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DNA methylation markers and serum α-fetoprotein level are prognostic factors in hepatocellular carcinoma

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ABSTRACT

Introduction. Hypermethylation of relevant genes may affect the prognosis of patients with cancer. The purpose of this study was to analyze whether methylation of the promoter regions of cell cycle regulators as well as elevated α-Fetoprotein (AFP) levels are useful prognostic factors for patients with hepatocellular carcinoma (HCC). **Material and methods.** Nested methylation-specific PCR (nested-MSP) was used to analyze methylation status of the promoter regions of *p15*, *p16*, *p21*, *p27*, and *ras-association domain family 1 (RASSF1A)* genes in tumor specimens from 50 patients with HCC. **Results.** Promoter methylation was most common in the *RASSF1A* gene (96%), followed by the *p16* gene (56%), the *p21* gene (44%), the *p15* gene (28%), and the *p27* gene (2%). Patients with a serum AFP level < 400 ng/mL and an unmethylated *p21* promoter had a better prognosis than patients with a serum AFP level ≥ 400 ng/mL and a methylated *p21* promoter (overall survival, p = 0.076; disease-free survival, p = 0.016). In addition, patients with full methylation of the promoter region of *RASSF1A* had a better prognosis than patients with a partially methylated or unmethylated *RASSF1A* promoter region if their serum AFP level was ≥ 400 ng/mL (overall survival, p = 0.028; disease-free survival, p = 0.078). **Conclusion.** A partially methylated or unmethylated *RASSF1A* promoter as well as elevated serum AFP level or methylation of *p21* in addition to elevated serum AFP level might be associated with poor prognosis in patients with hepatocellular carcinoma.

Key word. Hepatocellular carcinoma (HCC). Methylation. α-fetoprotein (AFP). Ras-association domain family 1(RASSF1A). p21.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and is the third most common cause of cancer-related death.[1] Approximately 600,000 people are diagnosed with hepatocellular carcinoma every year. Furthermore, a marked difference has been found in the geographic distribution of HCC, especially in the Far East and Southeast Asia where viral hepatitis is more prevalent.[2] Despite improvements in the detection and treatment of HCC, the prognosis of patients with HCC is still very poor.

Promoter hypermethylation has been found in tumor suppressor genes involved in many different signaling pathways in different tumor types.[3-6] Epigenetic modification has been identified as a crucial event in carcinogenesis.[7] Aberrant methylation of CpG islands in promoters is associated with transcriptional inactivation of genes involved in all aspects of tumor development. Genes involved in DNA damage response, cell cycle control, apoptosis signaling, drug metabolism, detoxification, angiogenesis, DNA repair, intercellular adhesion, and tissue invasion can frequently become methylated and epigenetically silenced in tumors.[8] Epigenetic silencing mediated by CpG island methylation is, therefore, a potential therapeutic target as well as a potential prognosticator.[8]

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Cyclin-dependent kinase inhibitors are potent negative regulators of G1/S transition. There are two families of CDK inhibitors. One is the INK4 family, which comprises *p15*, *p16*, *p18* and *p19*, and the other is the KIP/CIP family, which comprises *p21*, *p27* and *p57*.[9] Hypermethylation of *p15* and *p16* is frequently detected in hepatocellular carcinoma.[10-12] Negative expression of *p21* protein has been shown to be associated with poor prognosis, and it was suggested that *p21* protein is an independent survival prognostic factor for HCC.[13] The expression of *p27* protein alone was shown to predict disease recurrence, indicating that it could be used as an independent prognostic marker for disease-free survival in HCC.[14] Mutations within the coding regions of the *p21* and *p27* genes were not detectable in a large series of human tumors.[15,16] Therefore, under-expression of both *p21* and *p27* proteins in tumor tissues might not be due to mutations in the structural genes. Rather, under-expression might be due to hypermethylation of the genes. *RASSF1A (Ras association domain family 1 isoform A)* is a tumor suppressor.[17] However, hypermethylation of the RASSF1A promoter has long been demonstrated in many liver diseases,[18-21] including HCC, cirrhosis, and hepatocellular nodules (HN).[22]

To the best of our knowledge, no studies have examined whether methylation status of cell cycle regulators and elevated serum α-Fetoprotein (AFP) levels are prognostic factors for patients with hepatocellular carcinoma. The purpose of this study was to analyze whether methylation of the promoter regions of cell cycle regulators (including *p15*, *p16*, *p21*, *p27* and *RASSF1A*) as well as elevated AFP levels are useful prognostic factors for patients with HCC.

MATERIALS AND METHODS

Patients and specimens

HCC tumor specimens were obtained by surgical excision from 50 patients (35 males and 15 females) between 2002 and 2004 at the Taichung Veterans General Hospital, Taichung, Taiwan. Institutional Review Board (IRB) approval and informed consent were obtained. The patients with HCC ranged in age from 36 to 80 years and had a mean age of 58.2 ± 11.9 years. Genomic DNA was extracted with Trizol® Reagent (Invitrogen) according to the manufacturer's instructions.

Sodium bisulfite modification

Genomic DNA was treated with sodium bisulfate using the MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures Pty Ltd) according to the manufacturer's instructions.

Nested methylation-specific polymerase chain reaction (Nested-MSP)

The *p15*, *p16*, *p21*, *p27* and *RASSF1A* promoter regions were subjected to nested methylation-specific polymerase chain reaction (nested-MSP) in a GeneAmp PCR system 2,400 thermal cycler (Applied Biosystems, Foster City, CA, USA). For the first-round, PCR reaction was carried out in a total volume of 50 μL with 100 ng modified DNA. The PCR mixture contained 200 μM dNTP, 5 μM primer, 100 ng DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1 unit of Platinum® Taq DNA Polymerase (Invitrogen™). The primers for amplification of the promoter regions were designed to distinguish between bisulfite-sensitive and bisulfite-resistant modifications of unmethylated and methylated cytosines, respectively (Table 1). Thermal cycling conditions were as follows: initial heat denaturing step was 5 minutes at 96 °C, the second step was 25 cycles of 96 °C for 30 sec, then 43-50 °C for 30 sec, then 72 °C for 30 sec, and the final extension at 72 °C for 10 minutes. The PCR products were purified with the PCR Clean Up Kit (GeneMark, Inc., Tai Chung Hsien, Taiwan, R.O.C) according to the manufacturer's instructions. For the second round, PCR reaction was carried out in a total volume of 25 μL with 50 ng purified PCR products. The PCR mixture contained 100 μM dNTPs, 2.5 μM primer, 50 ng DNA, 10 mM Tris-HCl (pH 8.4), 25 mM KCl, and 0.5 unit Super-Therm Taq DNA Polymerase (Hoffman-La-Roche). Thermal cycling conditions were as follows: initial heat denaturing step was 5 minutes at 96 °C, the second step was 30 cycles of 96 °C for 30 sec, then 55-60 °C for 30 sec, then 72 °C for 30 sec, and the final extension at 72 °C for 10 minutes. The second PCR products were separated by 2.5% agarose gel electrophoresis with 0.5 X TBE and stained with SYBR® Green I solution for visualization under UV illumination. EpiTect® control DNA (human), methylated DNA, and unmethylated DNA (QIAGEN®, Taipei, Taiwan) were used as positive and negative controls. Water was also used as a negative control in the nested-MSP.

Table 1. Primer sequences used for nested-MSP analysis.

Primer	Forward (5'→3')	Reverse (5'→3')	Size (bp)	Anneal (°C)	Ref.
p15 mod	AGTTTAAGGGGGTGGGGAGA	CCCCCACTAAACATACCCTTATT	459	52	
p15 met	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	148	55	Dong, *et al*., 2002
p15 unmet	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	154	55	
p16 mod	TTTTAGAGGATTTGAGGGATAGGG	CTAATTCCAATTCCCCTACAAACTT	387	49.2	
p16 met	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	60	Dong, *et al*., 2002
p16 unmet	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60	
p21 mod	GTGAGTTAGAAAGGGGGTTTATTTT	CTCTCTCACCTCCTCTAAATACCTC	456	49.2	
p21 met	TACGCGAGGTTTCGGGATC	CCCTAATATACAACCGCCCCG	174	60	Zhang, *et al*., 2008
p21 unmet	GGATTGGTTGGTTTGTTGGAATTT	ACAACCCTAATATACAACCACCCCA	164	60	
p27 mod	GGATTTGGAGAAGTATTGTAGAGA	TCAATCTTTAAATCCACCAAA	279	49.2	
p27 met	AAGAGGCGAGTTAGCGT	AAAACGCCGCCGAACGA	195	55	Nakamura, *et al*., 2001
p27 unmet	ATGGAAGAGGTGAGTTAGT	AAAACCCCAATTAAAAACA	212	55	
RASSF1A mod	GGGTTTTATAGTTTTTGTATTTAGGTT	AACTCAATAAACTCAAACTCCCC	200	43	
RASSF1A met	CGGTTTTTTTTAGTTTTTTTTCGTCG	TAACTTTAAACGCTAACAAACGCGAA	111	60	Gioia, *et al*., 2006
RASSF1A unmet	TGTGTGGTTTTTTTTAGTTTTTTTTTGTTG	CCCAACATAACCCAATTAAACCCA	147	60	

mod: modification. met: methylation. unmet: unmethylation.

Statistical analysis

The χ^2 test, hazard ratios (HR), and the Fisher's exact test were used to compare differences in methylation of the promoters of the *p15*, *p16*, *p21*, *p27* and *RASSF1A* genes. The overall survival and disease-free survival of HCC patients were examined by the Kaplan-Meier method and the log-rank test. Two-tailed p values of < 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS 17.0 software.

RESULTS

Frequency of methylation status of tumor suppressor genes in patients with HCC

Nested-MSP was performed on HCC specimens from 50 patients with HCC to investigate methylation of the promoter regions of cell cycle regulators, namely *p15*, *p16*, *p21*, *p27* and RASSF1A. The nested-MSP results were defined as follows: methylation was defined in products that showed evidence of complete methylation, partial methylation was defined in products that showed evidence of both methylated and unmethylated PCR products, and no methylation was defined in products that showed no evidence of methylation. Representative examples of nested-MSP results are presented in figure 1 and the overall results are summarized in figure 2. The frequency of promoter methylation of five genes in 50 HCC specimens varied from 2% to 96%. Promoter methylation was most common in the RASSF1A gene (96%), followed by the *p16* gene (56%), the *p21* gene (44%), the *p15* gene (28%), and the *p27* gene (2%).

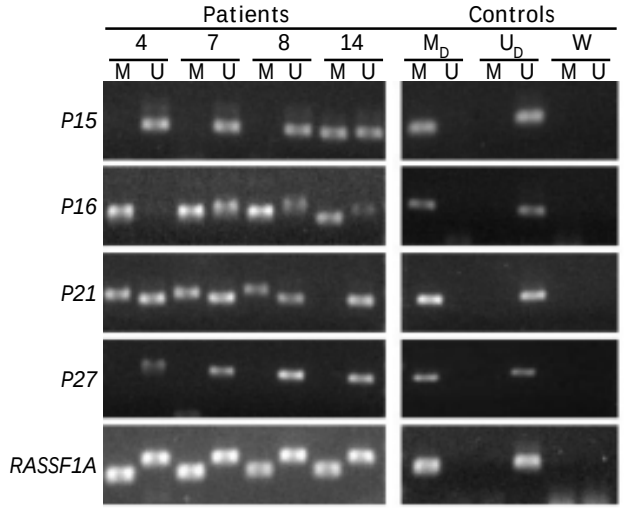

Figure 1. Representative nested-MSP for methylation analysis of p15, p16, p21, p27 *and* RASSF1A *genes. PCR products amplified with methylated (M) and unmethylated (U) sequence-specific primers. Methylated DNA (MD) and unmethylated DNA (UD) were used as positive controls. Distilled water (W) without DNA was used as a negative control. Positive and negative controls were used for each PCR run.*

Association between clinicopathologic characteristics and promoter methylation of tumor suppressor genes

The results of our analysis of the association between methylation status and clinicopathological parameters in patients with HCC are presented in table 2. We found that the frequency of p16 methylation was significantly higher in patients with HCV-related HCC than in patients with other types of HCC ($p = 0.01$) and that the frequency of unmethylated *p16* was significantly higher in patients with HBV-related HCC than in patients with other types of HCC ($p = 0.02$). There was no association between clinicopathological parameters in patients with HCC and the frequency of methylation of promoter regions of *p15*, *p21*, *p27* and *RASSF1A* genes (Table 2).

Univariate analysis of prognostic factors for patients with HCC

Cox proportional hazards analysis was used to analyze the significance of clinicopathologic characteristics (Tables 3 and 4) and *p15*, *p16*, *p21*, *p27* and *RASSF1A* methylation status (Tables 5 and 6) in predicting overall survival and disease-free survival. We found that age ≥ 58 years was associated with lower rates of overall survival than age < 58 years for patients with HCC (HR, 2.42; 95% CI, 1.04 to 5.63; $p = 0.04$; Table 3). Although the overall mortality rate among individuals with serum AFP levels ≥ 400ng/mL was twice as high as that among individuals with serum AFP levels < 400 ng/mL, there was no significant difference in overall survival between the two groups (HR, 2.16; 95% CI, 0.98 to 4.73; $p = 0.06$; Table 3). We also found that there was no significant difference in disease-free survival between patients with serum AFP levels ≥ 400 ng/mL and patients with serum AFP levels < 400 ng/mL (HR, 2.04; 95% CI, 0.98 to 4.23; $p = 0.06$; Table 4). In addition, no significant associations were found between methylation status of the five genes and clinicopathologic characteristics and overall survival and disease-free survival rates (Table 5 and Table 6).

Univariate analysis of p21 methylation status and AFP level for overall survival and disease-free survival

Patients were divided into four groups based on serum AFP levels and *p21* methylation status: group A_{p21} (serum AFP level < 400 ng/mL and *p21* unmethylation, n = 14), group B_{p21} (serum AFP

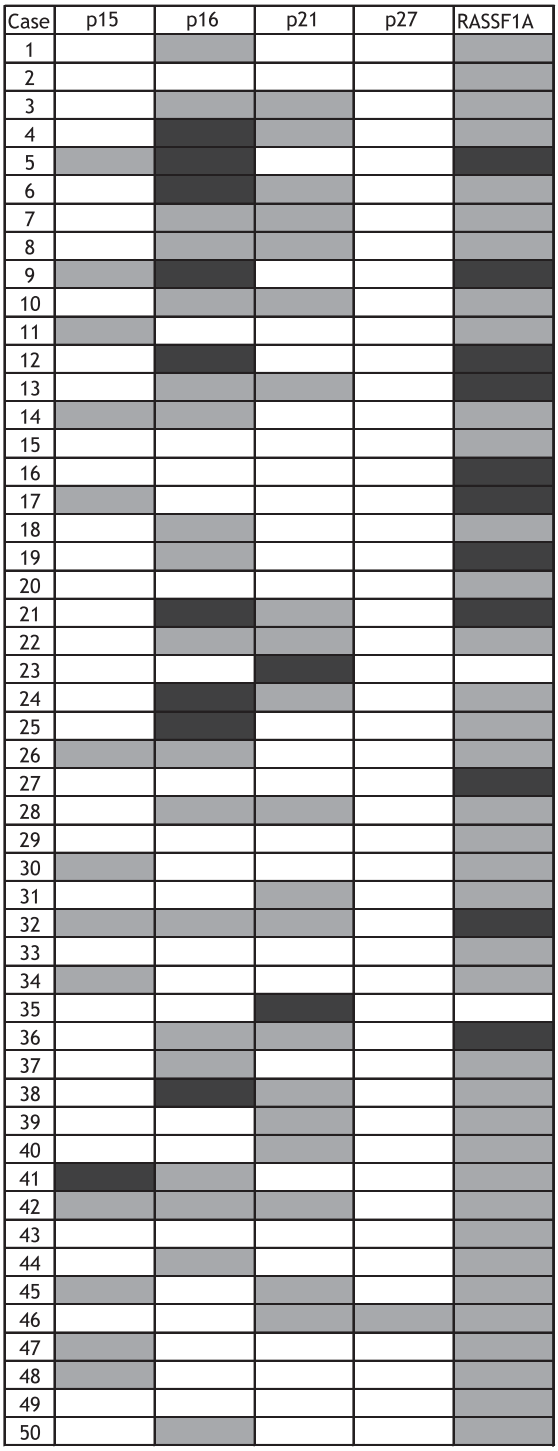

Figure 2. Summary of methylation of p15, p16, p21, p27 *and* RASSF1A *in 50 HCC samples. The frequency of proximal promoter methylation of five genes in 50 HCC specimens varied from 2% to 96%. Methylation was detected in 28% for p15, 56% for p16, 44% for p21, 2% for p27 and 96% for RASSF1A. Patient identification numbers are given. Filled boxes, presence of methylation; open boxes, presence of unmethylation; shadow boxes, presence of partial methylation.*

Table 2. Association between methylation status and clinical characteristics of 50 HCC patients.

Characteristic	Patients	*p15*			*p16*			*p21*			*p27*			*RASSF1A*		
		M (n = 14)	U (n = 36)	P	M (n = 28)	U (n = 22)	P	M (n = 22)	U (n = 28)	P	M (n = 1)	U (n = 49)	P	M (n = 48)	U (n = 2)	P
Age (years)*																
< 58	25	5	20	0.34	13	12	0.57	12	13	0.57	1	24	1.00	24	1	1.00
≥ 58	25	9	16		15	10		10	15		0	25		24	1	
Sex																
Male	35	9	26	0.73	18	17	0.49	17	18	0.49	1	34	1.00	33	2	1.00
Female	15	5	10		10	5		5	10		0	15		15	0	
HBV																
Positive	22	4	18	0.50	8	14	0.02	11	11	0.59	1	21	0.46	21	1	1.00
Negative	26	8	18		19	7		11	15		0	26		25	1	
Unknow	2	2	0		1	1		0	2		0	2		2	0	
HCV																
Positive	21	6	15	0.75	17	4	0.01	8	13	0.59	0	21	0.57	21	0	0.32
Negative	28	8	20		11	17		14	14		1	27		26	2	
Unknow	1	0	1		0	1		0	1		0	1		1	0	
AFP (ng/mL)																
< 400	29	8	21	0.90	17	12	0.97	15	14	0.46	1	28	1.00	28	1	1.00
≥ 400	20	5	15		11	9		7	13		0	20		19	1	
Unknow	1	1	0		0	1		0	1		0	1		1	0	
Cell differentiation																
Well	3	1	2	0.96	1	2	0.64	1	2	0.26	0	3	0.71	3	0	0.85
Moderately	30	8	22		18	12		16	14		1	29		29	1	
Poorly	17	5	12		9	8		5	12		0	17		16	1	
Size (cm)																
< 5	25	10	15	0.16	17	8	0.09	11	14	1.00	0	25	0.50	24	1	1.00
≥ 5	25	4	21		11	14		11	14		1	24		24	1	

M: methylation (included partially methylation). U: unmethylation. HBV: hepatitis B virus (according to appearance of serum HBsAg). HCV: hepatitis C virus (according to appearance of serum anti-HCV). * Discriminated by means.

Table 3. Univariate analysis of clinicopathologic characteristics for overall survival.

Characteristic	HCC patients (n = 50)	Overall survival 5-y rate (%)	Overall survival Median (mo)	HR (95% CI)	*p value*
Age, (years)*	58.2 ± 11.9				
< 58	25	68.0	66.0		0.04
≥ 58	25	44.0	45.0	2.42(1.04-5.63)	
Sex					
Male	35	54.0	59.0		0.53
Female	15	60.0	66.0	0.75(0.31-1.81)	
HBV					
Negative	26	62.0	66.0		0.34
Positive	22	45.0	44.0	1.46(0.67-3.22)	
HCV					
Negative	28	53.6	61.0		0.90
Positive	21	57.1	59.0	1.05(0.48-2.31)	
AFP (ng/mL)					
< 400	29	65.5	65.0		0.06
≥ 400	20	40.0	38.0	2.16(0.98-4.73)	
Cell differentiation					
Well	3	66.7	75.0		
Moderately	30	63.3	65.0	1.31(0.17-10.07)	0.79
Poorly	17	41.2	44.0	2.17(0.28-16.91)	0.46
Size (cm)					
< 5	25	56.0	65.0		0.61
≥ 5	25	56.0	59.0	1.23(0.56-2.70)	

HBV: data are missing for 2 patients. HCV: data are missing for 1 patient. AFP: data are missing for 1 patient. * Discriminated by means.

Table 4. Univariate analysis of clinicopathologic characteristics for overall survival.

Characteristic	HCC patients (n = 50)	Disease-free survival 5-y rate (%)	Disease-free survival Median (mo)	HR (95% CI)	p value
Age, (years)*	58.2 ± 11.9				
< 58	25	56.0	59.0		0.21
≥ 58	25	40.0	24.0	1.61(0.77-3.37)	
Sex					
Male	35	46.0	36.0		0.32
Female	15	53.0	55.0	0.66(0.29-1.50)	
HBV					
Negative	26	50.0	55.0		0.54
Positive	22	40.9	12.0	1.26(0.60-2.63)	
HCV					
Negative	28	50.0	43.0		0.73
Positive	21	42.9	28.0	1.14(0.60-2.37)	
AFP (ng/mL)					
< 400	29	58.6	59.0		0.06
≥ 400	20	30.0	7.0	2.04(0.98-4.23)	
Cell differentiation					
Well	3	66.7	77.0		
Moderately	30	53.3	56.0	0.95(0.21-4.24)	0.94
Poorly	17	42.0	12.0	1.31(0.28-6.06)	0.73
Size (cm)					
< 5	25	44.0	41.0		0.85
≥ 5	25	52.0	58.0	0.93(0.45-1.94)	

HBV: data are missing for 2 patients. HCV: data are missing for 1 patient. AFP: data are missing for 1 patient. * Discriminated by means.

Table 5. Univariate analysis of methylation status of the five genes for overall survival.

Characteristic	HCC patients (n = 50)	Overall survival 5-y rate (%)	Median (mo)	HR (95% CI)	p value
p15					
U	36	52.8	59.0		0.33
M	14	64.3	66.0	0.62(0.23-1.64)	
p16					
U	22	63.6	64.0		0.16
M	28	50.0	53.0	1.82(0.79-4.23)	
p21					
U	28	60.7	64.0		0.24
M	22	50.0	52.0	1.60(0.73-3.50)	
p27					
U	49	55.1	64.0		0.61
M	1	100.0	59.0	0.05(0-5894.83)	
*RASSF1A**					
U	39	51.3	58.0		0.11
M	11	72.7	71.0	0.37(0.11-1.24)	

* U included partially methylation.

Table 6. Univariate analysis of methylation status of the five genes for disease-free survival.

Characteristic	HCC patients (n = 50)	Disease-free survival 5-y rate (%)	Median (mo)	HR (95% CI)	p value
p15					
U	36	44.4	32.0		0.81
M	14	57.1	60.0	0.90(0.40-2.04)	
p16					
U	22	54.5	59.0		0.35
M	28	42.9	26.0	1.43(0.68-3.04)	
p21					
U	28	57.1	59.0		0.13
M	22	36.4	18.0	1.77(0.85-3.70)	
p27					
U	49	51.0	41.0		0.57
M	1	100.0	60.0	0.05(0-1591.58)	
*RASSF1A**					
U	39	46.2	36.0		0.42
M	11	54.5	66.0	0.68(0.27-1.71)	

* U included partially methylation.

level < 400 ng/mL and $_{p21}$ methylation, n = 15), and group C $_{p21}$ (serum AFP level ≥ 400 ng/mL and *p21* unmethylation, n = 13) and group D $_{p21}$ (serum AFP level ≥ 400 ng/mL and *p21* methylation, n = 7). The median overall survival rate in groups B $_{p21}$ (64.0%), C $_{p21}$ (48.0%), and D $_{p21}$ (8%) was shorter than that in groups A $_{p21}$ (65.0%). No significant difference in overall survival rate was found between groups A $_{p21}$ and B $_{p21}$ or between groups A $_{p21}$ and C $_{p21}$ ($p > 0.05$); however, a significant difference in overall survival was found between groups A $_{p21}$ and D $_{p21}$ ($p = 0.02$). The median disease-free survival rate in groups B $_{p21}$ (36.0%), C $_{p21}$ (28.0%), and D $_{p21}$ (3%) was also shorter than that in group A $_{p21}$ (60.0%). No significant difference in disease-free survival rate was found between groups A $_{p21}$ and B $_{p21}$ or between groups A $_{p21}$ and C $_{p21}$ ($p > 0.05$); however, a significant difference in disease-free survival was found between groups A $_{p21}$ and D $_{p21}$ ($p = 0.004$) (Table 7).

Table 7. Univariate analysis of *p21* methylation status and AFP level for overall survival and disease-free survival.

Characteristic	HCC patients (n = 50)	Overall survival 5-y rate (%)	Median (mo)	HR (95% CI)	p value	Disease-free survival 5-y rate (%)	Median (mo)	HR (95% CI)	p value
P21 and AFP (ng/mL)									
AFP < 400 and *p21* U	14	71.4	65.0			71.4	60.0		
AFP < 400 and *p21* M	15	60.0	64.0	1.99(0.60-6.63)	0.26	46.7	36.0	1.70(0.60-4.82)	0.32
AFP ≥ 400 and *p21* U	13	46.2	48.0	2.64(0.80-8.78)	0.11	38.5	28.0	1.97(0.68-5.71)	0.21
AFP ≥ 400 and *p21* M	7	28.6	8.0	5.07(1.35-19.05)	0.02	14.3	3.0	5.49(1.74-17.30)	0.004

AFP: data are missing for 1 patient.

Table 8. Univariate analysis of *RASSF1A* methylation status and AFP level for overall survival and disease-free survival.

Characteristic	HCC patients (n = 50)	Overall survival 5-y rate (%)	Median (mo)	HR (95% CI)	p value	Disease-free survival 5-y rate (%)	Median (mo)	HR (95% CI)	p value
RASSF1A and AFP (ng/mL)									
AFP < 400 and *RASSF1A* M	7	71.4	71.0			57.1	71.0		
AFP < 400 and *RASSF1A* U*	22	63.6	62.0	1.83(0.40-8.40)	0.44	59.1	59.0	1.00(0.31-3.20)	1.00
AFP ≥ 400 and *RASSF1A* M	4	75.0	70.0	0.89(0.08-9.78)	0.92	50.0	59.0	0.91(0.17-5.01)	0.92
AFP ≥ 400 and *RASSF1A* U*	16	31.3	19.0	4.58(1.02-20.59)	0.05	25.0	5.0	2.57(0.82-8.09)	0.11

AFP: data are missing for 1 patient. * U included partially methylation.

Univariate analysis of RASSF1A methylation status and AFP level for overall survival and disease-free survival

Patients were divided into four groups based on serum AFP levels and *RASSF1A* methylation status: group A $_{RASSF1A}$ (serum AFP level < 400 ng/mL and methylated *RASSF1A*, n = 7), group B $_{RASSF1A}$ (serum AFP level < 400 ng/mL and partially methylated or unmethylated RASSF1A, n = 22), and group C $_{RASSF1A}$ (serum AFP level ≥ 400 ng/mL and methylated *RASSF1A*, n = 4) and group D $_{RASSF1A}$ (serum AFP level ≥ 400 ng/mL and partially methylated or unmethylated *RASSF1A*, n = 16). The median overall survival rates in groups A $_{RASSF1A}$, B $_{RASSF1A}$, C $_{RASSF1A}$ and D $_{RASSF1A}$ were 71.0%, 62.0%, 70.0% and 19.0%, respectively. No significant differences in overall survival were found between groups A $_{RASSF1A}$ and B $_{RASSF1A}$ or between groups A $_{RASSF1A}$ and C $_{RASSF1A}$ ($p > 0.05$); however, a significant difference in overall survival was found between groups A $_{RASSF1A}$ and D $_{RASSF1A}$ ($p = 0.05$). The median disease-free survival rates in groups A $_{RASSF1A}$, B $_{RASSF1A}$, C $_{RASSF1A}$ and D $_{RASSF1A}$ were 71.0%, 59.0%, 59.0% and 5.0%, respectively. No significant differences in disease-free survival were found between groups A $_{RASSF1A}$ and B $_{RASSF1A}$, between groups A $_{RASSF1A}$ and C $_{RASSF1A}$ or between groups A $_{RASSF1A}$ and D $_{RASSF1A}$ ($p > 0.05$) (Table 8).

Kaplan-Meier survival curves analysis of serum AFP level and promoter methylation status

The results of the Kaplan-Meier analysis revealed that serum AFP level was associated with overall survival. We found that the five-year overall survival rate among patients with serum AFP levels < 400 ng/mL was 65.5% and that the five-year overall survival rate among patients with serum AFP levels ≥ 400 ng/mL was 40.0% ($p = 0.049$; Figure 3A). However, the five-year disease-free survival rate was 58.6% among patients with serum AFP levels < 400 ng/mL and 30.0% among patients with serum AFP levels ≥ 400 ng/mL ($p = 0.051$; Figure 3B). We also found that low serum AFP level combined with unmethylated *p21* status was associated with disease-free survival. Patients with serum AFP levels < 400 ng/mL and unmethylated *p21* promoters had a better prognosis than patients with serum AFP level ≥ 400 ng/mL and methylated *p21* promoters (overall survival, $p = 0.076$; disease-free survival, $p = 0.016$; Figure 4). In addition, patients with fully methylated *RASSF1A* promoter regions had a better prognosis than patients with partially methylated or

un-methylated *RASSF1A* promoters if their serum AFP level was ≥ 400 ng/mL (overall survival, p = 0.028; disease-free survival, p = 0.078; Figure 5).

DISCUSSION

The rate of survival of patients with HCC is low, mainly because of the high rate of recurrence after curative surgical resection. Therefore, it is important to identify groups at a higher risk for recurrence. Although there have been many reports on the prognostic significance of various factors associated with HCC, the results of these studies are controversial. Some groups have reported that methylation of *p15* or *p16* genes is not an indicator of prognosis for patients with HCC.[23,24] However, Wong *et al*. found that 9 of 12 (75%) patients with methylation of *p15* and *p16* genes were more likely

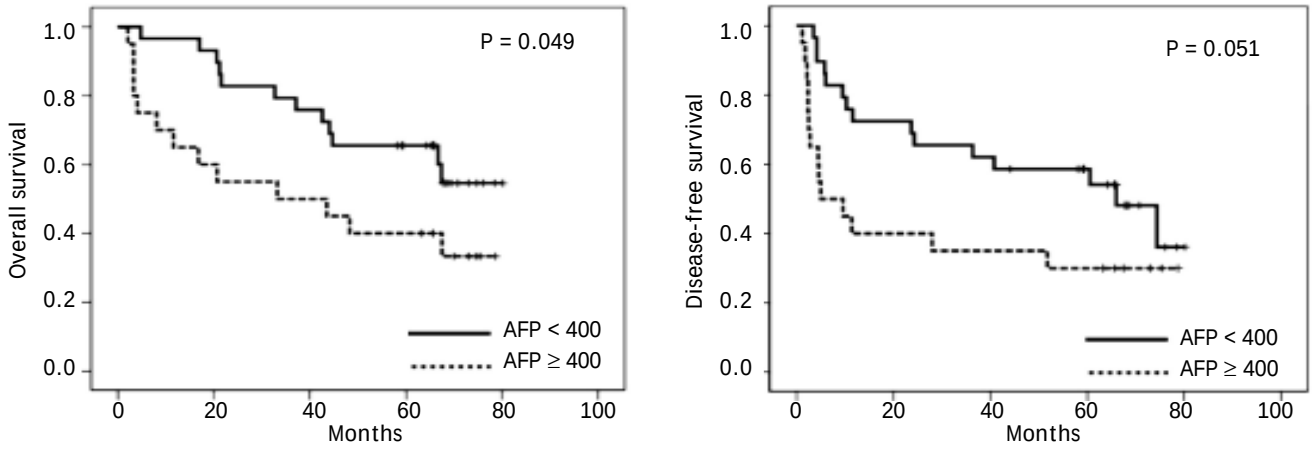

Figure 3. Kaplan-Meier survival analysis for all patients according to serum AFP level. Survival time was defined as the time from diagnosis to death or last known follow-up. Crosses represent censored values. The log-rank method was used to test for differences between groups. (A) Five-year overall survival was 65.5% and 40.0%, respectively (log-rank test, p = 0.049). (B) Five-year disease-free survival was 58.6% and 30.0%, respectively (log-rank test, p = 0.051).

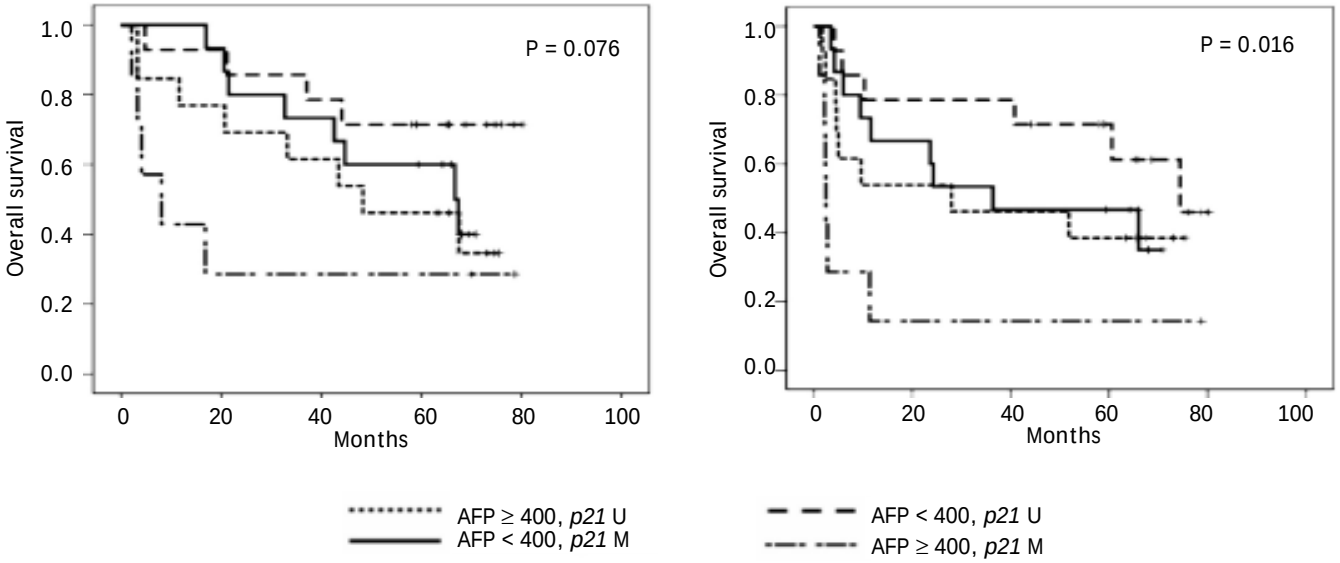

Figure 4. Kaplan-Meier survival analysis for all patients according to AFP level and methylation status of the p21 *proximal promoter region. Survival time was defined as the time from diagnosis to death or last known follow-up. Crosses represent censored values. The log-rank method was used to test for differences between groups. (A) Five-year overall survival rates were 71.4%, 60.0%, 46.2%, and 28.6% respectively (log-rank test, p = 0.076). (B) Five-year disease-free survival rates were 71.4%, 46.7%, 38.5%, and 14.3% respectively (log-rank test, p = 0.016).*

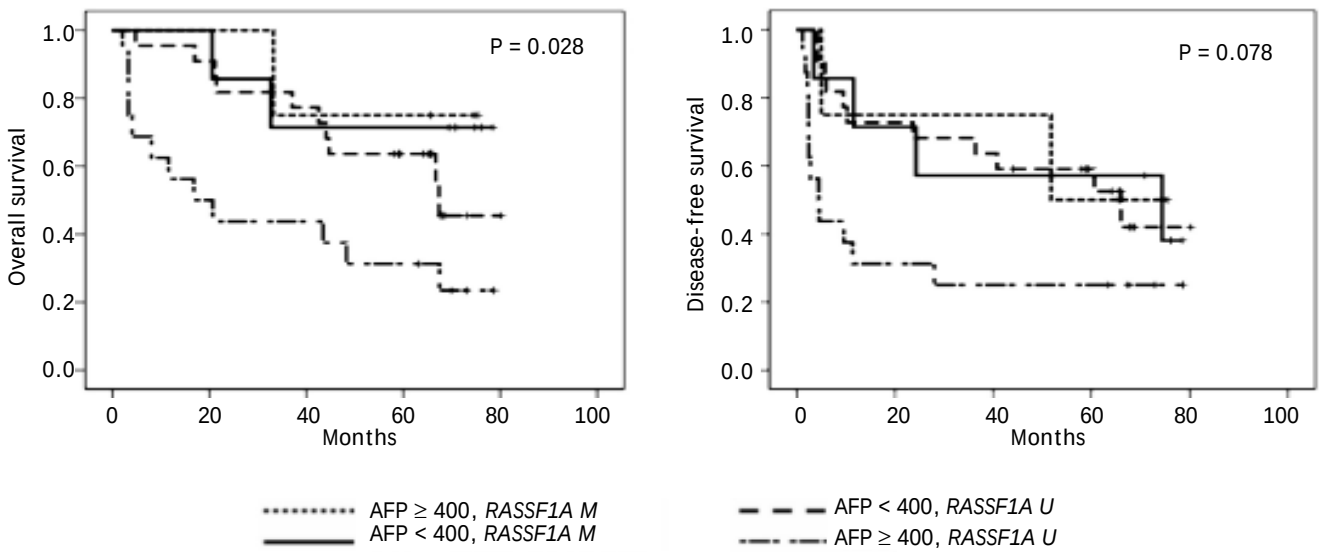

Figure 5. Kaplan-Meier survival analysis for all patients according to AFP level and methylation status of the RASSF1A proximal promoter region. Survival time was defined as the time from diagnosis to death or last known follow-up. Crosses represent censored values. The log-rank method was used to test for differences between groups. (A) Five-year overall survival rates were 75.0%, 71.4%, 63.6%, and 31.3% respectively (log-rank test, p = 0.028). (B) The five-year disease-free survival rates were 59.1%, 57.1%, 50.0%, and 25.0% respectively (log-rank test, p = 0.078).

to develop recurrent disease following resection.[25] In addition, Ko, *et al*. reported that promoter hypermethylation of the *p16* gene was associated with poor prognosis in recurrent early-stage hepatocellular carcinoma.[26] However, in the present study, *p15* and *p16* methylation status was not associated with poor prognosis of patients with hepatocellular carcinoma (data not shown).

Kao, *et al*. reported that methylation of the *p21* promoter was an independent predictor of survival for patients with hepatocellular carcinoma after resection.[13] In our study, methylation of the *p21* promoter and elevated serum AFP levels were found to be associated with poor prognosis in patients with hepatocellular carcinoma (Figure 4). Roman-Gomez showed that *p21* methylation status was associated with poor disease-free survival of patients with acute lymphoblastic leukemia ($p = 0.0001$).[27] The result suggested that under-expression of *p21* protein might be due to hypermethylation of the promoter of *p21* and that hypermethylation plays an important role in the progression of certain tumors.

We found that serum AFP level ≥ 400 ng/mL and promoter methylation of *RASSF1A* were associated with better overall survival in patients with HCC ($p < 0.028$). Similar results have been reported in HCC patients in Thailand, although no significant differences in overall survival were observed ($p = 0.12$).[28] However, Chan, *et al*. found that patients with higher serum *RASSF1A* methylation concentrations at diagnosis or at 1-year follow-up after tumor resection showed poorer disease-free survival ($p < 0.01$).[29] These conflicting observations may be due to differences in etiologic and underlying antecedent factors of hepatocellular carcinoma. It may be that a prognostic marker for HBV-related HCC is not a prognosticator for HCV-related HCC. Therefore, a study containing a mixed population may fail to take account of this unless appropriate sub analysis is performed.[9] In addition, these conflicting observations may be due to the fact that DNA methylation is associated with environmental exposure and dietetic habits.[30] Zhang, *et al*. reported that hypermethylation of *RASSF1A* and *p16* promoter regions is common and that hypermethylation is associated with aflatoxin B1-DNA adduct levels in patients with HCC.[31] These conflicting observations may also be due to the different methodologies between studies.

In conclusion, our data indicate that partially methylated or un-methylated *RASSF1A* promoters as well as elevated serum AFP levels or methylation of the promoter of the *p21* gene and elevated serum AFP levels might be associated with poor prognosis among patients with hepatocellular carcinoma. Methylation of *RASSF1A* and *p21* as well as serum AFP level may serve as potential prognosticators in patients with HCC.

REFERENCES

1. Bosch FX, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; 127: S5-S16.
2. Kao JH, Chen DS. Changing disease burden of hepatocellular carcinoma in the Far East and Southeast Asia. *Liver Int* 2005; 25: 696-703.
3. Goto T, Mizukami H, Shirahata A, Sakata M, Saito M, Ishibashi K, Kigawa G, et al. Aberrant methylation of the p16 gene is frequently detected in advanced colorectal cancer. *Anticancer Res* 2009; 29: 275-7.
4. Berg T, Steigen SE. DNA methylation in breast and colorectal cancer'. *Mod Pathol* 2008; 21: 1063-4.
5. Wang Y, Yu Z, Wang T, Zhang J, Hong L, Chen L. Identification of epigenetic aberrant promoter methylation of RASSF1A in serum DNA and its clinicopathological significance in lung cancer. *Lung Cancer* 2007; 56: 289-94.
6. Yeo W, Wong N, Wong WL, Lai PB, Zhong S, Johnson PJ. High frequency of promoter hypermethylation of RASSF1A in tumor and plasma of patients with hepatocellular carcinoma. *Liver Int* 2005; 25: 266-72.
7. Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol* 2004; 22: 4632-42.
8. Teodoridis JM, Strathdee G, Brown R. Epigenetic silencing mediated by CpG island methylation: potential as a therapeutic target and as a biomarker. *Drug Resist Updat* 2004; 7: 267-78.
9. Mann CD, Neal CP, Garcea G, Manson MM, Dennison AR, Berry DP. Prognostic molecular markers in hepatocellular carcinoma: a systematic review. *Eur J Cancer* 2007; 43: 979-92.
10. Narimatsu T, Tamori A, Koh N, Kubo S, Hirohashi K, Yano Y, Arakawa T, et al. p16 promoter hypermethylation in human hepatocellular carcinoma with or without hepatitis virus infection. *Intervirology* 2004; 47: 26-31.
11. Zhang C, Li Z, Cheng Y, Jia F, Li R, Wu M, Li K, et al. CpG island methylator phenotype association with elevated serum alpha-fetoprotein level in hepatocellular carcinoma. *Clin Cancer Res* 2007; 13: 944-52.
12. Zhang C, Guo X, Jiang G, Zhang L, Yang Y, Shen F, Wu M, et al. CpG island methylator phenotype association with upregulated telomerase activity in hepatocellular carcinoma. *Int J Cancer* 2008; 123: 998-1004.
13. Kao JT, Chuah SK, Huang CC, Chen CL, Wang CC, Hung CH, Chen CH, et al. P21/WAF1 is an independent survival prognostic factor for patients with hepatocellular carcinoma after resection. *Liver Int* 2007; 27: 772-81.
14. Ito Y, Matsuura N, Sakon M, Miyoshi E, Noda K, Takeda T, Umeshita K, et al. Expression and prognostic roles of the G1-S modulators in hepatocellular carcinoma: p27 independently predicts the recurrence. *Hepatology* 1999; 30: 90-9.
15. Shiohara M, el-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, et al. Absence of WAF1 mutations in a variety of human malignancies. *Blood* 1994; 84: 3781-4.
16. Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, et al. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 1996; 85: 707-20.
17. Donninger H, Vos MD, Clark GJ. The RASSF1A tumor suppressor. *J Cell Sci* 2007; 120: 3163-72.
18. Agathanggelou A, Honorio S, Macartney DP, Martinez A, Dallol A, Rader J, Fullwood P, et al. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene* 2001; 20: 1509-18.
19. Burbee DG, Forgacs E, Zöchbauer-Müller S, Shivakumar L, Fong K, Gao B, Randle D, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 2001; 93: 691-9.
20. Dammann R, Takahashi T, Pfeifer GP. The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. *Oncogene* 2001; 20: 3563-7.
21. Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, Chow LS, et al. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. *Cancer Res* 2001; 61: 3877-81.
22. Di Gioia S, Bianchi P, Destro A, Grizzi F, Malesci A, Laghi L, Levrero M, et al. Quantitative evaluation of RASSF1A methylation in the non-lesional, regenerative and neoplastic liver. *BMC Cancer* 2006; 6: 89.
23. Li X, Hui AM, Sun L, Hasegawa K, Torzilli G, Minagawa M, Takayama T, et al. p16INK4A hypermethylation is associated with hepatitis virus infection, age, and gender in hepatocellular carcinoma. *Clin Cancer Res* 2004; 10: 7484-9.
24. Lee S, Lee HJ, Kim JH, Lee HS, Jang JJ, Kang GH. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *Am J Pathol* 2003; 163: 1371-8.
25. Wong IH, Lo YM, Yeo W, Lau WY, Johnson PJ. Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. *Clin Cancer Res* 2000; 6: 3516-21.
26. Ko E, Kim Y, Kim SJ, Joh JW, Song S, Park CK, Park J, et al. Promoter hypermethylation of the p16 gene is associated with poor prognosis in recurrent early-stage hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev* 2008; 17: 2260-7.
27. Roman-Gomez J, Castillejo JA, Jimenez A, Gonzalez MG, Moreno F, Rodriguez M del C, Barrios M, et al. 5' CpG island hypermethylation is associated with transcriptional silencing of the p21(CIP1/WAF1/SDI1) gene and confers poor prognosis in acute lymphoblastic leukemia. *Blood* 2002; 99: 2291-6.
28. Saelee P, Wongkham S, Chariyalertsak S, Petmitr S, Chuensumran U. RASSF1A Promoter Hypermethylation as a Prognostic Marker for Hepatocellular Carcinoma. *Asian Pac J Cancer Prev* 2010; 11: 1677-81.
29. Chan KC, Lai PB, Mok TS, Chan HL, Ding C, Yeung SW, Lo YM. Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. *Clin Chem* 2008; 54: 1528-36.
30. Shen L, Ahuja N, Shen Y, Habib NA, Toyota M, Rashid A, Issa JP. DNA methylation and environmental exposures in human hepatocellular carcinoma. *J Natl Cancer Inst* 2002; 94: 755-61.
31. Zhang YJ, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, Lee PH, et al. High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. *Mol Carcinog* 2002; 35: 85-92.