Am J Cancer Res 2016;6(9):2098-2108
www.ajcr.us /ISSN:2156-6976/ajcr0035823

Original Article

Methylation of serum *SST* gene is an independent prognostic marker in colorectal cancer

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Received July 14, 2016; Accepted July 22, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: There is an increasing demand for accurate prognostication for colorectal cancer (CRC). This study sought to assess prognostic potentials of methylation targets in the serum of CRC patients. A total of 165 CRC patients were enrolled in this prospective study. Promoter methylation levels of seven genes in pre-operative sera and matched tumor tissues were evaluated by quantitative methylation-specific PCR. Kaplan-Meier test, and univariate and multivariate Cox proportional hazards regression models were used for survival analyses. After a median follow-up of 56 months, 43 patients (28.7%) experienced tumor recurrence. In univariate survival analyses, serum methylation levels of *SST* and *MAL* were significantly predictive of cancer-specific death ($P<0.005$ for both). The former was also a significant predictor for tumor recurrence ($P=0.007$). Independent prognostic effects of serum methylation levels of *SST* were revealed by multivariate Cox regression model ($P=0.031$ and $P=0.003$ for cancer death and recurrence, respectively). When focusing on stage II and III patients, prognostication with serum methylated *SST* remained significant. Methylated *SST* detected in all serum samples can be traced back to the matched primary tumor tissues. We believe that methylated *SST* detected in the pre-operative sera of CRC patients appear to be a novel promising prognostic marker and probably can be auxiliary to tumor staging system and serum carcinoembryonic antigen towards better risk stratification.

Keywords: Blood biomarker, cell free DNA, DNA methylation, *SST*, colorectal cancer, prognosis, disease-free survival

Introduction

The utility of staging systems in cancer treatment allows rationalisation of adjuvant treatments, stratifies risk of recurrence and provides accurate prognostications. Pathologic staging of colorectal cancer (CRC) is currently performed according to American Joint Committee on Cancer (AJCC) Staging Manual, 7th edition [1] with review of the resected specimen and investigations of distant metastases. In stage III CRC, routine adjuvant treatment after surgical resection is suggested. In Stage II CRC, AJCC TNM staging currently lacks sensitivity to discriminate high- from low-risk patients and recurrence rates occur around 20-30% [2, 3]. Therefore, patients with clinicopathological features such as clinical bowel obstruction or perforation, histopathological features of lymphovascular or perineural invasion, fewer than 12 lymph nodes harvested during surgery as well as close, indeterminate or positive margins have been classified as high-risk stage II patients [4], and may require adjuvant chemotherapy. However, recurrence is also observed in stage II patients with low-risk features [5]. This thus emphasises the need to develop a more reliable prognostication algorithm.

Carcinoembryonic antigen (CEA) is currently the only blood marker recommended for CRC surveillance and prognostication in established guidelines [6]. CEA however has poor specificity or sensitivity, and normal pre-operative CEA values can be found in almost 50% of CRC patients. Many other molecular classifiers have been suggested. These include microsatellite instability, chromosomal instability, mutations of cancer driver genes like *KRAS*, *BRAF*, *TP53*, *PIK3CA*, certain proteins, miRNAs and gene expression signatures [7-10]. To date, none of these have been recommended for routine clinical use [4].

Epigenetic silencing of tumor-related genes by promoter hypermethylation is a common event in various cancers including CRC. Tumor-specific methylated DNA is detectable in the bloodstream of CRC patients. A small number of circulating methylated genes appear to have diagnostic potential for CRC [11], but far fewer exhibit convincing prognostic relevance [12, 13]. There are conflicting reports on circulating methylated p16 [12, 14]. Serum methylated helicase-like transcription factor (*HLTF*) was initially suggestive of CRC recurrence [13, 15], but a validation study by same authors failed to replicate such prognostic significance [16]. In our previous pilot study on 37 CRC and 20 healthy donors, 12 genes were selected for investigation in serum samples following comprehensive literature search. Seven out of 12 genes investigated [*MAL*, septin 9 (*SEPT9*), tachykinin-1 (*TAC1*), nel-like type 1 (*NELL1*), cellular retinoic acid binding protein 1 (*CRABP1*), somatostatin (*SST*) and eyes absent homolog 4 (*EYA4*)] seemed to be more methylated in CRC patients as compared to healthy controls [17]. This was based on differential methylation frequencies or magnitude between case and control groups. The aim of this prospective study is to explore prognostic potential of these methylated genes present in serum via an independent cohort.

Materials and methods

Patients and sample collection

This prospective study included 165 consecutive patients with sporadic CRCs who underwent elective curative surgical resection in a single institution (Singapore General Hospital) between Oct 2003 and June 2005. Curative surgical treatment was defined as absence of gross residual tumor after resection and negative margins confirmed pathologically. Patients who underwent neoadjuvant chemotherapy or radiotherapy, with inflammatory bowel diseases or family history suggestive of Lynch syndrome or familial adenomatous polyposis were excluded. Written informed consent was obtained from all study participants and the study was approved by the Institutional Review Board of the Singapore General Hospital.

Post-operative CRC surveillance was via an established protocol. Local recurrence was defined as the first clinical, radiological and/or pathologically evident tumor of the same histological type at or in the region of anastomosis. Distant recurrence was defined as clinical or radiological evidence of systematic spread outsider the primary tumor basin. Mortality dates and causes of death were obtained from the Singapore Cancer Registry.

Sample processing and DNA isolation

Peripheral blood was obtained within one week before surgery. Fresh tumor tissues were snap-frozen in liquid nitrogen immediately upon surgical removal and stored at -80°C. All tumor specimens were carefully microdissected with tumor cells of at least 85% achieved. Tissue processing and DNA isolation were carried out as previously described [17].

Methylation analysis

DNA was converted by bisulfate and subjected to fluorescence-based quantitative PCR (qPCR) as previously described [17]. qPCR on seven target genes and one control gene b-actin (*ACTB*) was carried out in a 7500 Sequencing Detection System (Applied Biosystems, Foster, CA). Each reaction was run in duplicates or triplicates. Every plate also included a positive control and a no template control. Quantities of genes of interest were normalized by dividing the gene/*ACTB* ratio of a sample by the gene/*ACTB* ratio of a positive control and multiplying by 1000. Normalized methylation value (NMV) was used as a measure representing the relative level of methylation in a particular sample.

Serum CEA measurement

Serum CEA was determined by a micro-particle enzyme immunoassay on the Abbott Axsym analyzer according to manufacturer's instructions (Abbott laboratories, Abbott Park, Illinois). Cutoff value was set to 3.5 ng/mL following the clinical setting at the hospital.

Statistical analysis

Serum methylation levels of seven genes and CEA were examined as both continuous and dichotomous variables. Disease-free survival (DFS) time and cancer-specific survival (CSS) time were calculated from date of surgery, to presentation of disease recurrence or death from disease, or to last follow-up before 31 Jan

Table 1. Clinicopathological characteristics of 165 colorectal cancer patients

Parameters		*N* (%)
Age at diagnosis	Median, 67 years	165
Gender	Male	91 (55.2)
	Female	74 (44.8)
TNM Staging (AJCC 5th edition)#	I	26 (15.8)
	II	62 (37.6)
	III	62 (37.6)
	IV	15 (9.1)
Depth of tumor invasion	T_1	7 (4.2)
	T_2	26 (15.8)
	T_3	115 (69.7)
	T_4	17 (10.3)
LN Status	No LN involved	92 (55.8)
	1-3 LN involved	39 (23.6)
	≥4 LN involved	34 (20.6)
Metastatic status	No	150 (90.9)
	Yes	15 (9.1)
Tumor differentiation	Well	26 (15.8
	Moderate	126 (76.4)
	Poor	13 (7.9)
Histological type	Adenocarcinoma	160 (97.0)
	Mucinous	5 (3.0)
Perineural invasion	No	140 (84.8)
	Yes	25 (15.2)
Lymphovascular invasion	No	1337 (80.6)
	Yes	32 (19.4)
No. of LN assessed	Median, 14.0	
	LN<12	54 (32.7)
	LN≥12	111 (67.3)
Tumor site†	Right colon	22 (13.3)
	Left colon	77 (46.7)
	Rectum	66 (40.0)
Pre-operative serum CEA	≤3.5 ng/mL	67 (40.6))
	>3.5 ng/mL	97 (58.8)
	Not tested	1 (0.6)
Recurrent site	LocalReginal	8 (4.8)
	Distant	35 (21.2)
Adjuvant therapy	No	112 (67.9)
	Yes	53 (32.1)

#, TNM stage is based on American Joint Commission on Cancer guidelines, 5th edition. †Right colon includes caecum through transverse colon, while left colon includes descending colon and sigmoid colon. Abbreviation: LN, lymph node.

2010. Associations of serum methylation levels of studied genes and other clinicopathological factors with CSS or DFS were assessed by univariate Cox proportional hazards regression model. Multivariate Cox regression model was then employed to estimate the independent prognostic effect of methylated genes, adjusting for other significant factors revealed in the univariate analyses. CSS or DFS survival curves based on serum methylation levels of *SST* gene were plotted by Kaplan-Meier method and compared using the log-rank test. Association between *SST* methylation levels in sera and tumors was evaluated by Spearman's correlation test. *SST* methylation magnitude in sera and matched tumors was compared via Wilcoxon rank sum test. Statistical analysis was performed using the Statistical Package for Social Sciences version 21.0 (SPSSÒ Inc, Chicago, Illinois). All statistical tests were two-sided and *P* values less than 0.05 were considered statistically significant.

Results

Clinicopathological characteristics

Clinicopathological characteristics were obtained from a prospectively maintained computerized database and are summarized in **Table 1**. Of 165 patients studied, median age at diagnosis was 67 years (range, 33-91 years). After a median follow-up of 56 months (range, 5-79 months), 58 patients had died and of which 54 (93.1%) deaths were from disease progression. Out of 150 cases with stage I-III tumors, 43 cases (28.7%) developed either local or distant recurrence.

Analysis of cancer-specific survival stratified by serum methylation levels

None of the seven serum methylation markers were normally distributed. The median serum NMV was 1.487 (range, 0-1.250) for *SST*, 1.419 (range, 0-40.303) for *MAL*, 1.230 (range, 0-2.610) for *TAC1*, 0.026 (range, 0-1.396) for *SEPT9*, 0.832 (range, 0-4.283) for *EYA4*, 0.002 (range, 0-5.316) for *CRABP1*, and 0 (range, 0-4.364) for *NELL1*. Seven methylation markers were thus dichotomized by the median values of individual genes, while the dichotomy of serum CEA

Table 2. Univariate and multivariate Cox analysis for cancer-specific survival (CSS) in 165 colorectal cancer patients

Univariate Cox					Multivariate Cox (n=163)	
Factor	No. of patients	No. of death#	HR (95% CI)†	*P*-value	Adjusted HR‡ (95% CI)	*P*-value
Age at diagnosis, continuous	165	54	1.03 (1.01, 1.06)	0.013	1.03 (1.01, 1.06)	0.009
Sex				0.421		
Male	91	28	1.0			
Female	74	26	1.25 (0.73, 2.13)			
TNM stage (AJCC 5th version)				<0.001		<0.001
I	26	4	1.0		1.0	
II	62	14	2.01 (0.65, 6.24)		2.19 (0.68, 7.10)	
III	62	22	3.70 (1.24, 10.98)		3.59 (1.17, 10.99)	
IV	15	14	32.68 (10.06, 106.19)		28.20 (8.15, 97.66)	
Differentiation				0.014		0.465
Well	26	8	1.0			
Moderate	126	37	1.02 (0.48, 2.20)			
Poor	13	9	3.50 (1.34, 9.12)			
Histological type				0.277		
Adenocarcinoma	160	51	1.0			
Mucinous	5	3	1.91 (0.59, 6.14)			
Tumor site				0.185		
Right colon	22	11	1.0			
Left colon	77	23	0.54 (0.26, 1.11)			
Rectum	66	20	0.53 (0.25, 1.12)			
Perineural invasion				<0.001		0.336
No	140	40	1.0			
Yes	25	14	3.07 (1.66, 5.68)			
Lymphovascular invasion				<0.001		0.004
No	133	36	1.0		1.0	
Yes	32	18	3.06 (1.73, 5.41)		2.53 (1.35, 4.72)	
No. of LN assessed						
LNs<12	54	21	1.39 (0.81, 2.41)	0.235		
LNs≥12	111	33	1.0			
Serum CEA				0.011		0.412
≤3.5 ng/mL	67	15	1.0			
>3.5 ng/mL	97	38	2.18 (1.20, 3.97)			
Serum methylation of *NELL1*§				0.543		
Low	110	34	1.0			
High	55	20	1.19 (0.68, 2.07)			
Serum methylation of *SEPT9*§				0.950		
Low	83	27	1.0			
High	82	27	1.02 (0.59, 1.74)			
Serum methylation of *EYA4*§				0.424		
Low	83	25	1.0			
High	82	29	1.24 (0.73, 2.12)			
Serum methylation of *CRABP1*§				0.730		
Low	83	26	1.0			
High	82	28	1.10 (0.64, 1.87)			
Serum methylation of *TAC1*§				0.612		

Low	83	25	1.0		
High	82	29	1.15 (0.67, 1.97)		
Serum methylation of *MAL*§				0.004	0.169
Low	83	19	1.0		
High	82	35	2.26 (1.29, 3.96)		
Serum methylation of *SST*§				0.003	0.031
Low	83	17	1.0	1.0	
High	82	37	2.40 (1.35, 4.28)	1.96 (1.06, 3.62)	

#, Cancer-specific death. †, Hazard ratio (95% confidence interval). ‡, Adjusted hazard ratio from multivariate regression analysis by adjusting for other significant factors revealed in univariate analysis. §, Methylation levels are dichotomized by median levels of corresponding genes. Many cases had extremely low levels of *NELL1* in serum and thus tied. Abbreviation: LN, Lymph node.

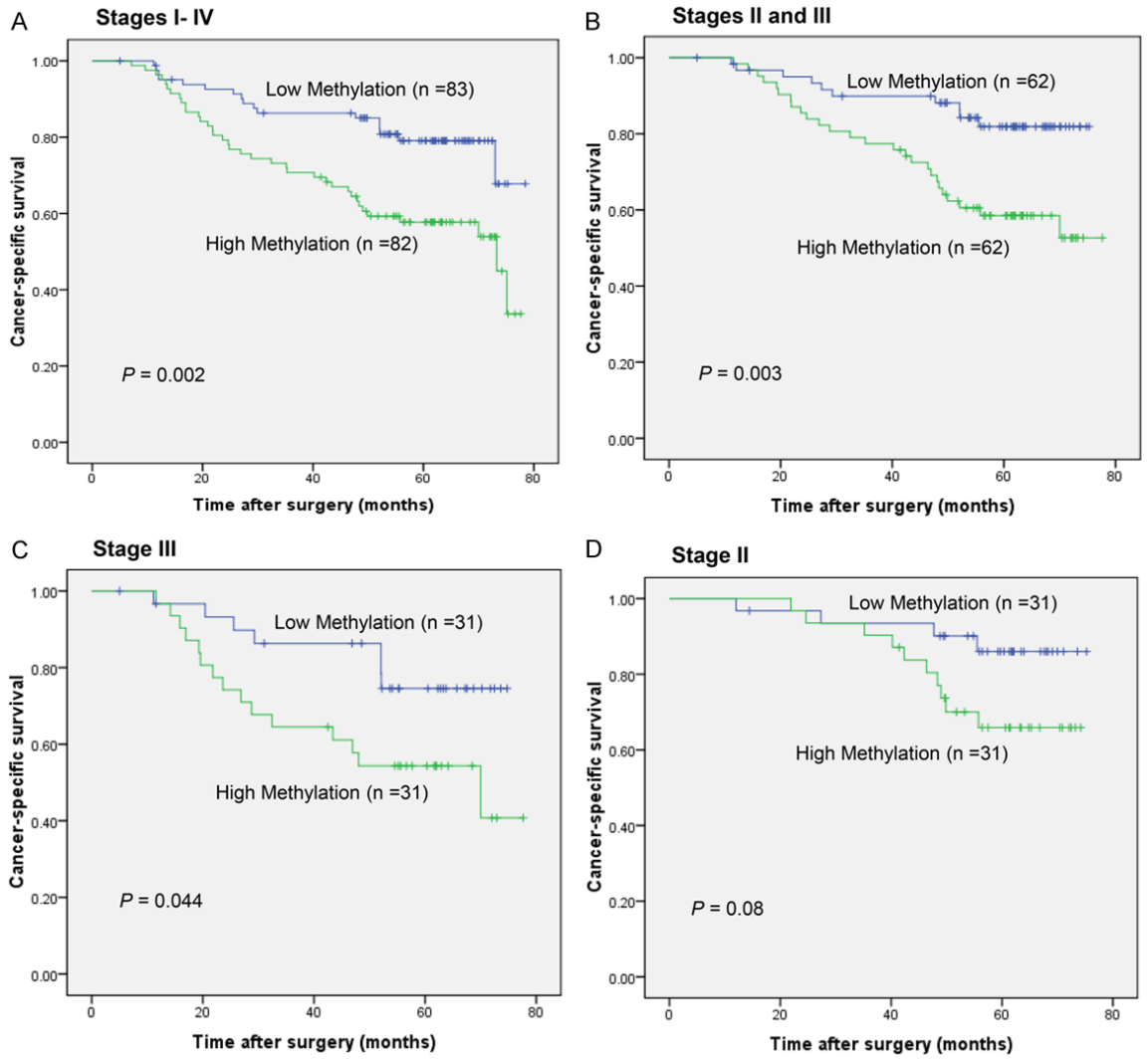

Figure 1. Kaplan-Meire estimations of cancer-specific survival according to serum methylation levels of *SST* in colorectal cancer patients of (A) Stages I-IV; (B) Stages II and III; (C) Stage III alone; and (D) Stage II alone. Serum *SST* methylation levels were dichotomized by medians of respective entities.

was generated based on the reference cutoff value of 3.5 ng/mL. In univariate analysis, cancer-specific survival was significantly influenced not only by traditional clinicopathological

Table 3. Univariate and multivariate Cox analysis for disease free survival (DFS) in 150 stage I-III colorectal cancer patients

Univariate Cox					Multivariate Cox (n=149)	
Factor	No. of patients	No. of Recurrence	HR† (95% CI)	*P* value	Adjusted HR (95% CI)‡	*P* value
Age at diagnosis, continuous	150	43	1.02 (0.99, 1.05)	.138		
Sex				.669		
Male	85	26	1.0			
Female	65	17	0.875 (0.48, 1.61)			
TNM stage (AJCC 5th version)				.005		0.280
I	26	3	1.0			
II	62	15	2.43 (0.70, 8.40)			
III	62	25	4.73 (1.43, 15.69)			
Differentiation				.098		
Well	25	6	1.0			
Moderate	114	31	1.21 (0.51, 2.90)			
Poor	11	6	3.37 (1.08, 10.49)			
Histological type				.529		
Adenocarcinoma	145	41	1.0			
Mucinous	5	2	1.58 (0.38, 6.52)			
Tumor site				.982		
Right colon	19	5	1.0			
Left colon	71	20	1.01 (0.38, 2.68)			
Rectum	60	18	1.07 (0.40, 2.87)			
Perineural invasion				.002		.039
No	131	33	1.0		1.0	
Yes	19	10	3.05 (1.50, 6.21)		2.27 (1.04, 4.93)	
Lymphovascular invasion				.001		.009
No	125	30	1.0		1.0	
Yes	25	13	2.93 (1.52, 5.62)		2.58 (1.27, 5.25)	
Number of LN assessed						
LNs<12	47	17	1.47 (0.79, 2.71)	0.218		
LNs≥12	103	26	1.0			
Serum CEA				.016		.016
≤3.5 ng/mL	66	13	1.0		1.0	
>3.5 ng/mL	83	30	2.23 (1.16, 4.28)		2.24 (1.16, 4.32)	
Serum methylation of *NELL1*§				.362		
Low	103	32	1.0			
High	47	11	0.73 (0.37, 1.44)			
Serum methylation of *SEPT9*§				.384		
Low	75	24	1.0			
High	75	19	0.77 (0.42, 1.40)			
Serum methylation of *EYA4*§				.592		
Low	75	20	1.0			
High	75	23	1.18 (0.65, 2.15)			
Serum methylation of *CRABP1*§				.647		
Low	75	20	1.0			
High	75	23	1.15 (0.63, 2.10)			
Serum methylation of *TAC1*§				.889		
Low	75	22	1.0			

High	75	21	0.96 (0.53, 1.74)		
Serum methylation of *MAL*§			.650		
Low	75	21	1.0		
High	75	22	1.15 (0.63, 2.09)		
Serum methylation of *SST*§			.007		.003
Low	75	14	1.0	1.0	
High	75	29	2.40 (1.27, 4.55)	2.60 (1.37, 4.94)	

†, Hazard ratio (95% confidence interval). ‡, Adjusted hazard ratio from multivariate regression analysis by adjusting for other significant factors revealed in univariate analysis. §, Methylation levels are dichotomized by median levels of corresponding genes. Many cases had extremely low levels of *NELL1* in serum and thus tied. Abbreviation: LN, Lymph node.

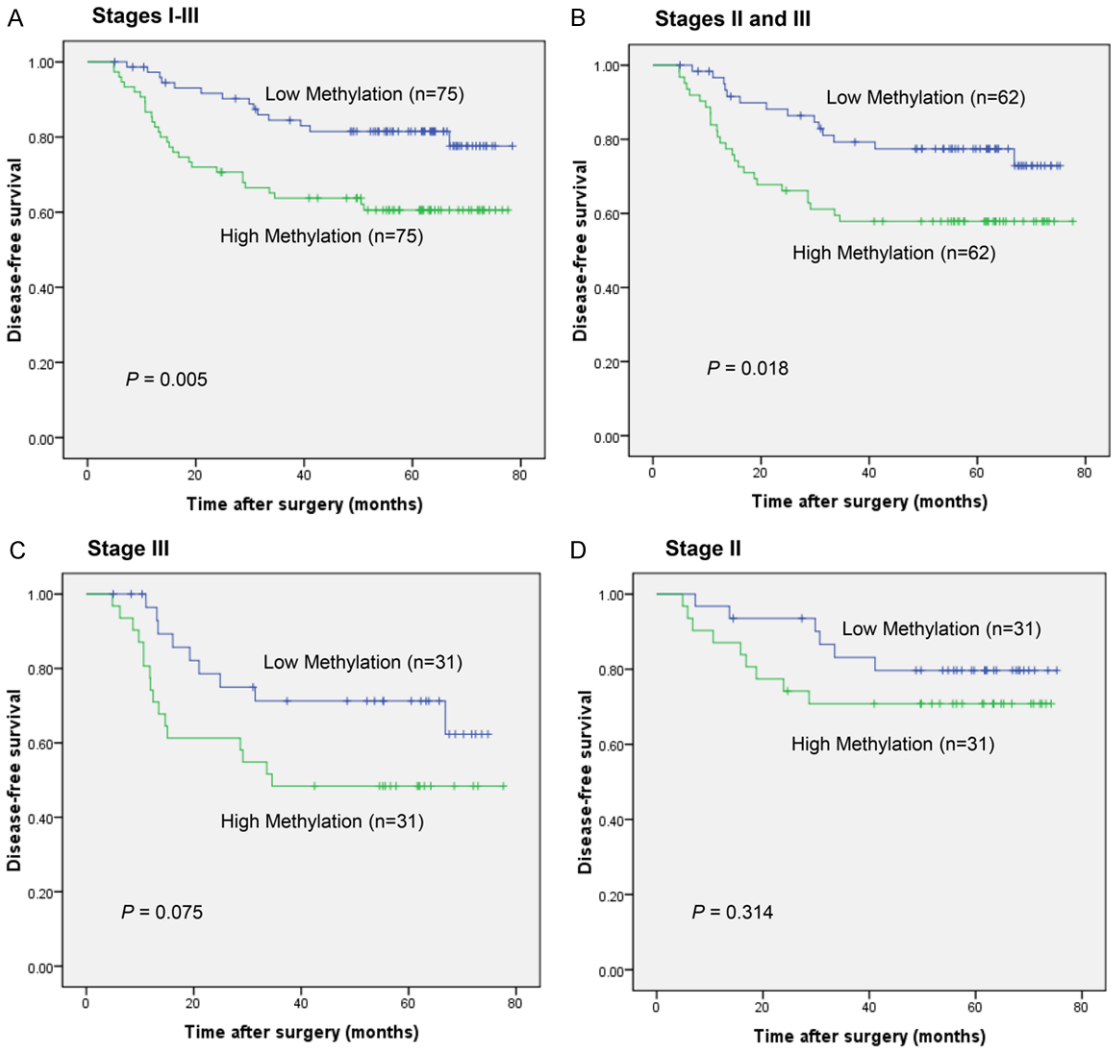

Figure 2. Kaplan-Meire estimations of disease-free survival according to serum methylation levels of *SST* in colorectal cancer patients with (A) Stages I-III; (B) Stages II and III; (C) Stage III alone, and (D) Stage II alone. Serum *SST* methylation levels were dichotomized by medians of respective entities.

parameters and serum CEA, but also by serum methylation levels of *MAL* and *SST* ($P<0.05$ for all parameters, **Table 2** and **Figure 1A**). In multivariate Cox analysis adjusting for all other significant factors, serum methylated *SST* (m*SST*) remained as a significant and independent predictor for poor CSS (**Table 2**). Patients with high serum m*SST* were noted to have a higher risk

for cancer-specific death [hazard ratio (HR)= 1.96, 95% CI: 1.06-3.62, *P*=0.031].

CSS was also assessed based on tumor stages. In the combined group (n=124) of stages II and III, cancer-specific death rate was 41.9% in the high serum m*SST* group, whilst it was 16.1% in the low m*SST* group. Kaplan-Meire curves based on serum m*SST* levels clearly separated two groups of patients exhibiting different survival (*P*=0.003, **Figure 1B**). In multivariate Cox analysis, serum m*SST* remained as a significant independent predictor for CSS (HR=2.797, 95% CI, 1.34-5.84, *P*=0.006). However, when each stage was individually analyzed, independent predictive significance was maintained only in the stage III subgroup (HR=2.52, 95% CI, 1.02-6.25; *P*=0.045 by multivariate Cox analysis; Kaplan-Meire curves were displayed in **Figure 1C**). Prognostication in the stage II subgroup was of borderline significance (*P*=0.075-0.080 in Kaplan-Meire test and multivariate Cox analysis, **Figure 1D**).

Analysis of disease-free survival stratified by serum methylation levels

Similar to the influence to CSS, traditional clinicopatholgocial factors including TNM stage, lymphovascular invasion, perineural invasion, and serum CEA were individually associated with risk of tumor recurrence (*P*<0.05 for all parameters, **Table 3**). Serum methylated *SST* was also significantly predictive of tumor recurrence (**Table 3**). The incidence of recurrence was significantly higher in the high m*SST* group than in the low m*SST* group (38.7% vs. 18.7%, *P*=0.005). The disease-free survival was also significantly shorter in the former group (**Figure 2A**). In multivariate analysis, it was noted that patients with high serum m*SST* had a higher risk for recurrence (HR=2.60, 95% CI: 1.37-4.94, *P*=0.003, **Table 3**).

DFS was similarly analyzed in subset groups. In combined group of stages II and III, serum m*SST* clearly separated two groups of patients with different DFS (*P*=0.018, **Figure 2B**). It remained as an independent recurrence indicator as revealed in multivariate Cox analysis (HR=2.15, 95% CI, 1.12-4.12; *P*=0.022). The same trends were observed in individual subsets of stage III or stage II alone, but statistical significance was no longer retained (**Figure 2C** and **2D**, respectively).

SST methylation levels in tumors and their association with serum methylation levels and patients' prognosis

Methylation levels of *SST* in sera and matched tumor tissues were subsequently compared in 162 patients with paired samples available. Methylation of *SST* (NMV>0) was noted in all tumor samples. Methylation magnitude in tumors was significantly higher as compared to that in sera (median levels in tumors and sera, NMV of 417.693 and 1.487, respectively, *P*<0.001). Particularly with diagnostic relevance, methylated *SST* in sera (n=154) could always be traced back to the corresponding tumors that were *SST* methylated. Notably, there was however no correlation that could be established between methylation levels in tumor tissues and sera (Spearman's correlation coefficient =0.103, *P*=0.192). *SST* methylation levels in tumors were also not associated with tumor recurrence nor cancer-specific survival (*P*>0.05 in both analyses, data not shown).

Discussion

It is evident that current clinical criteria are suboptimal to accurately estimate patient prognosis and outcomes. This report describes for the first time, the prognostic potential of serum methylated *SST* in CRC. The significant and independent prognostic effects of serum methylated *SST* have also never been reported in other cancers [18]. This novel prognostic factor may provide additional information beyond the use of traditional serum CEA, high-risk clinicopathological features (e.g., lymphovascular or perineural invasion) or well-utilised staging criteria. In this prospectively-designed study, we have noted that high methylation levels of *SST* in sera, rather than in tumors, were associated with high risk of tumor recurrence and unfavorable outcomes. Although the mechanisms leading to the presence of cell-free tumor-specific DNA in the circulation of cancer patients are not fully understood, it is postulated that this may have arose from lysis of circulating cancer cells, or by DNA leakage from tumor necrosis or apoptosis [19]. It may thus be hypothesized that differential *SST* methylation levels displayed in our series of sera may reflect a more aggressive biology of the primary tumor, or the presence of systemic micrometastasis that are yet to be readily detected by current technologies in clinical settings. The utility of

serum *SST* methylation assay may thus be an additional adjunct to predict poorer outcome and higher incidence of tumor recurrence.

In our study, serum *SST* methylation levels were clearly significant independent predictors for overall CSS and DFS (**Figures 1A**, **1B**, **2A** and **2B**). When individual stages were analysed, high serum methylation of *SST* was the only significant predictor for cancer-specific death in stage III. The same trends were observed for CSS and DFS in the stage II subgroup, but statistical significance was not achieved. One possible explanation is that in our study cohort, the majority of Stage II patients were stage IIA (93.5%, n=58), while stage IIB and IIC accounted for the remaining four cases (6.5%). With a predominance of stage IIA cases, there are possibly insufficient events of recurrence or cancer-specific death to provide for a statistical difference thus leading to a type II error. The full prognostic potential of serum SST methylation in stage II disease will require evaluation in futures studies of larger cohorts with more stage IIB and IIC cases.

There are several clinical applications that can be suggested with our findings. Firstly, methylated *SST* may help to identify patients who may need intensive surveillance after surgery. Despite recommended adjuvant therapy for stage III patients [4], recurrence rates still exceed 30-40%. Recurrence rates are high at 20-30% for stage II cancers as well [3]. One of the main problems is that surveillance protocols in various countries after curative surgery, require a prudent balance in assessing cost-conscious programmes against the chance of cancer recurrence. The limitations thus prevent standardisation and there is lack of consensus of the type of imaging, frequency of imaging as well as colonoscopy surveillance which are often left to the individual surgeon or oncologist. These surveillance regimes are also universally applied across all stages of disease and may limit early detection of recurrences. It would therefore be extremely useful if there were suitable indicators to highlight a higher risk of recurrence and suggest a more intensive follow-up. The surveillance protocols for stage II are even more unclear as they are still classified as “early cancers”. In our institution, we have reported a recurrence rate of 18.1% in stage II patients [20] and biomarkers such as serum m*SST* may be helpful by providing an indication of the need for aggressive surveillance. Secondly, serum m*SST* may help to select stage II patients who may benefit from adjuvant therapy. One factor that may account for the lack of meaningful benefit of adjuvant therapy for stage II patients is the inability of the current prognostication system to identify those who are truly high risk. Therefore, the incorporation of serum *SST* methylation into existing standard prognostication modules may allow better risk stratification, with possible improvement in survival.

Functional annotations have suggested that *SST*, encoding a well-characterized gastrointestinal neuroendocrine and growth-regulatory peptide, acts as a tumor suppressor gene and possesses potent antitumor abilities. It exerts antitumor effects by multiple mechanisms. Indirect effects on cell growth include suppressing the synthesis and/or secretion of growth factors (e.g. *IGF-1*) and growth-promoting hormones as well as inhibiting neoplastic angiogenesis that is essential for tumor growth and spread [21]. Direct antitumor effects include causing cell cycle arrest; controlling cell proliferation; inducing apoptosis; and inhibiting cell invasion [21]. Clinical studies also provide corroborative evidences with reduced expression of SST protein and mRNA in colorectal tumor tissues [22]. Epigenetic involvement of *SST* gene silencing in CRC was initially suggested by observations that *SST* methylation levels in tumor tissues were significantly higher than that in adjacent normal mucosa of CRC patients or normal mucosa from healthy controls. This was further strengthened by the results that administration of demethylation agents into *SST* methylated CRC cell lines triggered up-regulation of *SST* expression [23]. The *SST* hypermethylation identified in 100% of tumors in our series may suggest that epigenetic silencing of *SST* gene is ubiquitously involved in CRC tumorigenesis. In fact, hypermethylation of *SST* gene has also been evinced in other human cancers like esophageal, gastric and renal cancers [24-26]. Although the purpose of this study is to identify novel molecular factors with prognostic potentials, regardless of their functions, and we did not explore the biological mechanisms of methylated *SST* in tumor development, future researches are warranted to better understand the underlying mechanisms that explain the association of serum m*SST* with tumor recurrence.

The repetitive independent influence of pre-operative serum (rather than tumor) m*SST* on DFS and CSS suggests potential possibility of use of serum m*SST* as a non-invasive prognostic biomarker for CRC. Furthermore, methylated *SST* gene is highly stable in the circulation. As there are limited loci to be assayed (compared with wide mutational spectra in frequently mutated genes such as *APC* and *TP53*), the analysis of this marker promises to be rapid and simple. It is also important to note that methylated *SST* detected in sera has a very high concordance with tumour methylation, suggesting high analytical specificity. All these features support the motivation towards developing a new blood-based prognostic assay.

We are aware of the limitations of this study. These include a relatively small sample size especially in stage II subgroup which might lead to underpower to detect more subtle differences in patients' outcomes. In addition, the study subjects in the present study are from a single population of Chinese origin. Utility of serum m*SST* as a prognostic biomarker in CRC must be validated in other ethnic populations as well.

In summary, advances in molecular biology provide opportunities to identify tumor-associated materials in blood which are associated with micrometastasis. We have identified pre-operative serum *SST* methylation as a novel promising prognostic marker. Its independent prognostic information could be complementary to standard prognostication modules, allowing better stratification of the patients who may receive tailored management for their disease and ultimately benefit with improved outcomes. Additional validation studies in large and independent cohorts are required to fully define the utility of this new marker and address whether modification of treatment/management decisions based on additional prognostic information from this methylation marker would yield improvement in DFS and CSS.

Acknowledgements

This work was supported by the National Medical Research Council of Singapore (No. NMRC/0697/2002). We thank Prof. Francis Seow-Choen for constructive suggestions at the initial stage of this study. We are also grateful to Ms. CTT Loi for accrual of clinical data.

Disclosure of conflict of interest

None.

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References

[1] Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A. AJCC Cancer Staging Manual, 7th Edition. New York (NY): Springer; 2010.

[2] Obrand DI and Gordon PH. Incidence and patterns of recurrence following curative resection for colorectal carcinoma. Dis Colon Rectum 1997; 40: 15-24.

[3] Uen YH, Lin SR, Wu DC, Su YC, Wu JY, Cheng TL, Chi CW and Wang JY. Prognostic significance of multiple molecular markers for patients with stage II colorectal cancer undergoing curative resection. Ann Surg 2007; 246: 1040-46.

[4] National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology, Colon Cancer, version 2.2014. Available at: http://www.nccn.org/professionals/physician_gls/f_guidelines.asp#site. Accessed, November 16, 2013.

[5] Sharif S and O'Connell MJ. Gene Signatures in Stage II Colon Cancer: A Clinical Review. Curr Colorectal Cancer Rep 2012; 8: 225-231.

[6] Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, Somerfield MR, Hayes DF, Bast RC Jr; ASCO. ASCO 2006 update of recommendations for the use of tumor markers in gastroenterology cancer. J Clin Oncol 2006; 24: 5313-27.

[7] Mouradov D, Domingo E, Gibbs P, Jorissen RN, Li S, Soo PY, Lipton L, Desai J, Danielsen HE, Oukrif D, Novelli M, Yau C, Holmes CC, Jones IT, McLaughlin S, Molloy P, Hawkins NJ, Ward R, Midgely R, Kerr D, Tomlinson IP and Sieber OM. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. Am J Gastroenterol 2013; 108: 1785-93.

[8] Toiyama Y, Miki C, Inoue Y, Okugawa Y, Tanaka K and Kusunoki M. Serum hepatocyte growth factor as a prognostic marker for stage II or III colorectal cancer patients. Int J Cancer 2009; 125: 1657-62.

[9] Stein U, Burock S, Herrmann P, Wendler I, Niederstrasser M, Wernecke KD and Schlag PM. Diagnostic and prognostic value of matastasis

inducer S100A4 transcripts in plasma of colon, rectal, and gastric cancer patients. J Mol Diagn 2011; 13: 189-198.
[10] Dong Y, Wu WK, Wu CW, Sung JJ, Yu J, Ng SS. MicroRNA dysregulation in colorectal cancer: a clinical perspective. Br J Cancer 2011; 104: 893-8.
[11] Herbst A and Kolligs FT. Detection of DNA hypermethylation in remote media of patients with colorectal cancer: new biomarkers for colorectal carcinoma. Tumor Biol 2012; 33: 297-305.
[12] Nakayama G, Hibi K, Kodera Y, Koike M, Fujiwara M and Nakao A. P16 methylation in serum as a potential marker for the malignancy of colorectal carcinoma. Anticancer Res 2007; 27: 3367-70.
[13] Wallner M, Herbst A, Behrens A, Crispin A, Stieber P, Göke B, Lamerz R and Kolligs FT. Methylation of serum DNA is an independent prognostic marker in colorectal cancer. Clin Cancer Res 2006; 12: 7347-52.
[14] Bazan V, Bruno L, Augello C, Agnese V, Calò V, Corsale S, Gargano G, Terrasi M, Schirò V, Di Fede G, Adamo V, Intrivici C, Crosta A, Rinaldi G, Latteri F, Dardanoni G, Grassi N, Valerio MR, Colucci G, Macaluso M, Russo A; Gruppo Oncologico dell'Italia Meridionale. Molecular detection of TP53, Ki-Ras and p16INK4A promoter methylation in plasma of patients with colorectal cancer and its association with prognosis. Results of a 3-year GOIM (GruppoOncologicodell'ItaliaMeridionale) prospective study. Ann Oncol 2006; 17 Suppl 7: vii84-90.
[15] Herbst A, Waller M, Rahmig K, Stieber P, Crispin A, Lamerz R and Kolligs FT. Methylation of helicase-like transcription factor in serum of patients with colorectal cancer is an independent predictor of disease recurrence. Eur J Gastroenterolhepatol 2009; 21: 565-9.
[16] Phillip AB, Stiber P, Nagel D, Neumann J, Spelsberg F, Jung A, Lamerz R, Herbst A and Kolligs FT. Prognostic role of methylated free circulating DNA in colorectal cancer. Int J Cancer 2012; 131: 2308-19.
[17] Liu Y, Tham CK, Ong SY, Ho KS, Lim JF, Chew MH, Lim CK, Zhao Y, Tang CL and Eu KW. Serum methylation levels of TAC1. SEPT9 and EYA4 as diagnostic markers for early colorectal cancers: a pilot study. Biomarkers 2013; 18: 399-405.
[18] Misawa K, Mochizuki D, Imai A, Endo S, Mima M, Misawa Y, Kanazawa T, Carey TE and Mineta H. Prognostic value of aberrant promoter hypermethylation of tumor-related genes in early-stage head and neck cancer. Oncotarget 2016; [Epub ahead of print].
[19] Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD and Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001; 61: 1659-65.
[20] Chew MH, Yeo SA and Tang CL. Prognostic Variables in 1814 Sporadic Colon Cancers: A Review of Experience from a Single Institution from 1999-2005. Proceedings of Singapore Health 2011; 20: 3-11
[21] Pyronnet S, Bousquet C, Najib S, Azar R, Laklai H and Susini C. Antitumor effects of somatostatin. Mol Cell Endocrinol 2008; 286: 230-7.
[22] Leiszter K, Sipos F, Galamb O, Krenács T, Veres G, Wichmann B, Fűri I, Kalmár A, Patai ÁV, Tóth K, Valcz G, Tulassay Z and Molnár B. Promoter hypermethylation-related reduced somatostatin production promotes uncontrolled cell proliferation in colorectal cancer. PLoS One 2015; 10: e0118332.
[23] Mori Y, Cai K, Cheng Y, Wang S, Paun B, Hamilton JP, Jin Z, Sato F, Berki AT, Kan T, Ito T, Mantzur C, Abraham JM and Meltzer SJ. A genome-wide search identifies epigenetic silencing of somatostatin, tachykinin-1, and 5 other genes in colon cancer. Gastroenterology 2006; 131: 797-808.
[24] Jin Z, Mori Y, Hamilton JP, Olaru A, Sato F, Yang J, Ito T, Kan T, Agarwal R and Meltzer SJ. Hypermethylation of the somatostatin promoter is a common, early event in human esophageal carcinogenesis. Cancer 2008; 112: 43-9.
[25] Jackson K, Soutto M, Peng D, Hu T, Marshal D and El-Rifai W. Epigenetic silencing of somatostatin in gastric cancer. Dig Dis Sci 2011; 56: 125-30.
[26] Shi X, Li X, Chen L and Wang C. Analysis of somatostatin receptors and somatostatin promoter methylation in human gastric cancer. Oncol Lett 2013; 6: 1794-8.