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Prognostic role of APC and RASSF1A promoter methylation status in cell free circulating DNA of operable gastric cancer patients

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ABSTRACT

Gastric carcinogenesis is a multistep process including not only genetic mutations but also epigenetic alterations. The best known and more frequent epigenetic alteration is DNA methylation affecting tumor suppressor genes that may be involved in various carcinogenetic pathways. The aim of the present study was to investigate the methylation status of *APC* promoter 1A and *RASSF1A* promoter in cell free DNA of operable gastric cancer patients.

Using methylation specific PCR, we examined the methylation status of *APC* promoter 1A and *RASSF1A* promoter in 73 blood samples obtained from patients with gastric cancer.

APC and *RASSF1A* promoters were found to be methylated in 61 (83.6%) and 50 (68.5%) of the 73 gastric cancer samples examined, but in none of the healthy control samples ($p < 0.001$). A significant association between methylated *RASSF1A* promoter status and lymph node positivity was observed ($p = 0.005$). Additionally, a significant correlation between a methylated *APC* promoter and elevated CEA ($p = 0.033$) as well as CA-19.9 ($p = 0.032$) levels, was noticed. The Kaplan–Meier estimates of survival, significantly favored patients with a non-methylated *APC* promoter status ($p = 0.008$). No other significant correlations between *APC* and *RASSF1A* methylation status and different tumor variables examined was observed.

Serum *RASSF1A* and *APC* promoter hypermethylation is a frequent epigenetic event in patients with early operable gastric cancer. The observed correlations between *APC* promoter methylation status and survival as well as between a hypermethylated *RASSF1A* promoter and nodal positivity may be indicative of a prognostic role for those genes in early operable gastric cancer. Additional studies, in a larger cohort of patients are required to further explore whether these findings could serve as potential molecular biomarkers of survival and/or response to specific treatments.

1. Introduction

Gastric cancer is one of the most frequent cancers in the world [1]. Despite recent therapeutic advances, it remains a major clinical challenge, due to its high prevalence and poor prognosis of patients at the advanced stage [2]. At this point, the only way to improve resectability and prognosis is early stage detection. Although endoscopy remains widely the method of choice, however, in some cases its cost is prohibitive and generally healthy individuals are unwilling to accept such an unpleasant procedure. There is, therefore, an urgent need for non-invasive, new molecular biomarkers that could be useful in diagnosis, but also, could improve prognosis and treatment prediction.

Gastric carcinogenesis is a multistep process including not only genetic mutations but also epigenetic alterations [3]. 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 [4]. Hypermethylation of

Abbreviations: MSP, methylation specific PCR; OS, overall survival; PS, performance status.

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CpG islands in the promoter region of tumor suppressor genes leads to their inappropriate silencing which affects negatively cancer initiation and progression [5]. The technical advantage of DNA methylation is that is chemically stable and can be detected with a very high sensitivity of up to 1:1000 molecules [6]. Many studies have also demonstrated that cancer specific, methylated DNA, can be found in body fluids, suggesting it could be used as a useful non-invasive marker [7,8]. Blood, plasma or sera, is the most easy-to-handle sample and also a great source of cell free circulating tumor DNA. The mechanism surrounding the origin of tumoral DNA that is released into the circulation is poorly understood, but it is believed that the DNA is released during the necrosis and/or apoptosis of tumor cells [9]. These circulatory molecules can be easily isolated and serve for the detection of the methylation status of certain genes [9–11]. Ras-association domain family 1 isoform A (*RASSF1A*) and Adenomatous Polyposis Coli (*APC*) are two very important tumor suppressor genes that are involved in different molecular pathways and are frequently epigenetically inactivated in a wide range of cancer types [12,13].

RASSF1A is known to regulate the activation of cell death [14], cell cycle progression [15], and microtubule formation [16]. The methylation of *RASSF1A* is thought to be one of the earliest cellular changes in tumorigenesis and its methylation frequency in different solid tumors varies widely [12,13,17,18]. Notably, it is not methylated in gastric adenoma, intestinal metaplasia and chronic gastritis, suggesting that methylation and inactivation of this gene may be a delayed event during the malignant transformation process. The effect of *RASSF1A* methylation abnormalities on prognosis has also been investigated. Studies have shown that methylated *RASSF1A* represents a poor prognostic indicator in lung cancer [18], something which was not confirmed in other tumor types. The prevalence and significance of *RASSF1A* promoter methylation in serum has not been extensively studied. Wang et al. recently reported serum *RASSF1A* methylation in 34% of patients with gastric adenocarcinoma and in 28.9% of those with colorectal carcinoma [19].

APC was first identified as the gene responsible for the familial adenomatous polyposis (FAP) syndrome. *APC* mutations have been reported infrequently in gastric cancer unlike in the colorectal cancer [20,21]. *APC* gene inactivation by hypermethylation leads to stabilization of b-catenin in the cytoplasm due to dysfunction of b-catenin protein degradation [22]. *APC* is expressed in the stomach as two isoforms originating from two promoters 1A and 1B [23]. Methylation in gastric tissue occurs predominantly in promoter 1A, and for this reason this promoter is mostly examined for hypermethylation in gastric cancer. Methylation at promoter 1A is not predominately tumor related, as it is frequently found in non-malignant gastric mucosa [24]. Consequently, methylation at promoter 1A in gastric mucosa is likely to be a passenger, rather, than a driver of carcinogenesis [25].

In this study, we investigated the methylation status of these two genes in the cell free circulating DNA of operable gastric cancer patients. Our aim was to primarily assess the methylation status of these two independent genes and secondary to explore their possible prognostic significance in patients with early operable gastric cancer.

2. Materials and methods

2.1. Study design

The study material consisted of 73 blood samples obtained from gastric cancer patients who underwent curative surgery with a known clinical outcome and a long follow up period (median: 56 months, range: 12–111). Patient's mean age ± SD was 67.07 ± 11.09 years (range: 28–82, median age: 70.5 years) and they all had a performance status (WHO scale) of 0–1. Patients were categorized in 2 groups: those with early stages (stage I and II) and those with advanced stages (IIIA and IIIB). All patients provided informed consent. Additionally, 20 blood samples taken from healthy individuals were used as a control group. All these control samples were taken from healthy friends and non-blood related family members of patients treated in the Department of Medical Oncology of the University Hospital of Alexandroupolis. The majority of them were men, all age-matched with our patient population and received no medical care at the time of the sample collection.

2.2. Sample collection and isolation of cell free DNA

Whole blood was drawn from patients pre-operatively. Blood was collected in serum clot activator tubes. Serum was obtained immediately through centrifugation at 3000 rpm 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, Germany). 200 μL of serum were mixed with 200 μL of working solution and 50 μL proteinase K (18 mg/mL) and incubated for 10 min at 72 °C; the DNA isolation was then processed as described in the manufacturer's protocol. DNA concentration was determined by a real-time PCR method using *GAPDH* gene as an amplifying target. Three μL of DNA elution were used as a template for the Sybr-green based real time PCR analysis. The amount of cell free DNA was quantified using a pair of primers as previously described [26]. The cell free DNA concentration was calculated according to a reproducible standard dilution curves using a known concentration of MCF-7 genomic DNA. The quantitative PCR assay demonstrated high linearity of product amplification assessed as the mean slope ($m = -3.31$) and the mean correlation coefficient ($R^2 = 0.99$) of all standard curves constructed. The specificity of the qPCR amplification product was verified by melting curve analysis. The DNA concentration ranged from 0.80 to 480 ng/mL. DNA samples were stored at −80 °C until used

2.3. CEA and CA 19-9 measurements

Carcinoembryonic antigen (CEA) levels and CA 19-9 levels were measured using the Cobas Elecys kit (Roche Diagnostics). The cut-offs that were used were 10 ng/mL for CEA and 37 U/mL for CA 19-9.

2.4. Sodium bisulfite conversion

Extracted DNA was modified with sodium bisulfite (SB), in order to convert all unmethylated, but not methylated-cytosines to uracil. Bisulfite conversion was carried out up to 500 ng of extracted DNA using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer's instructions. The converted DNA was stored at −80 °C until used.

2.5. Methylation specific PCR (MSP)

The methylation status of *APC* and *RASSF1A* in cell free circulating serum DNA samples was detected by Methylation Specific PCR (MSP) using specific primer pairs for both the methylated and unmethylated promoter sequences. The primer sequences are shown in Table 1. Each MSP reaction was performed in a total volume of 25 μL. One μL of sodium bisulfite converted DNA was added into a 24 μL reaction mixture that contained 0.1 μL of Taq DNA polymerase (5 U/μL, hot start Go Taq Polymerase; Promega, USA), 5 μL of the supplied 10× PCR buffer, 2.0 μL of $MgCl_2$ (50 mmol/L), 0.5 μL of dNTP's (10 mmol/L; Fermentas) and 1 μL of the corresponding forward and reverse primers (10 μmol/L); finally dH_2O

Table 1
Primer sequences used in MSP analyses.

Gene	Direction	Sequence	bp
Unmethylated			
RASSF1A	Forward	5′-GGTTGTATTTGGTTGGAGTG-3′	180
	Reverse	5′-CTACAAACCTTTACACACAACA-3′	
APC	Forward	5′-GTGTTTTATTGTGGAGTGTGGGTT-3′	108
IA	Reverse	5′-CCAATCAACAAACTCCCAACAA-3′	
Methylated			
RASSF1A	Forward	5′-GTTGGTATTCGTTGGGCGC-3′	160
	Reverse	5′-GCACCACGTATACGTAACG-3′	
APC	Forward	5′-TATTGCGGAGTGCGGGTC-3′	98
1A	Reverse	5′-TCGACGAACTCCCGACGA-3′	

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 and one for unmethylated promoter sequences. Human placental genomic DNA (gDNA; Sigma Aldrich) methylated in vitro with SssI methylase (NEB, Ipswich, MA) was used, after sodium bisulfite conversion, as fully methylated (100%) MSP positive control. The same unmethylated placental gDNA, was used, after sodium bisulfite conversion, as a negative MSP control. Thermocycling conditions used for the methylated and the unmethylated reaction were as follows: (i) *APC*, one cycle at 95 °C for 5 min, followed by 39 cycles of 95 °C for 45 s, 55 °C for 60 s and 72 °C for 60 s, with a final extension cycle of 72 °C for 10 min. (ii) *RASSF1A*, one cycle at 95 °C for 5 min, followed by 39 cycles of 95 °C for 30 s, 57 °C for 45 s and 72 °C for 45 s, 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 EDTA (pH = 8) and visualized by ethidium bromide staining. Our criterion for methylation positivity was as follows: Samples with equal or stronger band intensity than the positive control in the methylation specific reaction were denoted as strongly methylated (++). Samples with less intense bands than the positive control were categorized as weakly methylated, whereas samples with very weak band intensity and samples with no visible PCR product were regarded as unmethylated (Fig. 1).

2.6. Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM). The methylation status of *APC* and *RASSF1A* and all other qualitative variables were expressed as frequencies and percentages (%). The chi-square test was used to evaluate any potential association of *APC* and *RASSF1A* methylation status with patients' demographic and clinicopathological characteristics. Odds ratios (OR) and their 95% confidence interval (CI) were estimated as a measure of association of *APC* and *RASSF1A* status with patients' characteristics. Survival rates were calculated with the Kaplan–Meier method and the statistical difference between survival curves was determined with both log-rank and Breslow tests. Multivariate Cox proportional hazards regression analysis was performed to explore the independent effect of *APC* and *RASSF1A* status on overall survival. Patients' gender, age, tumor site, differentiation, lymph node infiltration, stage, CEA and CA19.9 levels were also included in the multivariate model as potential confounders. All tests were

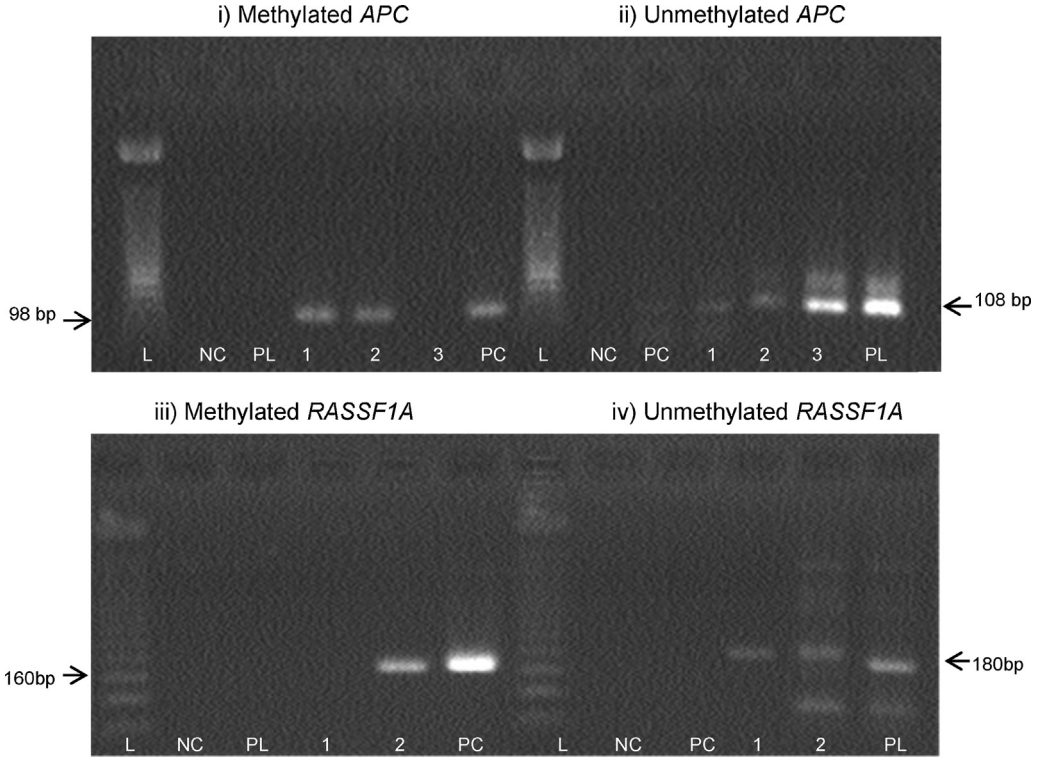

Fig. 1. Agarose gel electrophoresis of methylation specific PCR products for (i) methylated *APC* (NC = negative control, no DNA, PL = placental DNA, 1-2-3 (patient's samples), PC = positive control, methylated in vitro DNA, 100% methylated); (ii) unmethylated *APC* (NC = negative control, no DNA, PC = positive control, methylated in vitro DNA, 100% methylated, 1-2-3 (patient's samples), PL = placental DNA); (iii) methylated *RASSF1A* (NC = negative control, no DNA, PL = placental DNA, 1-2 (patient's samples), PC = positive control, methylated in vitro DNA, 100% methylated); (iv) unmethylated *RASSF1A* (NC = negative control, no DNA, PC = positive control, methylated in vitro DNA, 100% methylated, 1–2 (patient's samples), PL = placental DNA).

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

Patient's characteristics	*n*	*APC* methylation	*p* value	*RASSF1A* methylation	*p* value
Gender			0.671		0.256
Females	22	19 (86.4)		13 (59.1)	
Males	51	42 (82.4)		37 (72.5)	
Age			0.984		0.406
≤60 years	17	14 (82.4)		10 (58.8)	
>60 years	56	46 (82.1)		39 (69.6)	
Stage			0.687		0.031
Early (I–II)	20	16 (80.0)		11 (55.0)	
Advanced (III_A–$IIIA_{B)}$	38	32 (84.2)		31 (81.6)	
Unknown	15				
Tumor site			0.366		0.671
Body	33	29 (87.9)		23 (69.7)	
Antrum	40	32 (80.0)		26 (65.0)	
Differentiation			0.096		0.403
Well	11	7 (63.6)		9 (81.8)	
Median–Poor	42	36 (85.7)		29 (69.0)	
Unknown	20				
Regional lymph nodes			0.578		**0.005**
N+	43	37 (86.0)		34 (79.1)	
N−	15	12 (80.0)		6 (40.0)	
Unknown	15				
CEA levels			**0.033**		0.422
≤10 ng/mL	37	28 (75.7)		23 (62.2)	
>10 ng/mL	25	24 (96.0)		18 (72.0)	
Unknown	11				
CA19.9 levels			**0.032**		0.490
≤37 U/mL	44	34 (77.3)		30 (68.2)	
>37 U/mL	17	17 (100.0)		10 (58.8)	
Unknown	12				

Statistically significant values are highlighted in bold.

two tailed and statistical significance was considered for *p* values <0.05.

3. Results

The methylation status of *APC* and *RASSF1A* was evaluated in serum cell free circulating DNA samples from 73 patients diagnosed with operable gastric cancer in double-blinded experiments. Patient's clinicopathological characteristics (Table 2) and clinical outcome data became available after the completion of the study for the statistical correlations. *APC and RASSF1A* promoters were found to be methylated in 61 (83.6%) and 50 (68.5%) of the 73 gastric cancer samples examined respectively, but in none of the control samples ($p < 0.001$).

3.1. Correlations between APC and RASSF1A promoter methylation status and different tumor parameters

Chi-square analysis revealed a significant association between a methylated *APC* promoter status and high CEA levels (96.0% vs. 75.7%, $p = 0.033$; OR = 7.7; 95% CI: 1.0–65.4) as well as high CA19.9 levels (100.0% vs. 77.3%, $p = 0.032$). A significant association of methylated *RASSF1A* promoter status with more advanced stages (81.6% vs. 55.0%, $p = 0.031$; OR = 3.6, 95% CI: 1.1–12.1) and lymph node positivity was observed (79.1% vs. 40.0%, $p = 0.005$; OR = 5.7, 95% CI: 1.6–20.1). No other significant association between *APC* and *RASSF1A* promoter methylation status and other tumor parameters examined was observed. The associations of demographic and clinicopathological features with *APC* and *RASSF1A* methylation status are given in Table 2. Co-expression of methylated *APC* and *RASSF1A* promoters were found in 42 (57.5%) of the 73 gastric cancer samples and its presence was associated with lymph node positivity (69.8% vs. 33.3%, $p = 0.013$; OR = 4.6; 95% CI: 1.3–16.2) and advanced stages (71.1% vs. 45.0%, $p = 0.052$; OR = 3.0, 95% CI: 1.0–9.2).

3.2. Correlations between APC and RASSF1A promoters methylation status and survival

After a median follow up period of 56 months (range, 12–111 months), 38 (52.1%) patients have died as a consequence of disease progression. *APC and RASSF1A* methylation was detected in 36 (94.7%) and 25 (65.8%) of these patients, respectively. The incidence of death was significantly higher in patients with a methylated than in patients with an unmethylated *APC* promoter status (59.0% vs. 16.7%, $p = 0.007$; HR = 5.5, 95% CI: 1.3–22.9), but it was independent of the *RASSF1A* methylation status (56.5% in unmethylated vs 50.0% in methylated, $p = 0.604$; HR = 0.9, 95% CI: 0.5–1.7).

Among the entire cohort, the mean survival time ± SE was 54.0 ± 5.7 mo (95% CI: 42.9–65.1 mo, median: 36.0 mo). The mean survival time ± SE of patients with an unmethylated *APC* promoter status was 85.0 ± 9.0 months (95% CI: 67.0–103.0) which was substantially longer to the mean survival ± SE of 46.0 ± 6.0 months (95% CI: 34.0–58.0; median survival, 27 mo) observed in those with a methylated *APC* promoter status. It should be noted that in patients with unmethylated *APC* promoter status the median survival time was not reached since less than 50% of these patients died during follow up period. The Kaplan–Meier estimates of survival rates, significantly favored patients with a non-methylated *APC* promoter status (Fig. 2, Log Rank test, $p = 0.008$; Breslow test, $p = 0.014$). Patients' survival was not significantly associated with *RASSF1A* methylation status (Fig. 3, Log Rank test, $p = 0.683$; Breslow test, $p = 0.791$). Mean survival time of patients with an unmethylated *RASSF1A* promoter status was 43.0 ± 7.0 mo (95% CI: 28.0–57.0, median survival: 27 mo) and 56.0 ± 7.0 mo (95% CI:

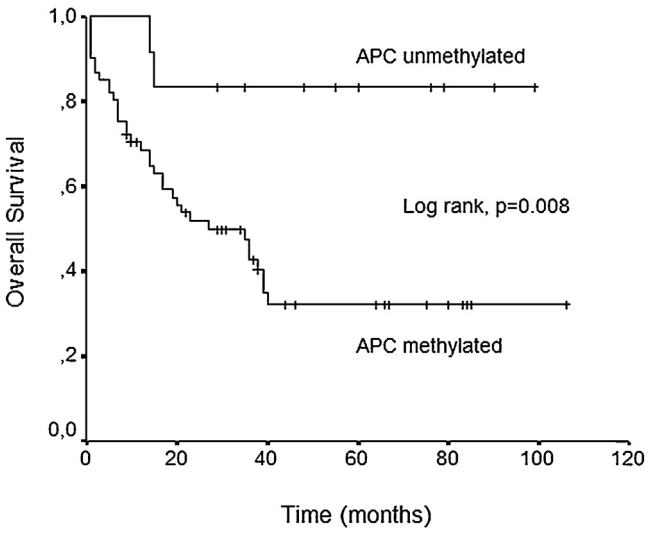

Fig. 2. Kaplan Meier estimate of overall survival (OS) for patients with early operable gastric cancer with or without *APC* promoter methylation ($p = 0.008$).

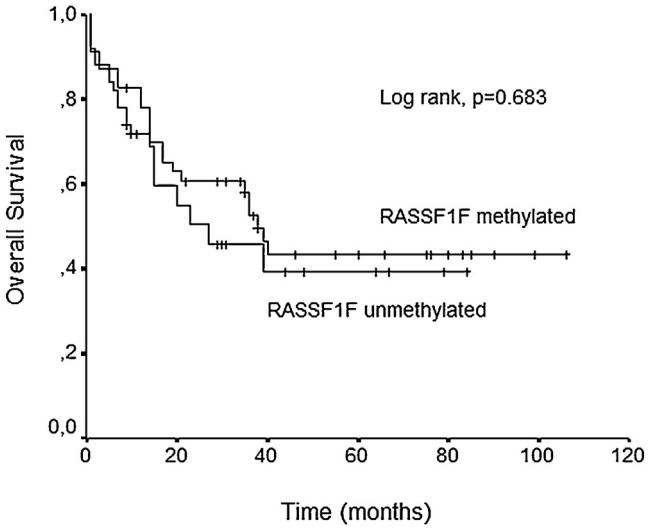

Fig. 3. Kaplan Meier estimate of overall survival (OS) for patients with early operable gastric cancer with or without *RASSF1A* promoter methylation ($p = 0.683$).

42.0–69.0, median survival: 38 mo) in those with a methylated *RASSF1A* promoter status. The association of survival with the co-expression of methylated *APC and RASSF1A* promoters was of marginal statistical significance (Log Rank test, $p = 0.089$; Breslow test, $p = 0.119$).

Further investigation with multivariate Cox proportional hazards regression analysis revealed that only methylated *APC* promoter status (aHR = 4.6, 95% CI: 1.1–20.3, $p = 0.046$) and tumor site (body) (aHR = 3.2, 95% CI: 1.5–6.8, $p = 0.031$ $p = 0.003$) remained the only statistically significant independent determinants for poor survival. Other parameters, such as gender ($p = 0.950$), age ($p = 0.075$), tumor differentiation ($p = 0.681$), lymph node status ($p = 0.080$), stage ($p = 0.868$), CEA levels ($p = 0.979$), CA19.9 ($p = 0.290$) and *RASSF1A* methylated status ($p = 0.209$) were not significantly associated with survival.

4. Discussion

Gastric cancer is a common aggressive malignancy. Although its incidence varies widely among different countries, gastric cancer represents a major health problem, worldwide. The etiology of gastric cancer has not been fully explored. However, it is well established that gastric cancer represents a multistep process involving genetic and epigenetic events, such as activation of oncogenes, overexpression of growth factors and receptors, and inactivation of tumor suppressor genes [3]. Promoter methylation of cancer-related genes is an important pathway in gastric carcinogenesis, with numerous factors involved in this process. Indeed, the inactivation of many tumor suppressor genes involved in gastric carcinogenesis, such as *E-cadherin* [27], *p16* [28], *hMLH1* [29], *RUNX3* [30], *SOX17* [31] and others, is more frequently caused by DNA hypermethylation, rather, than by gene mutations. Therefore, it is presumed that this frequent molecular event could also serve as a useful marker, instead of gene mutation analysis, for the early diagnosis and prognosis of this type of cancer.

In the present study, we explored the promoter methylation status of *APC* and *RASSF1A* genes in cell free DNA from patients with early operable gastric cancer and examined their possible correlations with different tumor parameters and survival. *RASSF1A* and *APC* are two very important tumor suppressor genes that are commonly epigenetically inactivated in gastric cancer [12,13]. *RASSF1A* protein is actively involved in microtubule regulation, genomic stability maintenance, cell-cycle regulation, apoptosis modulation, cell motility and invasion control [14–16]. Its methylation frequency in gastric cancer has been reported to vary within 30 and 50% [13,32]. The adenomatous polyposis coli (*APC*) tumor suppressor gene, a negative regulator of WNT signaling is known to be methylated in 34–83% of gastric cancers while its mutations are very rare [20]. *APC* gene inactivation by hypermethylation leads to stabilization of β-catenin in the cytoplasm as a result of dysfunction of b-catenin protein degradation [22]. *APC* is expressed in the stomach as two isoforms originating from two promoters 1A and 1B. Methylation in gastric tissue occurs predominantly in promoter 1A, and for this reason this promoter is more frequently examined for hypermethylation [23].

We detected *RASSF1A* promoter methylation in 50 (68.5%) out of the 73 examined cases, which is indicative of a high methylation frequency. It may also be indicative of its crucial role in gastric carcinogenesis that is played at early disease stages, as in the population included in our study. Methylation of *RASSF1A* was significantly correlated with lymph node positivity which is in accordance with its known role as a tumor suppressor gene. *RASSF1A* encodes a protein similar to the RAS effector proteins, and functions as a tumor suppressor whose epigenetic inactivation is closely associated with the development of gastric cancer. Methylation-induced inactivation of *RASSF1A* possibly associates with a more aggressive tumor phenotype, and thus, the observed correlation with lymph node positivity in early operable gastric cancer. This finding is also in accordance with previous studies showing association between hypermethylation of *RASSF1A* and poor survival [33]. However, no significant correlation between *RASSF1A* methylation status and survival was observed in our study. We further explored its possible relation with other tumor characteristics such as age, differentiation and/or serum markers but no significant correlation was observed. These results are also in keeping to those reported by Wang et al. [19] Indeed, in this study no correlation between several tumor parameters examined and *RASSF1A* hypermethylation was found. Notably, in that study as well as in another study by Tan et al. [34], the incidence of *RASSF1A* promoter hypermethylation in the serum of patients with gastric carcinomas was found to be 34% and 25%, respectively, which is considerably lower to the 68.5% observed in our study. However, these deviations between studies may reflect ethnic, environmental and/or geographical reasons that have established gastric cancer as a different disease in Asia and Europe. Furthermore, the number of cases examined in that study ($n = 47$) as well as in our study ($n = 73$) is limited for safe statistical considerations. In our study,

all healthy donors had a non-methylated *RASSF1A* promoter status, something which has also been reported previously, showing that *RASSF1A* promoter hypermethylation status may be used as a potential biomarker for gastric cancer diagnosis. This is additionally supported by studies showing *RASSF1A* promoter hypermethylation status being identical in both the serum and in available paired tumor genomic DNA from patients with gastric cancer.

Regarding *APC* and specifically its 1A promoter, this was found to be methylated in 61 (83.6%) of our cases, which is also suggestive of a pivotal role in gastric carcinogenesis. A significant correlation between methylated *APC* promoter status and higher serum tumor marker levels (CEA and CA19-9) was also observed. A similar correlation has not been reported previously and we do not have a clear explanation on this finding. No other significant correlations with different tumor variables examined was seen. The survival analysis revealed that a methylated *APC* promoter was significantly associated with a worst clinical outcome. Indeed, patients with an unmethylated *APC* promoter status had a mean survival of 86 months which is considerably better as compared to the 26 month survival of those with a methylated one. These differences in survival are possibly relevant to the methylation-induced inactivation of the *APC* gene. It has been reported that, although *APC* mutations are rare in gastric tumors, the nuclear accumulation of β-catenin is detected in almost 39% of human gastric cancers [22]. Methylation-induced down-regulation of *APC* and subsequent activation of the WNT/β-catenin pathway may be indicative for the presence of an aggressive tumor behavior associating with poor survival and metastatic potential.

In conclusion, we found that serum *RASSF1A* and *APC* promoter hypermethylation is a frequent epigenetic event in patients with early operable gastric cancer. We also observed a significant correlation between *APC* promoter methylation status and survival as well as between a hypermethylated *RASSF1A* promoter and nodal positivity. Additional studies, in a larger cohort of patients are required to further explore whether this findings could establish methylation status of *APC* and *RASSF1A* as potent biomarkers for early detection and prognosis in gastric cancer patients.

Conflict of interest statement

The authors of this paper agree for its publication in your journal and declare no conflict of interest.

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