RESEARCH COMMUNICATION

RASSF1A Promoter Hypermethylation as a Prognostic Marker for Hepatocellular Carcinoma

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Abstract

This study was performed to determine whether epigenetic aberrant methylation of RASSF1A might be associated with hepatocarcinogenesis. Methylation specific-PCR was performed to identify RASSF1A promoter hypermethylation in 29 tumors and corresponding normal liver tissues. In addition, RASSF1A mRNA levels were analyzed by quantitative real-time reverse transcription-PCR. Aberrant methylation of RASSF1A was detected in 25 of 29 cases (86%), with loss of RASSF1A expression evident in 8 of 22 cases (36%). No correlation between loss of RASSF1A mRNA and promoter hypermethylation of the RASSF1A gene was observed. There was a significant correlation between the methylation status of RASSF1A and hepatocellular carcinoma (HCC) patients who did not undergo chemotherapy (P = 0.03). Multivariate analysis, adjusted for tumor size, treatment, RASSF1A hypermethylation, and RASSF1A under-expression, showed RASSF1A hypermethylation to be assocaited with a better prognosis for HCC patients (HR= 0.089, 95% CI = 0.013-0.578; P = 0.012). Our findings showed that RASSF1A promoter hypermethylation occurs frequently, and may serve as a good prognostic factor.

Keywords: Hepatocellular carcinoma - RASSF1A - DNA methylation - methylation-specific PCR - Thailand

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Introduction

Hepatocellular carcinoma (HCC) is the leading cause of cancer-related death worldwide, especially in sub-Saharan Africa and Asia (Parkin, 1999). Important risk factors for HCC have included hepatitis C virus infection (HCV), hepatitis B virus infection (HBV), and cirrhosis caused by alcoholic liver disease (Motola-Kuba et al., 2006). Recently, several genetic and epigenetic alterations have been implicated with hepatocarcinogenesis, including mutations of p53 (Nishida et al., 1993; Zhu et al., 2004; Elmileik et al., 2005), Rb1 (Zhang et al., 1994), β-catenin (de La Coste et al., 1998), IGF2R (De Souza et al., 1995), SMAD2/4 (Yakicier et al., 1999), DNA amplification and over-expression of cyclin D1 gene (Nishida et al., 1994), and frequent promoter hypermethylation of several tumor suppressor genes such as p16 (Kaneto et al., 2001), GSTP1 (Zhong et al., 2002; Zhang et al., 2005), E-cadherin (Lee et al., 2003), SOCS1 (Okochi et al., 2003; Yang et al., 2003; Yoshida et al.,2004) and RASSF1A (Zhang et al., 2007).

The Ras Association domain Family 1A (RASSF1A) tumor suppressor gene is located in 3p21.3; inactivation is involved with the development of many human cancers including HCC (Zhong et al., 2003; Donninger et al., 2007; Zhang et al., 2007). It is implicated in the ras signaling pathway and has been shown to play a critical role in cell-cycle regulation, apoptosis, and microtubule stability (Agathanggelou et al., 2005; Donninger et al., 2007). However, an important mechanism of RASSF1A promoter hypermethylation in hepatocarcinogenesis still remains largely unknown.

In this study, we evaluated whether gene expression and the promoter hypermethylation of RASSF1A might play a role in hepatocarcinogenesis, and correlated RASSF1A hypermethylation and gene expression with clinico-pathological data of patients with HCC.

Materials and Methods

Sample collection

Twenty-nine paired samples of HCC and normal liver tissues were collected from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. The study was approved by the Institutional Review Board. No therapeutic treatment (chemotherapy or radiation) was instituted before the operation. All tissues samples were frozen in liquid nitrogen at -80°C for extraction of DNA and RNA. Hematoxylin & eosin-stained samples from each tumor block were examined microscopically. Differential grading was performed, according to Edmonson and Steiner (1954), with classification into 3 groups--well differentiated (grade I), moderately differentiated (grades

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II-III), and poorly differentiated (grade IV). Tissues with > 80% tumor cells and corresponding normal liver tissues from the same patients were used in this study.

DNA isolation and sodium bisulfite treatment

Genomic DNA was isolated by Proteinase K digestion and salting-out method (Miller et al., 1988). DNA was loaded in agarose gel electrophoresis, and stained with ethidium bromide to check purity. Treatment of DNA with sodium bisulfite would result in the unmethylated cytosines being converted into uracil, while the methylated cytosines remained unchanged. Bisulfite conversion was carried out using the reagents provided in an EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). One μg of DNA was treated with sodium bisulfite according to the manufacturer's directions. The bisulfite-converted DNA was eluted in a total volume of 25 μl and stored at -20°C until used.

RNA extraction and cDNA synthesis

Total RNA was extracted from 22 HCC and their corresponding normal liver tissues using Trizol reagent, according to the instruction manual (Invitrogen, Carlsbad, CA, USA). mRNA was isolated by Oligotex mRNA purification kit (Qiagen, Gmbh, Germany). Reverse transcription reactions were conducted according to the manufacturer's instructions, using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (Invitrogen, Carlsbad, CA, USA).

Methylation analysis

The methylation status of the promoter CpG islands of the RASSF1A gene in 29 tumors, and their corresponding normal liver tissues, were analyzed by methylation specific-PCR on sodium bisulfite modified DNA. The primers for the methylated sequences were FM-RASSF1A (5'-GCG TTG AAG TCG GGG TTC-3') and RM-RASSF1A (5'-CCC GTA CTT CGC TAA CTT TAA ACG-3'). The primers for unmethylated sequences were FU-RASSF1A (5'-GTG TTG AAG TTG GGG TTT-3') and RU-RASSF1A (5'-CCC ATA CTT CAC TAA CTT TAA ACA-3') (Lehmann et al., 2002). The reactions were carried out in a total volume of 25 μl, containing 100 ng genomic DNA, 1X PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.4 μM of sense and antisense primers, 0.5X GC-rich solution and 1 unit of FastStart TaqDNA Polymerase (Roche Diagnostics, Mannheim, Germany). Reaction mixtures were hot-started at 95°C for 5 min. Amplification was performed in a Mastercycler gradient (Eppendorf) for 30 cycles (1 min at 95°C, 30 sec at 65°C (methylated sequence) and 62°C (unmethylated sequence) and 30 sec at 72°C, followed by a final extension of 5 min at 72°C. Twenty-five microliters of PCR product were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light. Normal lymphocyte DNA treated with SssI methylase according to the manufacturer's instructions (New England Biolabs, Beverly, MA) was used as a positive control after modification with sodium bisulfite, and a negative control derived from normal lymphocyte DNA, after treatment with sodium bisulfite (Figure 1).

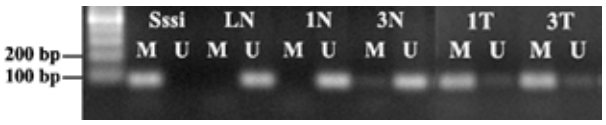

Figure 1. Methylation Analysis. RASSF1A in HCC (T) and corresponding normal samples (N) was assessed by MS-PCR. Normal Lymphocyte (Sssi) Treated with SssI methylase was used as a positive and normal lymphocytes (LN) as a negative control; M, methylated; U, unmethylated

Quantitative real-time reverse transcription-PCR

The gene expression level of RASSF1A was further analyzed by iQ5 Real Time PCR Detection System (BioRad, Hercules, CA), with primers designed using the Primer 3 program. The primer sequences were forward F-RASSF1A (5'-CCT TTT ACC TGC CCA AGG AT-3') and reverse R-RASSF1A (5'-GGT CAT CCA CCA CCA AGA AC-3'). The β-globin gene was used as an endogenous reference to obtain relative expression values. The reaction mixture was reacted using 20 ng of template cDNA, 2x FastStart SYBR Master (Roche Diagnostics, Mannheim, Germany), and 0.5 μM forward and reverse primers, in a final volume of 25 μl. The PCR was then started at 95°C for 5 min (to activate the FastStartTaq), followed by 50-cycle amplification (95°C for 10 s, 62°C for 30 s and 72°C for 30 s). After the PCR, each amplification reaction was checked using a dissociation curve. All real-time assays were performed in duplicate.

The relative level of gene expression was determined as previously described (Livak et al., 2001). RASSF1A ranged between 0.07-14.01 (median 1.04, mean 2.90, SD 3.88). In this study, the cutoff values for gene expression were adopted from the median expression levels. Tumor gene expression <1.0-fold was assigned as reduced expression for RASSF1A, and >1.0 served as positive.

Statistical analysis

The correlation between the methylation status of RASSF1A, RASSF1A mRNA expression and clinico-pathological characteristics--age, gender, tumor differentiation, tumor size, metastasis, treatment, serum HBsAg and anti-HCV--were examined by Fisher's exact test. Survival analysis was analyzed with patients who were followed-up for at least 200 weeks or until death, after surgery. One patient who died within four weeks, and one patient who was lost to follow-up, were excluded. Thus, only 27 patients were available for survival analysis. Kaplan-Meier analysis and log-rank tests were used to analyze overall survival. Cox regression analysis was utilized to estimate the prognostic effect for aberrant methylation of the RASSF1A gene on the overall survival of HCC patients. A significant correlation was considered where P value < 0.05.

Results

The methylation status of RASSF1A was evaluated in 29 tumors and corresponding normal liver tissues by MS-PCR. The methylation status of RASSF1A was detected in the tumors of 25 of 29 cases (86%). Meanwhile, only 3

Table 1. The Association Between Methylation Status of RASSF1A and Clinico-Pathological Data of 29 HCCs, and Reduced Expression of RASSF1A

Parameter	RASSF1A		P
	M n (%)	U n (%)	
Gender			0.47
Male	22 (88)	3 (12)	
Female	3 (75)	1 (25)	
Age			0.59
<50	13 (93)	1 (7)	
≥50	12 (80)	3 (20)	
Histological type			-
MD	9(100)	-	
PD	2(100)	-	
Unknown	14 (78)	4 (22)	
Tumor size (cm)			1.00
≤5	9 (90)	1 (10)	
>5	16 (84)	3 (16)	
Metastasis			1.00
Negative	12 (86)	2 (14)	
Positive	13 (87)	2 (13)	
Treatment			0.03*
CMT	9 (69)	4 (31)	
No CMT	16(100)	0	
HBsAg			0.56
Negative	8 (80)	2 (20)	
Positive	12 (92)	1 (8)	
Unknown	5 (83)	1 (17)	
Anti-HCV			0.46
Negative	12 (86)	2 (14)	
Positive	2 (67)	1 (33)	
Unknown	11 (92)	1 (8)	
RASSF1A			0.12
Positive	13 (93)	1 (7)	
Reduced	5 (63)	3 (38)	
Unknown	7(100)	0	

CI, confidence interval; MD, moderate differentiation; PD, poorly differentiation; CMT, chemotherapeutic treatment; HBsAg, hepatitis B surface antigen; anti-HCV, antibody to hepatitis C virus; M, methylated; U, unmethylated; * statistically significant association (P < 0.05)

Table 2. The Association Between RASSF1A Under-Expression and Clinico-Pathological data of 22 HCCs

Parameter	RASSF1A under-expression		P
	RASSF1A+ n (%)	RASSF1A- n (%)	
Gender			1.00
Male	7 (39)	11 (61)	
Female	1 (25)	3 (75)	
Age			0.38
<50	5 (50)	5 (50)	
≥50	3 (25)	9 (75)	
Histological type			0.33
MD	1 (20)	4 (80)	
PD	1(100)	0	
Unknown	6 (37)	10 (63)	
Tumor size (cm)			0.31
≤5	3 (60)	2 (40)	
>5	5 (29)	12 (71)	
Metastasis			1.00
Negative	5 (36)	9 (64)	
Positive	3 (37)	5 (63)	
Treatment			0.67
CMT	5 (42)	7 (58)	
No CMT	3 (30)	7 (70)	
HBsAg			0.59
Negative	4 (40)	6 (60)	
Positive	1 (16)	5 (83)	
Unknown	3 (50)	3 (50)	
anti-HCV			0.09
Negative	3 (23)	10 (77)	
Positive	2(100)	0	
Unknown	3 (43)	4 (57)	

CI, confidence interval; MD, moderate differentiation; PD, poorly differentiation; CMT, chemotherapeutic treatment; HBsAg, hepatitis B surface antigen; anti-HCV, antibody to hepatitis C virus; +, under-expression; -, no under-expression

of 29 cases (10%) were observed in normal liver tissues. RASSF1A promoter hypermethylation did not correlate with patient age, gender, tumor differentiation, tumor size, metastasis, serum HBsAg or anti-HCV. Interestingly, our findings demonstrated that the aberrant methylation of RASSF1A was associated with HCC patients who did not undergo chemotherapy (P = 0.03) as shown in Table 1.

To assess the methylation status of RASSF1A-influenced gene expression, we examined mRNA RASSF1A expression level on 22 HCCs by quantitative real-time reverse transcription-PCR. Our results showed 8 of 22 cases (36%) had reduced expression, of whom 5 showed RASSF1A hypermethylation as well; the other 3 cases were nonhypermethylated RASSF1A. However, in this study, no correlation was found between reduced expression and hypermethylation of the RASSF1A gene (P= 0.12), as shown in Table 1. In addition, no significant correlation was found between loss of RASSF1A mRNA and clinico-pathological characteristics (Table 2).

Univariate analysis by Kaplan-Meier and log-rank test revealed that the existence of methylated RASSF1A has an important positive impact on prolonged overall survival than unmethylated RASSF1A. The median survival period was 102.9 weeks in patients with RASSF1A hypermethylation, and 14 weeks in cases without (P = 0.12), as shown in Figure 2. Multivariate Cox regression analysis showed that the existence of RASSF1A hypermethylation was related to a better prognosis for HCC patients (HR = 0.089, 95%CI = 0.013-0.578; P =

Table 3. Multivariate Analysis of Prognostic Factors for Survival of HCC Patients, by Cox Regression Analysis

Variable	Hazard ratios	95%CI	P
Tumor size; ≥5 vs <5	4.936	0.77-31.50	0.091
Metastasis; positive vs negative	1.300	0.35-4.85	0.697
Treatment; No CMT vs CMT	0.254	0.05-1.21	0.085
RASSF1A hypermethylation status; + vs -	0.087	0.01-0.58	0.012*
RASSF1A under-expression, + vs-	0.451	0.09-2.24	0.330

Abbreviations: CI, confidence interval; CMT, chemotherapeutic treatment; * Statistically significant association (P < 0.05)

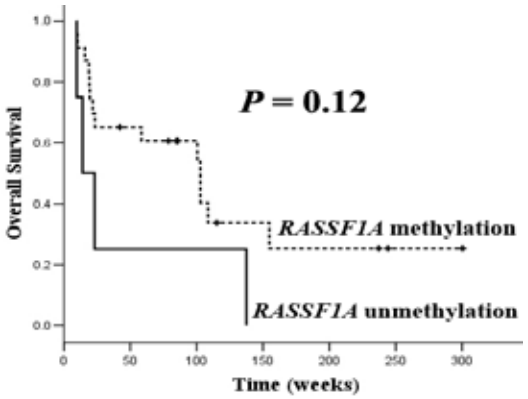

Figure 2. Kaplan-Meier Survival Curves for HCC Patients with RASSF1A Methylation (dotted line) and Unmethylation (bold line). The Log-Rank Test was Used to Compare the Survival Times Among Groups

0.012) as shown in Table 3.

Discussion

The aberrant methylation status of RASSF1A has been reported in many cancers, including HCC. RASSF1A methylation status was very frequent (70%) among HCC patients (Zhang et al., 2007), and was associated with HBV-related HCC and level of AFB(1)-DNA adducts in tumor tissues (Zhang et al., 2002; Zhong et al., 2003). Moreover, earlier studies reported the methylated RASSF1A gene in ovarian (Yoon et al., 2001), thyroid (Nakamura et al., 2005), gastric (Byun et al., 2001), bladder (Lee et al., 2001) and breast cancers (Agathanggelou et al., 2001; Liu et al., 2002).

Previous studies demonstrated that RASSF1A hypermethylation was associated with tumor size among HCC patients (Yeo et al., 2005), and mostly detected in tumors with vascular invasion or pleural involvement, poorly differentiated tumors, late-stage in lung cancer (Tomizawa et al., 2002; Wang et al., 2007) and tumor-stage in patients with esophageal squamous-cell carcinoma (Kuroki et al., 2003).

The current study identified the aberrant methylation of RASSF1A by MS-PCR. The findings showed that the methylation status of RASSF1A was very frequent (86%) in HCC patients, and correlated significantly with the non-chemotherapy patients group (P= 0.03). Similarly, Muller et al reported that RASSF1A DNA methylation was a prognostic marker in breast-cancer patients who did not undergo adjuvant therapy. Moreover, the incidence of RASSF1A methylation is higher in cisplatin-resistant tumors than in cisplatin-sensitive tumors of the male germ cell (Koul et al., 2004).

The correlation between RASSF1A hypermethylation and survival was analyzed by univariate analysis using Kaplan-Meier and log-rank test. RASSF1A hypermethylation had an important longer overall survival in HCC patients than those with RASSF1A non-hypermethylation. Multivariate Cox regression analysis revealed that HCC patients with RASSF1A methylation had a better prognosis. This is the first report for RASSF1A hypermethylation showing the impact on longer overall survival for HCC patients. One previous study showed that patients with a methylated RASSF1A gene survived longer than those with a wild-type allele in non-small cell lung cancer (Fischer et al., 2007). Conversely, several studies have reported that RASSF1A promoter hypermethylation was associated with a poor prognosis in certain cancers, such as prostate (Liu et al., 2002), thyroid (Schagdarsurengin et al., 2002) and lung cancers (Burbee et al., 2001; Kim et al., 2003; Wang et al., 2004).

Loss of gene expression related to hypermethylation of RASSF1A was demonstrated in previous studies (Dammann et al., 2000; Agathanggelou et al., 2001; Lo et al., 2001; Liu et al., 2002; Macheiner et al., 2006). In contrast, our study found no association between reduced expression and hypermethylation of RASSF1A. One study revealed that loss of expression in the absence of hypermethylation could be due to genetic alterations (deletion or mutation) or regulatory mechanisms (Lehmann et al., 2007). However, a further study with larger samples is needed to confirm these findings.

In conclusion, we showed that RASSF1A hypermethylation was associated with HCC patients who did not received chemotherapy, and that hypermethylation was associated with a good prognosis.

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