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Review Article

The Methylation Status of *RASSF1A* and *APC* Represents a Valuable Prognostic Indicator in Gastrointestinal Malignancies

Ioanna Balgkouranidou[1]*, Biziota E[1], Koukaki T[1], Bolanaki H[2], Karayanakis A[2], Chelis L[1], Amarantidis K[1], Xenidis N[1], Lambropoulou M[3], Chatzaki E[4], Lianidou E[5] and Kakolyris S[1]

[1]*Department of Medical Oncology, Medical School, Democritus University of Thrace, Greece*

[2]*Second Department of Surgery, Medical School, Democritus University of Thrace, Greece*

[3]*Department of Histology and Embryology, Medical School, Democritus University of Thrace, Greece*

[4]*Department of Pharmacology, Medical School, Democritus University of Thrace, Greece*

[5]*Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece*

***Corresponding Author:** Ioanna Balgkouranidou, Department of Medical Oncology, Medical School, Democritus University of Thrace, Greece.

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Abstract

Gastrointestinal 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.

Using methylation specific PCR, we examined the methylation status of promoter 1A and *RASSF1A* promoter in cell free (cfDNA) DNA from blood samples obtained from patients with operable gastric cancer as well as operable and metastatic colorectal cancer.

We found that serum *RASSF1A* and *APC* promoter hypermethylation is a frequent epigenetic event in gastrointestinal malignancies in both early and advanced disease. The observed correlations between *APC* and *RASSF1A* promoter methylation status and survival may be indicative of a prognostic role for those genes in patients with gastric and colorectal cancer.

Additional studies, in a larger cohort of patients are required to further explore whether the methylation status of *APC* and *RASSF1A* detected in cell free DNA of patients with gastric and colorectal cancer could serve as potential molecular biomarkers of survival and/or response to specific treatments.

Keywords: *Gastrointestinal Cancers; DNA Methylation; Cell-Free DNA; APC; RASSF1A; Biomarkers*

Abbreviations

GICs: Gastrointestinal Cancers; CRC: Colorectal Cancer; GC: Gastric Cancer; cfDNA: Cell Free DNA; MSP: Methylation Specific PCR; OS: Overall Survival

Introduction

Cancer is a complex group of diseases caused by interactions of multiple factors. During the multistep carcinogenesis, many genetic and epigenetic events occur [1] that rend every single step specific and unique. The classic genetic alterations are the mutations in key

tumor suppressor genes or oncogenes that defect the protein function or deregulate the gene expression. On the other hand, epigenetic events affect gene expression without any changes in DNA sequence. The multitude of genetic and epigenetic variations may act synergistically in the malignant transformation process. Recent evidence focus to a crosstalk between these two mechanisms in cancer formation and it is now well established that epigenetic events can be driver events in the pathogenesis of cancer and that these epigenetic alterations cooperate with gene mutations in cancer formation and progression [2,3].

The best-characterized epigenetic modification is DNA methylation, a covalent addition of a methyl group to cytosines within CG dinucleotides by specific enzymes called DNA methyltransferases (DNMTs) [4]. Among them, DNMT1 is responsible for the maintenance of the existing methylation while DNMT3a and DNMT3b catalyze DNA methylation in a de novo fashion [5,6]. CpG sequence is the substrate for the DNMTs and the regions rich in such sequences are called CpG islands. CpG islands mainly exist in the promoter region of genes and play a key role in gene expression. Methylation of CpG islands in the promoter region is correlated with transcriptional silencing. This is mainly due to Methyl-binding proteins (MBPs) that bind with high affinity to methylated DNA and indirectly block the access of transcription factors to the promoter region [7]. Cancer cells are characterized by genome wide hypomethylation and hypermethylation in the CpG islands of gene promoters. Promoter hypermethylation is commonly associated with gene silencing as well as demethylation with genome instability and gene expression [8]. In the process of tumor development, demethylation and hypermethylation in the CpG islands of gene promoters occur simultaneously [9]. DNA methylation is chemically stable and is easy to detect in different subtracts and assays for the detection of promoter hypermethylation. Additionally, it may have a higher sensitivity than microsatellite analyses, and can have advantages over mutation analyses. These characteristics, rend DNA methylation an optimal potential biomarker [10,11]. The ever-growing number of genes that have been found hypermethylated in various cancers emphasizes the crucial role of DNA methylation for future diagnosis, prognosis and prediction of response to therapy.

Gastrointestinal cancers (GICs) mainly including, gastric cancer (GC) and colorectal cancer (CRC), account for a great proportion of human malignancies which are related to a high cancer mortality rate worldwide [12]. The understanding of the molecular pathways of tumorigenesis is like unmasking cancer's secret identity and define its molecular complexity. In GICs, there are many genes that seem to be more commonly methylated in the multi-step process leading from normal epithelium to an adenocarcinoma. Some of them are frequently methylated in the passage from an aberrant crypt focus to polyp/adenoma. These genes could be optimal diagnostic or prognostic candidate biomarkers, respectively. Nowadays, there is an increasing interest in the development of biomarkers that will facilitate diagnosis, improve prognostication as well as prediction for the use of personalized therapies. Due to accessibility, blood is invariably the most ideal analyte for a cancer biomarker. More specifically, blood based biomarkers developed using cell free circulating DNA in plasma are starting to emerge. Cell free circulating DNA (cfDNA) is released into the blood by apoptosis or necrosis of cancer cells in the tumor microenvironment. Secretion has also been suggested as a potential source of cfDNA [13]. Nevertheless, cell free DNA maintains all the molecular characteristics of the tumor, such as tumor specific mutations or epigenetic alterations.

Several oncogenes have been recognized as potential prognostic biomarkers according to their methylation status in GI malignancies. Indeed, the most established methylated DNA blood biomarker for CRC patients is methylated Septin 9 (*SEPT9*), which belongs to gene family that encodes a group of GTP-binding and filament-forming proteins involved in cytoskeletal formation [14]. Lofton-Day., *et al.* first identified methylated *SEPT9* as a noninvasive diagnostic biomarker for CRC, with 69% sensitivity and 86% specificity for discriminating CRC patients from healthy individuals [15]. Subsequent studies validated the clinical significance of methylated SEPT9 as a potential biomarker for CRC screening, which is now commercially offered as a blood based screening test in various assays including EpiproColon® 1.0 (Epigenomics, Seatle, WA), ColoVantage® (Quest Diagnostics, Madison, NJ) and RealTime mS9 (Abbott Laboratories, Des Plaines, IL). In addition, several other blood based methylation diagnostic or prognostic biomarkers have been identified for GICs tumors. Optimal diagnostic candidates are, *ALX4* which is frequently methylated in adenocarcinomas of the gastrointestinal tract with 83% sensitivity and 70% specificity in colorectal cancer [16] and *NEUROG1*, which was found to be methylated in the serum of colorectal cancer patients

independent of tumor stage [17]. Among prognostic biomarker candidates, CIMP status has been the most promising indicator for prognostication, especially in CRC patients. CIMP-positive cancers have been found to correlate with an overall unfavorable prognosis [18,19]. Similarly, according to the 'one gene' candidate methylation biomarker approach, we have proposed *APC* and *RASSF1A* as possible prognostic biomarkers both in gastric and in colorectal cancer [20,21].

RASSF1A

The RAS-association domain family (RASSF) consists of 10 members implicated in a variety of key biological processes, including cell cycle regulation, apoptosis and microtubule stability. Furthermore, they have been implicated in tumorigenesis and several family members are now thought to be tumor suppressors. As opposed to the KRAS oncogene, for which mutational inactivation is frequent in colorectal cancer (CRC), the members of the RASSF family are found to be silenced mainly by aberrant promoter methylation [22]. *RASSF1A* is one of the most frequently epigenetically inactivated tumor-suppressor genes in sporadic human malignancies [23,24]. It is a component of key cancer pathways, namely Ras/PI3K/AKT, RAS/RAF/MEK/ERK and Hippo pathways and its inactivation is an important factor contributing to pathogenesis and progression of solid tumors [25]. 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 [26,27]. Methylation of *RASSF1A* gene is rare in normal tissue and is one of the highest described in lung cancer [28]. Correlation of *RASSF1A* methylation with cancer risk has been validated in several GI cancers. In a study of 112 esophageal squamous cell carcinomas (ESCC), 116 gastric cardia adenocarcinomas (GCA) and 235 normal controls Zhou., *et al.* reported that *RASSF1A* promoter methylation associates with increased risk for the development of ESCC and for GCA [29]. Furthermore, gastric malignancies with inactivated *RASSF1A* appear to have clinicopathological characteristics that indicate more aggressive phenotype, such as advanced stage [30] and regional lymph node metastasis [31]. In colorectal cancer (CRC), the methylation status of *RASSF1A* strongly associated with the pathogenesis of CRC [32]. Nilsson., *et al.* reported that the methylation status of *RASSF1A* in 111 CRC specimens defines a poor prognosis subset of CRC patient's independently of both tumor stage and differentiation [33]. Table 1 summarizes the literature on the prognostic, predictive or diagnostic role of the methylation status of *RASSF1A* gene in gastrointestinal malignancies.

Cancer type	Number of Cases	Gene	Clinicopathol Features	Risk	Poor DFI	Poor OS	Metastasis	REF
Colorectal Cancer	111	*APC/RASSF1A*						Nilsson., *et al*.
	630	*RASSF1a*						Wang., *et al*.
	67, 88	*APC/RASSF1A*						Matthaios., *et al*.
Gastric cancer	116	*RASSF1A*						Zhou., *et al*.
	1215 (metanalysis)	*RASSF1A*						Shi., *et al*.
	92	*RASSF1A*						Guo., *et al*.
	62	*RASSF1A*						Sinha., *et al*.
	73	*APC/RASSF1A*						Balgkouranidou., *et al*.

Table 1: *Clinical associations of RASSF1A and APC methylation in Gastrointestinal malignancies.*

APC

APC was first identified as the gene responsible for the familial adenomatous polyposis (FAP) syndrome. The *APC* gene encodes a multifunctional protein with important role in the Wnt signaling pathway, inter cellular adhesion, cytoskeleton stabilization, cell cycle regulation and apoptosis [34]. *APC* mutations have been reported infrequently in gastric cancer unlike in the colorectal cancer. *APC* gene inactivation by hypermethylation leads to stabilization of b-catenin in the cytoplasm due to dysfunction of b-catenin protein degradation [35,36]. *APC* is expressed in the stomach as two isoforms originating from two promoters 1A and 1B [37]. 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 [38]. Consequently, methylation at promoter 1A in gastric mucosa is likely to be a passenger, rather, than a driver of carcinogenesis [39]. *APC* gene is one of the most prominent methylation biomarkers in GICs since it has been identified for CRC detection [40] and prognosis [21]. It has been shown that DNA methylation of *APC* defines a poor prognosis subset of CRC patients independently of tumor stage and differentiation [33]. Liu., *et al.* 2013, reported 48% *APC* methylation in gastric tissues and 30.6% in GC serum [41]. In this study, *APC* along with other methylated genes (CIMP+)/ *H. pylori+*, were associated with higher rates of metastasis [41]. (Table 1 summarizes the literature on the prognostic, predictive or diagnostic role of the methylation status of *APC* gene in gastrointestinal malignancies.

We present our recent findings on the prognostic role of the methylation status of *APC* and *RASSF1A* in the cfDNA of patients with CRC and gastric cancer. Differences in the methylation status of these genes in early and advanced disease are also discussed. We have also sum up the literature on the prognostic, predictive or diagnostic role of the methylation status of these genes in gastrointestinal malignancies.

Materials and Methods

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 155 blood samples obtained from patients with CRC, suffering from either early operable (67/155, 43.2%) or metastatic disease (88/155, 56.8%).

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.

Sample collection and Isolation of Cell Free DNA

Blood was collected in serum clot activator tubes. Serum was obtained immediately through centrifugation at 3,000 rpm for 10 minutes 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) according to 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 cell free DNA concentration was calculated according to a reproducible standard dilution curves using a known concentration of MCF-7 genomic DNA.

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.

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. 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.

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

Results

Correlation of *APC* and *RASSF1A* methylation status with different tumour parameters and survival in patients with early operable gastric cancer

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. *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 ($p < 0.001$).

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). We observed a significant association of methylated *RASSF1A* promoter status with more advanced disease stages (81.6% vs. 55.0%, p = 0.031; OR = 3.6, 95% CI: 1.1 - 12.1) and lymph node positivity (79.1% vs. 40.0%, p = 0.005; OR = 5.7, 95% CI: 1.6 - 20.1). 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 bad prognostic features such as lymph node positivity (69.8% vs. 33.3%, p = 0.013; OR = 4.6; 95% CI: 1.3 - 16.2) and more advanced disease stages (71.1% vs. 45.0%, p = 0.052; OR = 3.0, 95% CI: 1.0 - 9.2). Table 2 summarises correlations between *RASSF1A/APC* methylation status with different tumor characteristics.

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 (100.0)		10 (58.8)	
Unknown	17				

Table 2: *Association of APC and RASSF1A methylation status with demographic and clinicopathological features of patients with operable gastric cancer.*

After a median follow up period of 56 months (range: 12 - 111 mo), 38 (52.1%) patients have died as a consequence of disease progression. 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). 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, p = 0.001) 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 (p = 0.008) (Figure 1). 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).

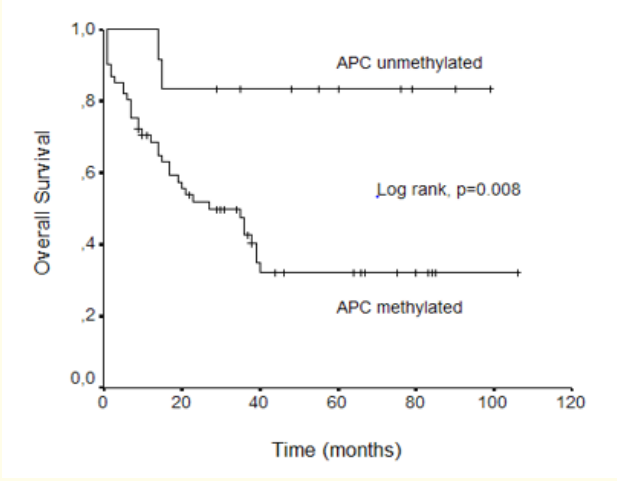

Figure 1: *Kaplan Meier estimate of overall survival (OS) for patients with early operable gastric cancer with or without APC promoter methylation (p = 0.008).*

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 anatomic tumor site (body of the stomach) (aHR = 3.2, 95% CI: 1.5 - 6.8, p = 0.031) 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), disease 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.

Correlation of *APC* and *RASSF1A* methylation status with different tumor parameters and survival in patients with early operable CRC

APC was methylated in 29 (43.3%) of the 67 patients with early operable CRC. Chi-square analysis revealed that higher frequency of methylated *APC* promoter status was associated with ages older than 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).

RASSF1A was methylated in 22 (32.8%) of the 67 patients with early operable CRC patients. Methylated *RASSF1A* promoter status was significantly associated with a higher disease stage (OR = 3.11; 95% CI: 1.16 - 8.36, p = 0.021). Table 3 summarises correlations between *RASSF1A/APC* methylation status with different tumor characteristics.

Patient's 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		0.012		0.267
≤ 70 years	8 (19.5)		8 (19.5)	
> 70 years	21 (44.7)		14 (29.8)	
Dukes		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)	
Median	14 (32.6)		9 (20.9)	
Poor	7 (46.7)		4 (26.7)	
Side		0.407		0.538
Right	16 (37.2)		12 (27.9)	
Left	13 (28.9)		10 (22.2)	
CEA levels (n = 82)		0.088		0.697
≤ 5 ng/mL	10 (25.0)		9 (22.5)	
> 5 ng/mL	18 (42.9)		11 (26.2)	
CA19.9 levels (n = 82)		0.083		0.372
Low (< 37 U/ml)	20 (29.9)		15 (22.4)	
High (> 37 U/ml)	8 (53.3)		5 (33.3)	
***APC* levels**				0.050
Unmethylated	-		11 (18.6)	
Methylated	-		11 (37.9)	
***RASSF1A* levels**		0.050		
Unmethylated	18 (27.3)		-	
Methylated	11 (50.0)		-	

Table 3: *Association of APC and RASSF1A methylation status with demographic and clinicopathological characteristics of patients with operable colorectal cancer (CRC).*

The mean survival time (± SE) of patients with an unmethylated *APC* promoter status was 81 ± 5 months (range: 71 - 91 mo) which was substantially longer to the mean survival (± SE) of 27 ± 4 months (range: 19 - 34 mo) observed in those with a methylated *APC* promoter status (Log Rank test, $p < 0.001$) (Figure 2a).

The mean survival time (± SE) of patients with an unmethylated *RASSF1A* promoter status was 71 ± 6 months (range: 60 - 81 mo) which was substantially longer to the mean survival (± SE) of 46 ± 8 months (range: 29 - 62 mo) observed in those with a methylated *RASSF1A* promoter status (Log Rank test, $p < 0.001$) (Figure 2b).

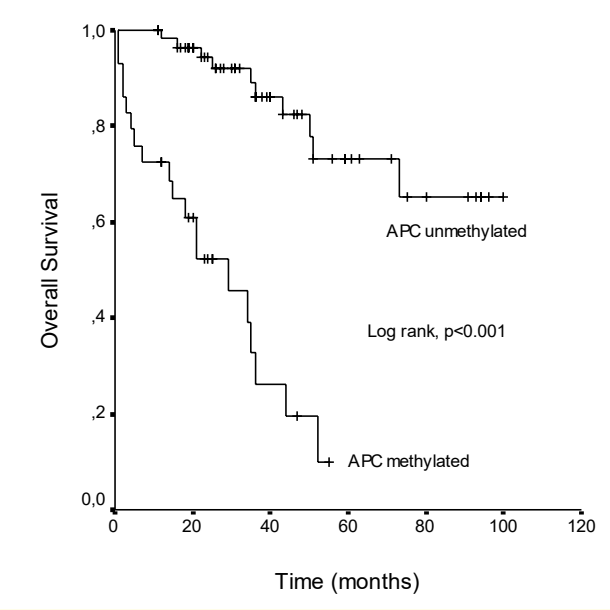

Figure 2a

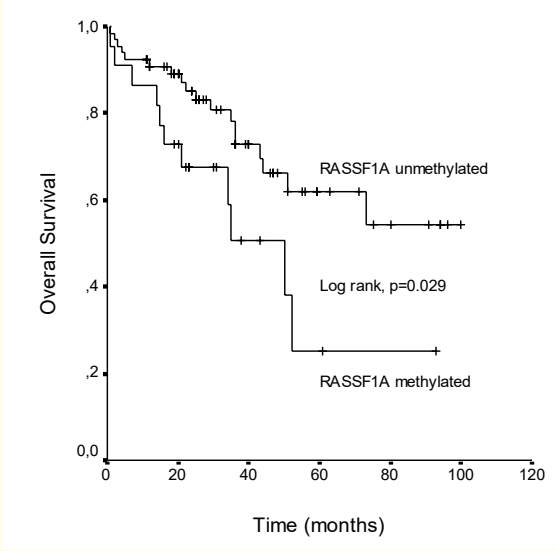

Figure 2b

Figure 2a and 2b: *Kaplan Meier estimate of overall survival (OS) for patients with early operable CRC with or without APC (a) and RASSF1A (b) promoter methylation.*

Correlation of *APC* and *RASSF1A* methylation status with different tumor parameters and survival in patients with metastatic CRC

APC was methylated in 36 (40.9%) of the 88 patients with metastatic CRC. Higher frequency of methylated *APC* promoter status was associated with methylated *RASSF1A* status (OR = 3.42; 95% CI: 1.23 - 9.49, p = 0.016) and male gender (OR = 2.43; 95% CI: 0.9 - 6.54, p = 0.076).

RASSF1A was methylated in 30 (34.1%) of the 88 patients with metastatic CRC patients. Methylated *RASSF1A* promoter status was significantly associated high CEA levels (OR = 4.21; 95% CI: 1.16-15.23, p = 0.023) and *APC* levels (OR = 3.21; 95% CI: 1.18 - 12.23, p = 0.016). Table 4 summarises correlations between *RASSF1A/APC* methylation status with different tumor characteristics.

Patient's 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		0.427		0.921
≤ 70 years	21 (50.0)		19 (45.2)	
> 70 years	15 (60.0)		11 (44.0)	
Dukes		-		-
A + B	-		-	
C	-		-	
D	36 (100.0)		30 (100.0)	
Differentiation		0.388		0.032
Well	10 (55.6)		12 (66.7)	
Median	17 (47.2)		11 (30.6)	
Poor	9 (69.2)		7 (53.8)	
Side		0.100		0.581
Right	20 (64.5)		15 (48.4)	
Left	16 (44.4)		15 (41.7)	
CEA levels (n = 56)		0.159		**0.023**
≤ 5 ng/mL	7 (41.2)		4 (23.5)	
> 5 ng/mL	24 (61.5)		22 (56.4)	
CA19.9 levels (n = 55)		0.883		0.898
Low (< 37 U/ml)	15 (53.6)		13 (46.4)	
High (> 37 U/ml)	15 (55.6)		13 (48.1)	
***APC* levels**				**0.016**
Unmethylated	-		9 (29.0)	
Methylated	-		21 (58.3)	
***RASSF1A* levels**		**0.016**		
Unmethylated	15 (40.5)		-	
Methylated	21 (70.0)		-	

***Table 4**: Association of APC and RASSF1A methylation status with demographic and clinicopathological characteristics of patients with metastatic colorectal cancer (CRC).*

The mean survival time (± SE) of patients with an unmethylated *APC* promoter status was 37 ± 7 months (range: 23 - 50 mo) which was substantially longer to the mean survival (± SE) of 15 ± 3 months (range: 9 - 20 mo) observed in those with a methylated *APC* promoter status (Log Rank test, $p < 0.001$) (Figure 3a).

The mean survival time (± SE) of patients with an unmethylated *RASSF1A* promoter status was 28 ± 4 (range: 19 - 36 mo) which was substantially longer to the mean survival (± SE) of 16 ± 3 (range: 9 - 22 mo) observed in those with a methylated *RASSF1A* promoter status (Log Rank test, $p < 0.001$) (Figure 3b).

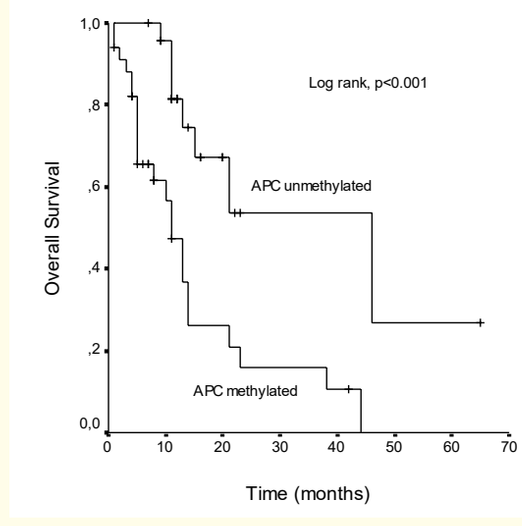

Figure 3a

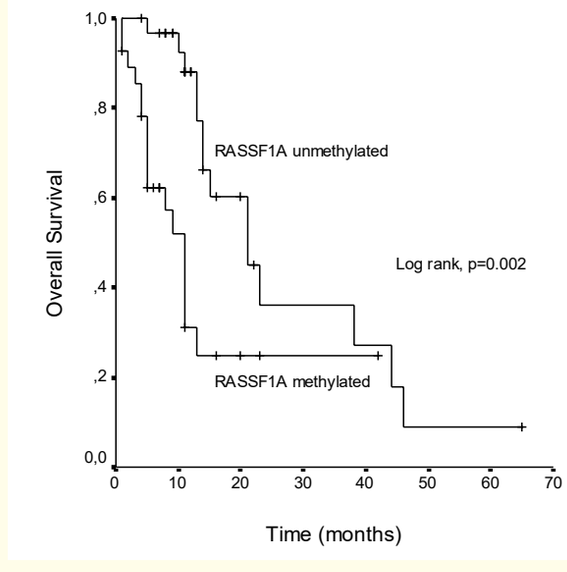

Figure 3b

Figure 3a and 3b: *Kaplan Meier estimate of overall survival (OS) for patients with metastatic CRC with or without APC (a) and RASSF1A (b) promoter methylation.*

Discussion

Gastrointestinal cancers (GICs), mainly including gastric and colorectal cancer, remain a global burden in world health. It is well established that GICs represent 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. Promoter methylation of cancer-related genes is an important pathway in gastric and colorectal carcinogenesis, with numerous factors involved in this process. Indeed, the inactivation of many tumor suppressor genes involved in gastrointestinal carcinogenesis, 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 our recent work we explored the promoter methylation status of *APC* and *RASSF1A* genes in cell free DNA from 73 patients with early operable gastric cancer as well as 67 patients with early operable and 88 patients with metastatic CRC 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 and in colorectal cancer. *RASSF1A* protein is actively involved in microtubule regulation, genomic stability maintenance, cell-cycle regulation, apoptosis modulation, cell motility and invasion control [42-44]. Its methylation frequency has been reported to vary within 30% - 50% in gastric cancer [26,45] and 20% - 45% in colorectal cancer [46,47]. 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 [48]. Germline mutations in the tumour suppressor *APC* cause FAP, and somatic mutations are common in sporadic CRCs. Hypermethylation of *APC* promoter has been reported in early steps of carcinogenesis in several tumours [49].

In gastric cancer we detected *RASSF1A* promoter methylation in 68.5% of the 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. It seems, that 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 [31]. Regarding *APC*, this was found to be methylated in 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 don't 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 remarkably 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 [50]. 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.

Similarly, we found that methylated *RASSF1A* was associated with higher disease stages in patients with early operable CRC, while in patients with metastatic disease methylated *RASSF1A* was significantly associated with high CEA levels and *APC* hypermethylated promoter status. Interestingly, in other studies *RASSF1A* methylation levels were significantly higher in the distal than the proximal CRCs [51,52] as well as in the normal-appearing mucosae, something which is also in accordance to the recent characterization of CRC cancers as left sided and right sided tumors with each side having different prognosis and different response to treatment [52]. Our data did not

support a difference in the profile of methylation between right (cecum, ascending colon and transverse colon) and left colon (descending colon, sigmoid colon and rectum). Finally, a significant difference was observed in the survival of patients with unmethylated *RASSF1A* promoter status compared with those with methylated *RASSF1A*. This was seen for patients with either early or metastatic disease. What was also notable in our results is that the negative impact of methylated *RASSF1A* promoter status on patients' survival was more pronounced in patients with metastatic disease. Despite many efforts, as the above mentioned reports, additional studies are required for a better characterization of the subsets of patients with *RASSF1A* promoter methylation and subsequently a better understanding of the role of *RASSF1A* during CRC development.

In patients with early operable CRC methylated *APC* promoter status was associated with ages older than 70 years and methylated *RASSF1A* status, while a tendency was found with high CEA levels and high CA19.9 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 survival compared to those with a methylated *APC* promoter status both in early and in metastatic setting. However, a significant and unexpected finding of our study was that the negative impact of methylated *APC* promoter status on patients' survival was more pronounced in earlier disease stages than in patients with metastases. This finding requires confirmation in further studies to establish *APC* methylation status as a prognostic marker with an additional value in early disease stages. The similar incidence of *APC* and *RASSF1A* promoter methylation in early and metastatic CRC possibly indicates that *APC* and *RASSF1A* methylation is a rather frequent event occurring independently of disease stage. *RASSF1A* methylation correlates with bad prognosis in both early and advanced disease and thus the association with poor survival seen in our study.

Conclusion

In conclusion, we found that *RASSF1A* and *APC* promoter methylation detected in cfDNA is a frequent epigenetic event in both operable gastric cancer as well as in early and metastatic CRC. *RASSF1A* methylation and subsequent inactivation associates with bad prognostic features, such as lymph node positivity in gastric cancer as well as poor survival in CRC. *APC* methylation was associated with a significantly poorer outcome in both early gastric cancer as well as early and metastatic CRC. Additional studies, in a larger cohort of patients are required to further explore whether this findings could establish methylation status of *APC* and *RASSF1A* in cell free DNA as potent biomarkers for early detection and prognosis in GIC's patients.

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