci [4,15].

Our findings showed that the promoter region of the RASSF1A gene was methylated in 75.9% of the non-cancerous liver tissues and 82.8% of HCC samples using a cutoff of 4% PMR, which indicates a high methylated frequency of the RASSF1A gene promoter in both non-cancerous tissues and HCC. This result may be related to the fact that the methylight assay is far more sensitive than the MSP protocol that was applied by others [13]. The high methylation rate in non-tumor liver tissues suggested that epigenetic change is involved in the early stage of liver carcinogenesis. When a significantly higher methylation rate cutoff of 20% was used, only 24.1% of non-tumor and 49.4% of HCC samples were considered to be methylated at the RASSF1A gene promoter. This result suggests that the paracancerous tissues of HCC have a moderate methylation degree of the RASSF1A gene promoter. Furthermore, the methylation rate of the RASSF1A promoter was significantly enhanced in the HCC samples compared with that of the corresponding non-tumor samples, indicating that this epigenetic change is involved in the development of liver carcinogenesis. The results of the present study were highly consistent with that of the previously published studies in this field [5–11], even though the detection methods differed in these studies.

Abnormal hypermethylated or hypomethylated genes in cancer can serve as biomarkers for clinical use in early detection, tumor classification, and prediction of treatment response [2]. Di Gioia et al. demonstrated that age-related methylation of the RASSF1A gene promoter takes place early in a small subpopulation of cells of the human liver [16]. Conversely, Zhong et al. and Feng et al. indicated that no association was apparent between methylation of the RASSF1A gene promoter and patient age [17,18]. In our study, we found that the methylation level of the RASSF1A gene promoter in the HCC samples was higher in younger patients when a cutoff of 50 years old was used. Of the 55 patients who were younger than 50 years old, the mean methylation level was 37.41%, which is slightly higher than the mean methylation level for the other 32 patients (25.32%).

We also found that the methylation level of the RASSF1A gene promoter in the HCC samples was correlated with tumor size, in concordance with the findings of other investigators [14]. The results showed that larger HCC tumors (>=6 cm) had higher methylation levels of RASSF1A. This result may be because RASSF1A is a cell cycle-related tumor suppressor protein that inhibits cyclin D1 and hence induces G1 phase arrest. RASSF1A methylation might

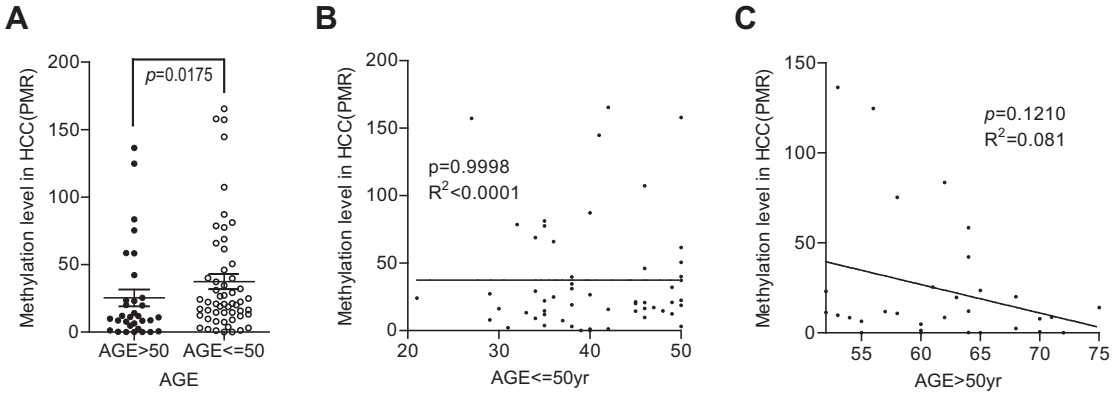

Fig. 3. Methylation level of RASSF1A promoter in HCC samples was correlated with patient age (A) Methylation level of RASSF1A gene promoter in HCC samples was higher in younger patients when the cutoff was set at 50 years old ($p = 0.0175$). Of the 55 patients who were younger than 50 years old, the mean methylation level was 37.41%, which was slightly higher than the mean methylation level of the other 32 patients (25.32%). (B) By linear regression analysis relationship, there was no apparent relationship between age and the methylation level of the RASSF1A gene promoter in HCC samples in patients younger than 50 years old ($p = 0.998$, $R^2 < 0.001$). (C) In patients older than 50 years old, the methylation level of the RASSF1A gene promoter in HCC samples had an inverse correlation trend with age ($p = 0.1210$, $R^2 = 0.081$).

allow damaged hepatocytes to proceed further into the cell cycle by escaping G1 phase arrest [5].

As RASSF1A hypermethylation will lead to low expression of RASSF1A in HCC [19], it is expected that a high frequency of RASSF1A methylation in HCC tissues should predict poorer prognosis. In the present study, the association between RASSF1A hypermethylation and survival was analysed by univariable analysis using the Kaplan–Meier and log-rank test. This is the first report for RASSF1A hypermethylation that showed no significant impact on overall survival; this results was inconsistent with one previous study [20].

5. Authors' contributions

XBY carried out methylight, analysis of data and drafted the manuscript. DJZ performed the statistical analysis. WZG and HXD participated in the experiment and coordination. LXY planned the experimental design, edited the manuscript and gave advice. ZQ participated in the study design and coordination. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.070.

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