Methylation status of the *APC* and *RASSF1A* promoter in cell-free circulating DNA and its prognostic role in patients with colorectal cancer

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Abstract. DNA methylation is the most frequent epigenetic alteration. Using methylation-specific polymerase chain reaction (MSP), the methylation status of the adenomatous polyposis coli (*APC*) and Ras association domain family 1 isoform A (*RASSF1A*) genes was examined in cell-free circulating DNA from 155 plasma samples obtained from patients with early and advanced colorectal cancer (CRC). *APC* and *RASSF1A* hypermethylation was frequently observed in both early and advanced disease, and was significantly associated with a poorer disease outcome. The methylation status of the *APC* and *RASSF1A* promoters was investigated in cell-free DNA of patients with CRC. Using MSP, the promoter methylation status of *APC* and *RASSF1A* was examined in 155 blood samples obtained from patients with CRC, 88 of whom had operable CRC (oCRC) and 67 had metastatic CRC (mCRC). The frequency of *APC* methylation in patients with oCRC was 33%. Methylated *APC* promoter was significantly associated with older age (P=0.012), higher stage (P=0.014) and methylated *RASSF1A* status (P=0.050). The frequency of *APC* methylation in patients with mCRC was 53.7%. In these patients, *APC* methylation was significantly associated with methylated *RASSF1A* status (P=0.016). The frequency of *RASSF1A* methylation in patients with oCRC was 25%. Methylated *RASSF1A* in oCRC was significantly associated with higher stage (P=0.021). The frequency of *RASSF1A* methylation in mCRC was 44.8%. Methylated *RASSF1A* in mCRC was associated with moderate differentiation (P=0.012), high levels of carcinoembryonic antigen (P=0.023) and methylated *APC* status (P=0.016). Patients with an unmethylated *APC* gene had better survival in both early (81±5 vs. 27±4 months, P<0.001) and advanced disease (37±7 vs. 15±3 months, P<0.001), compared with patients with methylated *APC*. Patients with an unmethylated *RASSF1A* gene had better survival in both early (71±6 vs. 46±8 months, P<0.001) and advanced disease (28±4 vs. 16±3 months, P<0.001) than patients with methylated *RASSF1A*. The observed significant correlations between *APC* and *RASSF1A* promoter methylation status and survival may be indicative of a prognostic role for these genes in CRC, which requires additional testing in larger studies.

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Abbreviations: MSP, methylation-specific polymerase chain reaction; OS, overall survival; PS, performance status; CRC, colorectal cancer; oCRC, operable colorectal cancer; mCRC, metastatic colorectal cancer

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Introduction

Colorectal cancer (CRC) is a leading cause of morbidity and mortality worldwide, with >600,000 associated mortalities annually (1). Of all CRC cases, 75-80% occur sporadically as the result of complex interactions between susceptibility genes and environmental factors (2). Despite the recent and continuous improvements in diagnosis and treatments, >50% of colon and rectal tumors metastasize to liver, lung and lymph nodes, and the 5-year survival rate remains low (~10%) for patients with metastatic CRC (mCRC) (3). There is, therefore, an urgent requirement for non-invasive, novel molecular biomarkers that could be useful in diagnosis and could also

improve prognosis and treatment prediction. The accumulation of *in vitro* and *in vivo* evidence suggests that epigenetics exerts a fundamental role in CRC pathogenesis (4). The best known and more frequent epigenetic alteration is DNA methylation, which affects tumor suppressor genes that may be involved in cell cycle control, DNA repair, metabolism of carcinogens, cell-cell interaction, apoptosis and angiogenesis (5). Hypermethylation of CpG islands in the promoter region of tumor suppressor genes leads to their inappropriate silencing, which may trigger cancer initiation and progression (6). The technical advantage of studying DNA methylation is its chemical stability, thus enabling its detection with very high sensitivity (≤1:1,000 molecules) (7). Numerous studies have demonstrated that cancer-specific, methylated DNA may be present in body fluids, suggesting that it could be used as a non-invasive marker (8,9). Blood, plasma or serum constitute the most easy-to-handle samples, and are also a great source of cell-free circulating tumor DNA (10). The mechanism surrounding the origin of tumoral DNA that is released into the circulation is poorly understood, but it is assumed that DNA is released during necrosis and/or apoptosis of tumor cells (11). These circulatory DNA molecules are easily isolated, and may serve for the detection of the methylation status of certain genes (12,13). Several genes with altered levels of methylation have been investigated in respect to their involvement in CRC initiation or progression. A number of those, including human mutL homolog 1, O(6)-methylguanine-DNA methyltransferase (MGMT) and thrombospondin 1, exhibited an increase in methylation during all the stages of the disease, while other genes such as Ras association domain family 1 isoform A (*RASSF1A*) or tissue inhibitor of metalloproteinase 3 have been reported to be methylated in later disease stage or in metastases (14).

RASSF members belong to a family of putative tumor suppressor RAS effectors, for which epigenetic silencing by promoter methylation has been reported to occur throughout the progression of several types of cancer, including CRC (15,16). Since its identification in 2000, the tumor suppressor *RASSF1A* gene has been extensively investigated (17). Transcriptional silencing of *RASSF1A* by inappropriate promoter methylation has been frequently observed in various types of human cancer, including lung, breast, colorectal, gastric and cervical cancer (18). *RASSF1A* methylation has been reported to range from 12 to 81% in CRC (19,20). In colon primary tumors, Yoon *et al* (19) detected methylation in the CpG island of the *RASSF1A* gene in 3 out of 26 (12%) tumor tissues, while none of the available normal tissues were methylated.

Mutations in the human tumor suppressor gene adenomatous polyposis coli (*APC*) are frequent in both sporadic and familial CRC (21). Wild-type APC protein contributes to the destabilization and degradation of β-catenin, which is a central effector molecule in the Wnt/β-catenin signaling pathway (22). Loss of *APC* function results in nuclear accumulation of β-catenin, which leads to the transcriptional activation (via the β-catenin/T-cell factor complex) of target genes that may contribute to colorectal tumorigenesis (23,24). The rate of *APC* promoter methylation in CRC and normal colorectal mucosa has been reported to range from 11 to 62% in different populations, and has been suggested to moderate the Wnt signaling pathway (25-29). Furthermore, hypermethylation of the *APC* promoter has been demonstrated to be relatively common in other gastrointestinal neoplasms, including those of the stomach, liver, pancreas and oesophagus (30).

In the present study, the methylation status of *APC* and *RASSF1A* were investigated in cell-free circulating DNA of patients with operable CRC (oCRC) and mCRC. The aim of the present study was to primarily assess the methylation status of the above genes and to explore their possible prognostic significance in early and advanced disease.

Materials and methods

Study design. The study material consisted of 155 blood samples obtained from patients with CRC between March 2010 and May 2014. Patients were suffering from either early operable (88/155, 56.8%) or metastatic disease (67/155, 43.2%). The clinicopathological data for all patients are presented in Table I. All patients had a performance status (PS) of 0-1 [World Health Organization scale (31)] and provided informed consent. Additionally, 20 blood samples obtained from healthy individuals [friends and non-blood related family members of patients treated at the Department of Medical Oncology of the University Hospital of Alexandroupolis (Alexandroupolis, Greece)] were used as a control group. The majority of controls were men, all age-matched with the patient population, who had no received medical care at the time of sample collection. All patients included in the study had signed an informed consent form along with an agreement to use biological material for experimental purposes.

Sample collection and isolation of cell-free DNA. Whole blood was extracted from patients pre-operatively. Blood was collected in serum clot activator tubes. Serum was obtained immediately through centrifugation at 3,000 x g for 10 min and stored at -80˚C until DNA extraction. Cell-free DNA from serum samples was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics GmbH, Mannheim, Germany). A total of 300 µl serum were mixed with 300 µl working solution and 60 µl proteinase K (18 mg/ml), and incubated for 10 min at 72˚C; DNA isolation was then processed as described in the manufacturer's protocol. DNA concentration was determined with an ND-100 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) measurements. CEA and CA 19-9 levels were measured using the Elecsys® CEA kit (Roche Diagnostics GmbH). The cut-offs used were 10 ng/ml for CEA and 37 U/ml for CA 19-9.

Sodium bisulfite conversion. Sodium bisulfite conversion of ≤200 ng cell-free DNA was performed using the EZ DNA Methylation-Gold™ kit (Zymo Research Corporation, Irvine, CA, USA), according to the manufacturer's protocol. The converted DNA was stored at -80˚C until used.

Methylation-specific polymerase chain reaction (MSP). The methylation status of *APC* and *RASSF1A* in cell-free circulating serum DNA samples was detected by MSP using specific primer pairs for both the methylated and unmethylated

Table I. Patients' characteristics in early (n=88) and advanced (n=67) disease.

Patients' characteristics	All patients, no. (%)	Patients with oCRC, no. (%)	Patients with mCRC, no. (%)
No. of patients	155	88	67
Gender			
Males	89 (57.4)	51 (58.0)	38 (56.7)
Females	66 (42.6)	37 (42.0)	29 (43.3)
Age, years			
≤70	83 (53.5)	41 (46.6)	42 (62.7)
>70	72 (46.5)	47 (53.4)	25 (37.3)
Dukes' stage			
A+B	58 (37.4)	58 (65.9)	-
C	30 (19.4)	30 (34.1)	-
D	67 (43.2)	-	67 (100.0)
Differentiation			
Well	48 (31.0)	30 (34.1)	18 (26.9)
Moderate	79 (51.0)	43 (48.9)	36 (53.7)
Poor	28 (18.0)	15 (17.0)	13 (19.4)
Presence of metastases			
No	88 (56.8)	-	-
Yes	67 (43.2)	-	-
Tumor location, colon side			
Right	74 (47.7)	43 (48.9)	31 (46.3)
Left	81 (52.3)	45 (51.1)	36 (53.7)
CEA levels, ng/ml (n=138)			
≤10	57 (41.3)	40 (48.8)	17 (30.4)
>10	81 (58.7)	42 (51.2)	39 (69.6)
CA 19.9 levels, U/ml (n=137)			
<37 (low)	95 (69.3)	67 (81.7)	28 (50.9)
>37 (high)	42 (30.7)	15 (18.3)	27 (49.1)

CRC, colorectal cancer; oCRC, operable colorectal cancer; mCRC, metastatic colorectal cancer; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9.

promoter sequences. Each MSP reaction was performed in a total volume of 25 μl. Sodium bisulfite-converted DNA (1 μl) was added into a 24-μl reaction mixture that contained 0.1 μl Taq DNA polymerase (5 U/μl; GoTaq® Hot Start Polymerase; Promega Corporation, Madison, WI, USA), 5 μl 10X buffer, 2.0 μl $MgCl_2$ (50 mmol/l), 0.5 μl deoxynucleotides triphosphate (10 mmol/l; Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) and 1 μl each of the corresponding forward and reverse primers (10 μmol/l); lastly, distilled H_2O was added to a final volume of 25 μl. Sodium bisulfite-treated DNA was amplified in two separate MSP reactions, one with a set of primers specific for methylated DNA, and one for unmethylated promoter sequences. The primer pairs used in this study are as follows: *RASSF1A unmethylated*, forward GGTTGTATTTGGTTGGAGTG and reverse CTACAAACC TTTACACACAACA; *RASSF1A methylated*, forward GTT GGTATTCGTTGGGCGC and reverse GCACCACGTATA CGTAACG; *APC unmethylated*, forward GTGTTTTATTGT GGAGTGTGGGTT and reverse CCAATCAACAAACTC CCAACAA; *APC methylated*, forward TATTGCGGAGTG CGGGTC and reverse TCGACGAACTCCCGACGA. Human placental genomic DNA (gDNA; Sigma-Aldrich, St. Louis, MO, USA) methylated *in vitro* with M.SssI methylase (New England BioLabs, Inc., Ipswich, MA, USA) was used, following sodium bisulfite conversion, as a fully methylated (100%) MSP positive control. The same unmethylated placental gDNA was used, following sodium bisulfite conversion, as a negative MSP control. The thermocycling conditions used were as follows: i) *APC*, 1 cycle at 95˚C for 5 min, followed by 39 cycles of 95˚C for 45 sec, 63˚C for 60 sec and 72˚C for 60 sec, with a final extension cycle of 72˚C for 10 min; and ii) *RASSF1A*, 1 cycle at 95˚C for 5 min, followed by 39 cycles of 95˚C for 30 sec, 58˚C for 45 sec and 72˚C for 45 sec, with a final extension cycle of 72˚C for 5 min. MSP products for methylated and unmethylated promoters were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM ethylenediaminetetraacetic acid (pH 8.0) and visualized by ethidium bromide staining.

Table II. Association of *APC* and *RASSF1A* methylation status with demographic and clinicopathological characteristics of patients with operable disease.

Patients' characteristics	*APC* methylation	P-value	*RASSF1A* methylation	P-value
Gender		0.711		0.170
Males	16 (31.4)		10 (19.6)	
Females	13 (35.1)		12 (32.4)	
Age, years		0.012		0.267
≤70	8 (19.5)		8 (19.5)	
>70	21 (44.7)		14 (29.8)	
Dukes' stage		0.014		0.021
A+B	14 (24.1)		8 (14.3)	
C	15 (50.0)		14 (34.1)	
Differentiation		0.403		0.670
Well	8 (26.7)		9 (30.0)	
Moderate	14 (32.6)		9 (20.9)	
Poor	7 (46.7)		4 (26.7)	
Tumor location, colon side		0.407		0.538
Right	16 (37.2)		12 (27.9)	
Left	13 (28.9)		10 (22.2)	
CEA levels, ng/ml (n=82)		0.088		0.697
≤5	10 (25.0)		9 (22.5)	
>5	18 (42.9)		11 (26.2)	
CA 19.9 levels, U/ml (n=82)		0.083		0.372
<37 (low)	20 (29.9)		15 (22.4)	
>37 (high)	8 (53.3)		5 (33.3)	
APC status		-		0.050
Unmethylated	-		11 (18.6)	
Methylated	-		11 (37.9)	
RASSF1A status		0.050		-
Unmethylated	18 (27.3)		-	
Methylated	11 (50.0)		-	

APC, adenomatous polyposis coli; *RASSF1A*, Ras association domain family 1 isoform A; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9.

Statistical analysis. Statistical analysis of the data was performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). The methylation status of *APC* and *RASSF1A* and all other qualitative variables were expressed as frequencies and percentages. The χ^2 test was used to evaluate any potential association of *APC* and *RASSF1A* status with patients' demographic and clinicopathological characteristics. Odds ratios (ORs) and their 95% confidence intervals (CIs) were estimated as a measure of the association between *APC* and *RASSF1A* status and patients' characteristics. Survival rates were calculated with the Kaplan-Meier method, and the statistical difference between the survival curves was determined with the log-rank test. Multivariate Cox proportional hazards regression (HR) analysis, using a backward selection approach, were performed to explore the independent effect of *APC* and *RASSF1A* status on overall survival. Patients' gender, age, clinical stage, tumor differentiation, lymph node status, CEA and CA 19.9 levels were also included in the multivariate model as potential confounders. All tests were two tailed, and P<0.05 was considered to indicate a statistical significant difference.

Results

Characteristics of the study population. The study population consisted of 155 patients with CRC with a median age of 70 years (range, 44-76 years; mean age ± standard deviation, 68.35±9.20 years), 57.4% of whom were males. The patient's clinicopathological characteristics are indicated in Table I. In total, 48 tumors (31.0%) were well differentiated, 79 (51.0%) were moderately differentiated and 28 (18.1%) were poorly differentiated carcinomas. The Dukes' system (32) was used for the classification of patients into different stages. Almost half

Table III. Association of *APC* and *RASSF1A* methylation status with demographic and clinicopathological characteristics of patients with metastatic disease.

Patients' characteristics	*APC* methylation	P-value	*RASSF1A* methylation	P-value
Gender		0.076		0.994
Males	24 (63.2)		17 (44.7)	
Females	12 (41.4)		13 (44.8)	
Age, years		0.427		0.921
≤70	21 (50.0)		19 (45.2)	
>70	15 (60.0)		11 (44.0)	
Dukes' stage		-		-
A+B	-		-	
C	-		-	
D	36 (53.7)		30 (44.8)	
Differentiation		0.388		0.032
Well	10 (55.6)		12 (66.7)	
Moderate	17 (47.2)		11 (30.6)	
Poor	9 (69.2)		7 (53.8)	
Tumor location, colon side		0.100		0.581
Right	20 (64.5)		15 (48.4)	
Left	16 (44.4)		15 (41.7)	
CEA levels, ng/ml (n=56)		0.159		0.023
≤5	7 (41.2)		4 (23.5)	
>5	24 (61.5)		22 (56.4)	
CA 19.9 levels, U/ml (n=55)		0.883		0.898
<37 (low)	15 (53.6)		13 (46.4)	
>37 (high)	15 (55.6)		13 (48.1)	
APC status		-		0.016
Unmethylated	-		9 (29.0)	
Methylated	-		21 (58.3)	
RASSF1A status		0.016		-
Unmethylated	15 (40.5)		-	
Methylated	21 (70.0)		-	

APC, adenomatous polyposis coli; *RASSF1A*, Ras association domain family 1 isoform A; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9.

of the cases (67 patients, 43.2%) were at stage D, 30 (19.3%) cases were at stage C and 58 (37.4%) were at stage A or B. *APC* and *RASSF1A* promoters were observed to be methylated in 65 (41.9%) and 52 (33.5%) of the 155 colon cancer samples examined, but in none of the normal control samples (both P<0.001). The association of the patients' demographic and clinicopathological features with *APC* and *RASSF1A* methylation status is presented in Tables II and III.

Correlation between APC methylation status and different tumor parameters

Patients with early oCRC. APC was methylated in 29 of 88 patients with early oCRC (33.0%). χ^2 analysis revealed that methylated *APC* promoter status was associated with age >70 years (OR=3.33, 95% CI=1.27-8.73, P=0.012), higher stage (OR=3.14, 95% CI=1.23-8.00, P=0.014) and methylated *RASSF1A* status (OR=2.67, 95% CI=1.00-7.22, P=0.050), while a tendency was noticed with high CEA levels (OR=2.25, 95% CI=0.88-5.77, P=0.088) and high CA 19.9 levels (OR=2.69, 95% CI=0.86-8.41, P=0.083) (Table II).

Patients with metastatic disease. APC was methylated in 36 of 67 patients with mCRC (53.7%). Methylated *APC* promoter status was associated with methylated *RASSF1A* status (OR=3.42, 95% CI=1.23-9.49, P=0.016) and marginally with male gender (OR=2.43, 95% CI=0.90-6.54, P=0.076). No other significant associations between *APC* methylation status and other tumor parameters were observed in patients with mCRC (Table III).

Correlation between RASSF1A methylation status and different tumor parameters

Patients with early oCRC. RASSF1A was methylated in 22 of 88 patients with early oCRC (25.0%). Methylated *RASSF1A*

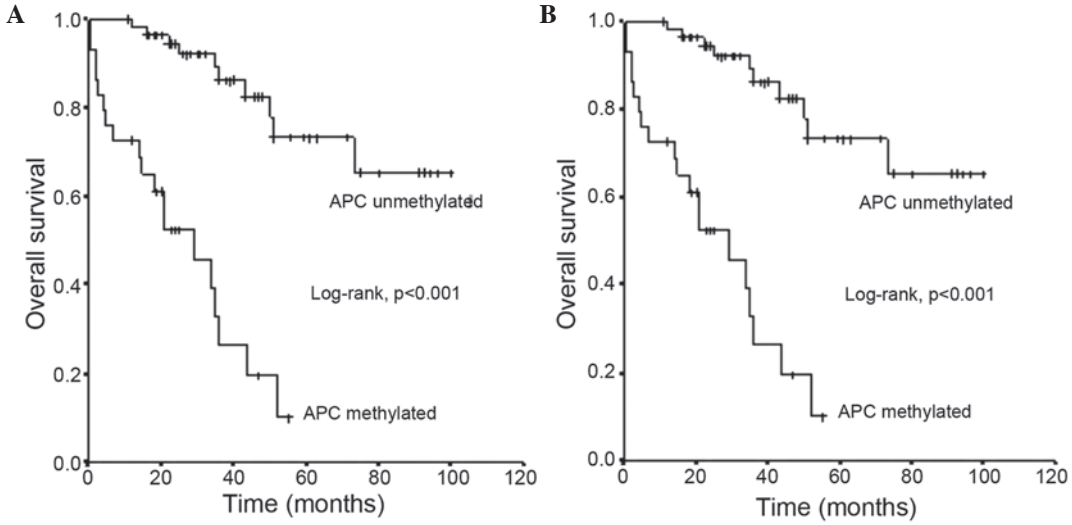

Figure 1. Overall survival of patients with CRC in relation to adenomatous polyposis coli methylation status in (A) early operable CRC and (B) metastatic CRC. CRC, colorectal cancer; *APC*, adenomatous polyposis coli.

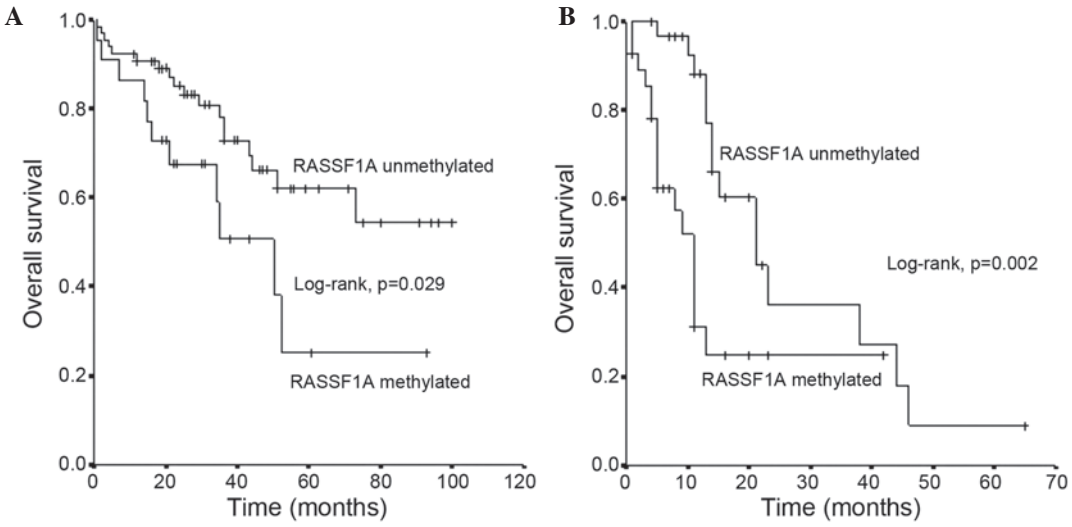

Figure 2. Overall survival of patients with CRC in relation to Ras association domain family 1 isoform A methylation status in (A) early operable CRC and (B) metastatic CRC. CRC, colorectal cancer; *RASSF1A*, Ras association domain family 1 isoform A.

promoter status was significantly associated with higher disease stage (OR=3.11, 95% CI=1.16-8.36, P=0.021). No other significant associations were observed (Table II).

Patients with metastatic disease. *RASSF1A* was methylated in 30 of 67 patients with mCRC (44.8%). Methylated *RASSF1A* promoter status was significantly associated with moderate differentiation (OR=3.60, 95% CI=1.31-9.91, P=0.012), high CEA levels (OR=4.21, 95% CI=1.16-15.23, P=0.023) and methylated *APC* status (OR=3.42, 95% CI=1.23-9.49, P=0.016) (Table III).

APC methylation status and survival in patients with early oCRC. The mean survival time ± standard error (SE) of patients with oCRC and an unmethylated *APC* promoter status was 81±5 months (95% CI=71-91), which was significantly longer than the mean survival ± SE of 27±4 months (95% CI=19-34) observed for those with a methylated *APC* promoter status (log-rank test, P<0.001) (Fig. 1A).

APC methylation status and survival in patients with mCRC. The mean survival time ± SE of patients with mCRC and an unmethylated *APC* promoter status was 37±7 months (95% CI=23-50), which was substantially longer than the mean survival ± SE of 15±3 months (95% CI=9-20) observed in those with a methylated *APC* promoter status (log-rank test, P<0.001) (Fig. 1B).

RASSF1A methylation status and survival in patients with early oCRC. The mean survival time ± SE of patients with oCRC and an unmethylated *RASSF1A* promoter status was 71±6 months (95% CI=60-81), which was substantially longer than the mean survival ± SE of 46±8 months (95% CI=29-62) observed in those with a methylated *RASSF1A* promoter status (log-rank test, P<0.001) (Fig. 2A).

RASSF1A methylation status and survival in patients with mCRC. The mean survival time ± SE of patients with

mCRC and an unmethylated *RASSF1A* promoter status was 28±4 months (95% CI=19-36) months, which was substantially longer than the mean survival ± SE of 16±3 months (95% CI=9-22) months observed in patients with a methylated *RASSF1A* promoter status (log-rank test, P<0.001) (Fig. 2B).

Multivariate Cox proportional HR analysis. Upon stratification of the analysis according to the presence or absence of metastases, the negative impact of methylated *APC* promoter status on patients' survival was more pronounced in patients without metastases [adjusted HR (aHR)=7.88, 95% CI=2.73-22.73, P<0.001] than in patients with metastases (aHR=3.47, 95% CI=1.35-8.92, P=0.017), while the negative impact of methylated *RASSF1A* promoter status on patients' survival was more pronounced in patients with metastases (aHR=5.76, 95% CI=2.44-14.82, P=0.001) than among patients without metastases (aHR=3.06, 95% CI=1.25-7.50, P=0.038).

Discussion

CRC remains a global burden on world health and economy, despite having a 90% 5-year survival rate when detected and treated early (1,33). Traditional methods cannot sufficiently predict the prognosis of single cancer cases, and clinicians may be not able to accurately decide which patients will be at high risk of recurrence and benefit from chemotherapy. Therefore, it is essential to identify novel biomarkers for improved prognosis, which would aid clinicians to decide which patients should receive adjuvant treatment. The questions of which patients should be treated and why certain patients respond better to chemotherapy than others must be solved, as adjuvant cancer therapy imposes unnecessary toxicity and a huge financial burden on patients. In the present study, the promoter methylation status of the *APC* and *RASSF1A* genes in cell-free DNA from patients with CRC was explored, and their incidence and potential correlations with different tumor parameters and survival were examined.

RASSF1A protein is actively involved in microtubule regulation, genomic stability maintenance, cell-cycle regulation, apoptosis modulation, cell motility and invasion control (34-36). A number of studies have identified a high percentage of *RASSF1A* methylation in CRC samples. Wagner *et al* (37) observed *RASSF1A* promoter methylation in 45% (13/29) of the primary CRC and in 80% (4/5) of the CRC cell lines analyzed. Contrarily, in a study with 222 sporadic CRC samples, van Engeland *et al* (38) detected *RASSF1A* methylation in 20% (45/222) of the samples, and a mutually exclusive association with the presence of Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations was suggested. Several other studies reported *RASSF1A* methylation in 17% (8/47), 36% (26/73) and 47% (17/36) of the CRC samples examined (39-41). Notably, the above different studies reporting *RASSF1A* methylation at different stages, as well as in the corresponding normal mucosa adjacent to the tumor, point to a role of the tumor suppressor gene *RASSF1A* early in the development of colorectal carcinogenesis. It may mean that methylation possibly occurs in proximal sites of cancer cells due to field effect phenomena; however, the mechanism remains to be determined. The present results are in accordance with the aforementioned reports, as 25% hypermethylated *RASSF1A* promoter was detected in patients with early oCRC, and 44.8% hypermethylated *RASSF1A* promoter was detected in patients with mCRC, suggesting that *RASSF1A* methylation is a frequent event in CRC, although more pronounced at later disease stages. This higher incidence of *RASSF1A* methylation in metastatic disease may be indicative of a more aggressive tumor behavior, which may explain the association with poorer prognosis observed in the present study. In our previous study, we performed a similar analysis of *RASSF1A* methylation status in patients with operable gastric cancer (42). In that study, a methylation rate of 68.5% was detected, which underlines the difference in the biological behavior of gastric cancer in comparison with CRC. Which of the multiple roles of *RASSF1A* is played at different disease stages according to its methylation status is unclear.

Wagner *et al* (37) investigated whether *RASSF1A* methylation correlated with tumor-node-metastasis status, but did not detect any significant associations. Similarly, in the study by Van England *et al* (38), *RASSF1A* methylation was not associated with gender, Duke's stage or location of the tumor. The only exception was the age at diagnosis, which was slightly higher in *RASSF1A* methylated CRC cases compared to non-methylated ones.

In a previous study with 36 CRC samples, a significant correlation was observed between *RASSF1A* methylation and gender, with *RASSF1A* being more frequently methylated in females (41) In the present study, methylated *RASSF1A* was associated with higher stages in patients with early oCRC, while in patients with metastatic disease, methylated *RASSF1A* was significantly associated with moderate differentiations, high CEA levels and *APC* hypermethylated promoter status. Notably, in other studies, *RASSF1A* methylation levels were significantly higher in distal CRC, compared with proximal CRC (43,44), as well as in normal mucosae (44). The present data did not support a difference in the profile of methylation between right and left colon. Additionally, a significant difference was observed in the survival of patients with unmethylated *RASSF1A* promoter status, compared with those with methylated *RASSF1A*, in patients with and without metastases. Furthermore, the negative impact of methylated *RASSF1A* promoter status on patients' survival was more pronounced in patients with metastases. Additional studies are required for a better characterization of the subsets of patients with *RASSF1A* promoter methylation and a better understanding of the role of *RASSF1A* in CRC development.

Germline mutations in the tumor suppressor *APC* gene cause familial adenomatous polyposis, and somatic mutations are common in sporadic CRC (45). Hypermethylation of the *APC* promoter has been reported in early steps of carcinogenesis in several tumors (46). A previous study reported that the frequencies of aberrant promoter methylation were 16% for cadherin 1, 2% for p16, 4% for MGMT and 24% for *APC* (47). An aberrant methylation of ≥1 of these genes was identified in 45 of 51 (88%) primary tumors (48). In the present cohort, *APC* was methylated in 33% of patients with early oCRC and in 53.7% of patients with mCRC. In patients with early oCRC, methylated *APC* promoter status was associated with ages older than 70 years and methylated *RASSF1A* status, while a tendency was observed with high CEA and CA 19.9 levels. In patients with metastatic disease,

APC methylation was associated with methylated *RASSF1A* status and male gender. Patients with an unmethylated *APC* promoter status had a substantially longer mean survival than those with a methylated *APC* promoter status, both in early and metastatic disease. A significant and unexpected finding of the present study was that the negative impact of methylated *APC* promoter status on patients' survival was more pronounced in patients without metastases than in patients with metastases. The present authors do not have a clear explanation for this observation. It appears that, although *APC* methylation is most frequently observed at later stages, when present at earlier stages it is indicative of an aggressive tumor phenotype associated with shorter survival. The present findings are in agreement with the proposed roles of *APC* and *RASSF1A* as tumor suppressor genes. Their silencing as a result of their methylation is indicative of a more aggressive tumor phenotype with shorter survival. This is also supported by the observed correlations with bad prognostic features, such as higher disease stages and older age for *APC*, and higher stages, moderate differentiation and high CEA levels for *RASSF1A*.

In conclusion, the present study demonstrated that serum *RASSF1A* and *APC* promoter hypermethylation is a frequent epigenetic event in patients with colon cancer, in both early and metastatic disease, which is indicative of a crucial role for both proteins in colorectal carcinogenesis. In addition, a significant correlation was observed between *APC* and *RASSF1A* promoter methylation status and survival. The high percentage of methylation of both proteins in the early and metastatic setting indicates that methylation represents a common event, and when present, it is possibly associated with a more aggressive tumor phenotype. Additional studies in a larger cohort of patients are required to further explore whether these findings could establish the methylation status of *APC* and *RASSF1A* as potential biomarkers for early detection and prognosis in CRC.

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