Original Article

Postoperative Serum Methylation Levels of *TAC1* and *SEPT9* Are Independent Predictors of Recurrence and Survival of Patients with Colorectal Cancer

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BACKGROUND: Serum carcinoembryonic antigen (CEA) is the only marker recommended for surveillance of colorectal cancer (CRC) recurrence; its sensitivity and specificity, however, are suboptimal. This study sought to evaluate the values of postoperative serum methylation levels of 7 genes for prognostication and especially for recurrence detection after curative resection. **METHODS:** This prospective cohort study included 150 patients with stage I-III CRC from whom 3 consecutive blood sampling was taken 1 week before, and 6 months and 1 year after operation. Methylation levels of 7 genes were evaluated via quantitative methylation-specific polymerase chain reaction. Serum CEA was measured in parallel. Univariate and multivariate survival analyses were followed by construction of receiver operating characteristic curves for recurrence detection. **RESULTS:** After a median follow-up of 59 months, 43 patients (28.7%) developed recurrent lesions. High serum methylation levels of *TAC1* in serum at 6-month follow-up (6M-FU), and *SEPT9* at 1-year follow-up (1Y-FU) were independent predictors for tumor recurrence and unfavorable cancer-specific survival (CSS) ($P < .05$ in all tests). Serum *NELL1* methylation levels were significant alone for CSS at both 6M-FU and 1Y-FU, but not for disease-free survival. Dynamic changes of *TAC1* and *SEPT9* with methylation increment were also independently predictive for recurrence ($P < .05$ in all tests). More importantly, *TAC1* at 6M-FU and *SEPT9* at 1Y-FU exhibited earlier detection of potential recurrences compared with concurrent serum CEA. **CONCLUSIONS:** Levels of *TAC1* and *SEPT9* methylation detected in postoperative sera of patients with CRC appear to be novel promising prognostic markers and may probably be considered for monitoring of CRC recurrence. ***Cancer* 2014;120:3131-41.** © *2014 American Cancer Society.*

KEYWORDS: blood markers, cancer recurrence, early diagnosis, epigenetics.

INTRODUCTION

Approximately 25% to 40% of patients who undergo curative resection of colorectal cancer (CRC) develop tumor recurrence with eventual demise.[1] Resection rates for recurrent lesions remain low (17.4%-54.8%) [2] although survival benefits have been described.[3,4] Resection rates may potentially be increased if recurrence can be diagnosed earlier, where lesions are small, localized, and clear resection margins are achievable. Alternatively, early institution of chemo or radiotherapy may maintain recurrent disease at small volumes with minimal or no symptoms.

Current modalities for postoperative CRC surveillance include thorax/abdominal/pelvic CT scans, PET scans, colonoscopies and serum carcinoembryonic antigen (CEA) measurement. CEA, the only blood marker recommended in established guidelines, however, has poor sensitivity or specificity.[5] Normal CEA values may be found in almost 50% of cancers before surgical resection and often do not rise during recurrences.[5] CEA elevation also has a slow lead time predating the clinically identifiable recurrence by approximately 5 months.[6] The other modalities have various limitations of costs, radiation exposure, and invasiveness with potential complications thus restricting repeated use.

Other putative recurrence markers present in postoperative blood have been suggested. These include circulating tumor cells (CTC),[7] carbohydrate antigen 19-9 (CA 19-9), mannan-binding lectin-associated serine protease-2[8] and S100A4 messenger RNA (mRNA).[9] None of these markers, however, are recommended in established guidelines[10] due to limitations of small sample size, presence of inconsistent results, or lack of verification in large, independent sample series.[7-9]

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Epigenetic silencing of tumor-related genes by promoter hypermethylation is a common event in various cancers including CRC. Although a small number of tumor-specific methylated genes detected in plasma or serum of CRC patients demonstrate diagnostic potentials for CRC, far fewer of these circulating features are investigated for prognostic relevance. The only promising candidate, serum methylated *HLTF*,[11,12] failed to replicate the prediction of CRC recurrence in a recently completed validation study.[13]

We have previously identified 7 genes (tachykinin-1 [*TAC1*], *MAL*, septin 9 [*SEPT9*], nel-like type 1 [*NELL1*], somatostatin [*SST*], cellular retinoic acid-binding protein 1 [*CRABP1*], and eyes absent homolog 4 [*EYA4*]) with higher methylation levels or frequencies in preoperative sera of CRC patients compared to age-matched healthy controls.[14] These genes demonstrate cancer-specific methylation in tumor tissues,[14] but their utility as a surveillance tool remains unknown. The aim of this study was to evaluate these 7 genes as CRC prognostic markers and their ability to detect cancer recurrence.

MATERIALS AND METHODS

Patients and Sample Collection

This prospective study included 150 consecutive patients with sporadic stage I-III CRCs in a single institution (Singapore General Hospital) between October 2003 and June 2005. Patients with inflammatory bowel disease, recurrent colorectal cancer, family history suggestive of Lynch Syndrome defined by Amsterdam criteria, or familial adenomatous polyposis were excluded. Curative surgical treatment was defined as absence of gross residual tumor after resection and negative margins confirmed pathologically. One of 150 patients had neoadjuvant treatment before surgery and other 45 patients received adjuvant chemotherapy and radiotherapy (Table 1). Informed consent was obtained and the study was approved by the Institutional Review Board of the Singapore General Hospital.

Postoperative CRC surveillance was via an established protocol. Briefly, postoperative patients were followed up at 3-month intervals for the first 2 years, 6-monthly for the next 2 years and then yearly thereafter. At each consultation, CEA levels were measured and full history and physical examination (including digital rectal examination) were performed. Colonoscopy was performed within 6 months of surgery if initial complete evaluation was not possible preoperatively due to tumor obstruction or stenosis. Those who had an initial complete evaluation underwent colonoscopy at first year follow-up, and again at 3-year intervals postoperatively. Patients with suspicious symptoms and signs or rising CEA trend on follow-up will be evaluated earlier with colonoscopy and/or radiological imaging including computerized tomography of the chest, abdomen and pelvis, bone scan and positron emission tomography scans where necessary.

Local recurrence was defined as 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 outside the primary tumor basin. Mortality dates and causes of death were obtained from the Singapore Cancer Registry.

Sample Processing and DNA Isolation

Blood samples are collected longitudinally at 3 time points: 1 week before surgery, 6-month follow-up (6M-FU), and 1-year follow-up (1Y-FU). Fresh tumor tissues were collected upon surgical removal. Sample processing and DNA isolation were performed as described.[14]

Methylation Analysis

Genomic DNA underwent bisulfite conversion by using Epi-Tech kit according to manufacturer's protocol (Qiagen, Hilden, Germany). Bisulfite-converted DNA was subjected to subsequent methylation-specific quantitative PCR (qPCR) as described.[14] Quantities of 7 target genes and 1 control gene β-actin (*ACTB*) were interpolated from respective standard curves constructed from 5 to 6 serial dilutions of a methylated DNA standard.[14] Each reaction was run in duplicate or triplicate. Every plate included a positive control and a no-template control. Methylation levels 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 relative levels of methylation in each sample.

Serum CEA Measurement

Serum CEA was determined by a microparticle enzyme immunoassay on the Abbott Axsym analyzer according to manufacturer's instructions (Abbott Laboratories, Abbott Park, Ill).

Statistical Analysis

Serum methylation levels of all 7 genes and CEA were examined as both continuous and dichotomous variables. Differences of methylation magnitude between groups were compared via Mann-Whitney U test followed by

TABLE 1. Summary of Neoadjuvant or Adjuvant Therapy in 46 Colorectal Cancer Patients

Patient	Chemo Aim	Primary Tx Type	Chemo Regimen[a]	Chemo Duration (mo)	RT Moment	RT Duration (wk)	RT Site
1	adjuvant	chemoRT	CI 5FU	6	Post-op RT	6	Rectum
2	adjuvant	chemo	Xeloda	6	No RT	0	
3	adjuvant	chemo	FOLFOX	6	No RT	0	
4	neoadj	chemoRT	XELOX	8	pre-op RT	6	Rectum
5	adjuvant	chemo	Xeloda	6	No RT	0	
6	adjuvant	chemoRT	XELOX	6	Post-op RT	6	Rectum
7	adjuvant	chemoRT	CI 5FU	6	Post-op RT	6	Rectum
8	adjuvant	chemo	Bolus 5FU	6	No RT	0	
9	adjuvant	chemo	Xeloda	2 (Stop due to toxicity)	No RT	0	
10	adjuvant	chemo	Xeloda	6	No RT	0	
11	adjuvant	chemo	XELOX	6	No RT	0	
12	adjuvant	chemoRT	XELOX	6	Post-op RT	6	Rectum
13	adjuvant	chemo	Xeloda	6	No RT	0	
14	adjuvant	chemoRT	Unknown	6	Post-op RT	6	Rectum
15	adjuvant	chemo	Bolus 5FU	0	No RT	0	
16	adjuvant	chemo	Xeloda	6	No RT	0	
17	adjuvant	chemoRT	Xeloda	6	Post-op RT	6	Rectum
18	adjuvant	chemoRT	CI 5FU	6	Post-op RT	6	Rectum
19	adjuvant	chemo	Bolus 5FU	6	No RT	0	
20	adjuvant	chemo	XELOX	6	No RT	0	
21	adjuvant	chemoRT	FOLFOX	6	Post-op RT	6	Rectum
22	adjuvant	chemo	Xeloda	6	No RT	0	
23	adjuvant	chemo	Xeloda	6	No RT	0	
24	adjuvant	chemo	XELOX	6	No RT	0	
25	adjuvant	chemo	Bolus 5FU	6	No RT	0	
26	adjuvant	chemoRT	Bolus 5FU	6	Post-op RT	6	Rectum
27	adjuvant	chemoRT	Bolus 5FU	6	Post-op RT	6	Rectum
28	adjuvant	chemoRT	Xeloda	6	Post-op RT	6	Rectum
29	adjuvant	chemoRT	CI 5FU	5 (Stop due to toxicity)	Post-op RT	6	Rectum
30	adjuvant	chemoRT	CI 5FU	6	Post-op RT	6	Rectum
31	adjuvant	chemoRT	Xeloda	6	Post-op RT	6	Colon
32	adjuvant	chemo	Xeloda	6	No RT	0	
33	adjuvant	chemo	Xeloda	6	No RT	0	
34	adjuvant	chemo	Bolus 5FU	6	No RT	0	
35	adjuvant	chemoRT	Xeloda	6	Post-op RT	6	Rectum
36	adjuvant	chemo	Xeloda	6	No RT	0	
37	adjuvant	chemoRT	CI 5FU	6	Post-op RT	0	Rectum
38	adjuvant	chemo	Xeloda	5 (Stop due to toxicity)	No RT	0	
39	adjuvant	chemo	Xeloda	1 (Stop due to toxicity)	No RT	0	
40	adjuvant	chemoRT	Xeloda	1 (Stop due to toxicity)	Post-op RT	6	Rectum
41	adjuvant	chemoRT	Bolus 5FU	6	Post-op RT	6	Rectum
42	adjuvant	chemo	Bolus 5FU	6	No RT	0	
43	adjuvant	chemoRT	FOLFOX	2 (Stop due to other reason)	Post-op RT	3 (Stop due to toxicity)	Rectum
44	adjuvant	chemo	XELOX	6	No RT	0	
45	adjuvant	chemo	Xeloda	6	No RT	0	
46	adjuvant	chemo	Xeloda	6	No RT	0	

Abbreviations: chemo, chemotherapy; RT, radiotherapy; Tx, treatment.
Chemotherapy treatments—CI 5FU: continuous infusion of 5-fluorouracil; bolus 5FU: bolus 5-fluorouracil; FOLFOX: a regimen made up of folinic acid (leucovorin), fluorouracil (5FU), and oxaliplatin; XELOX: a regimen made up of Xeloda and oxaliplatin.
[a] In statistical analysis, patients who received regimen of CI 5FU, bolus 5FU, or Xeloda were reclassified as one group, whereas those with FOLFOX or XELOX as another group.

Bonferroni correction. Disease free survival (DFS) time was calculated from the date of surgery to presentation of clinical or pathological evidence of disease recurrence, or to the last contact on or before January 31, 2010 (if no distant recurrence was recorded during this follow-up period). Cancer-specific survival (CSS) was calculated from the date of surgery to the date of death or the last contact on or before January 31, 2010 (if no death was recorded during this follow-up period). Kaplan-Meier survival curves were compared using the log-rank test. Hazards ratio (HR) was computed by univariate Cox proportional hazards regression model followed by multivariate Cox model analysis with stepwise backward procedure to remove variables from the regression model. ROC curves for recurrence detection were constructed and optimal cutoff values of serum methylation markers were

TABLE 2. Clinicopathological Characteristics of 150 Colorectal Cancer Patients

Parameter		Cases N (%)
Sex	Male	85 (56.7)
	Female	65 (43.3)
TNM staging[a]	I	26 (17.3)
	II	62 (41.3)
	III	62 (41.3)
Depth of tumor invasion	T1	7 (4.7)
	T2	24 (16.0)
	T3	107 (71.3)
	T4	12 (8.0)
Lymph nodal status	No lymph node involved	8 (58.7)
	1-3 lymph node involved	36 (24.0)
	≥4 lymph node involved	26 (17.3)
Tumor differentiation	Well	25 (16.7)
	Moderate	114 (76.0)
	Poor	11 (7.3)
Histological type	Adenocarcinoma	145 (96.7)
	Mucinous carcinoma	5 (3.3)
Perineural invasion	Yes	18 (12.0)
	No	128 (85.3)
	Not recorded	4 (2.7)
Vascular embolism	Yes	25 (16.7)
	No	120 (80.0)
	Not recorded	5 (3.3)
Tumor site[b]	Right colon	19 (12.7)
	Left colon	71 (47.3)
	Rectum	60 (40.0
Preoperative serum CEA	≤3.5 ng/mL	66 (44.0)
	>3.5 ng/mL	83 (55.3)
	Not tested	1 (0.7)
Neoadjuvant therapy	Yes	1 (0.7)
Adjuvant therapy	Yes	45 (30.0)
	No	104 (69.3)

[a] Based on American Joint Commission on Cancer guidelines, 5th edition.
[b] Right colon includes cecum through transverse colon, whereas left colon includes splenic flexure, descending colon, and sigmoid colon.

determined based on Youden index. Assay sensitivity was defined as the proportion of recurrent cases that had serum levels above cutoff values. Sensitivity differences were examined by McNemar's test. Association between methylation levels in sera and tumors was evaluated using Spearman's correlation test. Statistical analysis was performed using the Statistical Package for Social Sciences, version 17.0 (SPSS Inc, Chicago, Ill). All statistical tests were 2-sided and *P* values less than .05 were considered statistically significant.

RESULTS

Characteristics of Clinicopathological Factors and Serum Markers

Clinicopathological characteristics of these 150 patients were obtained from a prospectively maintained computerized database and are summarized in Table 2. Median age at diagnosis was 67 years (range, 33-88 years).

After a median follow-up period of 59 months (range, 5-79 months), 43 patients (28.7%) developed either local or distant recurrence (8 and 35 cases, respectively) as defined by clinical diagnostic criteria. Forty (90.9%) patients had died from disease progression.

All serum markers (methylation markers and CEA) were non-normally distributed at any of the 3 sampling time points. Similar to CEA, the overall methylation levels of all 7 genes decreased with time (the highest before surgery and the lowest at 1Y-FU; Fig. 1).

Association of Serum Methylation Levels With Cancer-Specific Survival

Serum methylation levels were dichotomized as "high" and "low" according to median levels at respective follow-up time-points. Dichotomized CEA status was generated according to the reference cutoff value of 3.5 ng/mL. At the preoperative time points, age, TNM stage, and vascular embolism were significantly associated with cancer-specific survival (CSS, $P = .012$, $P = .043$, and $P = .020$, respectively). Except for *SST* gene, none of other serum methylation markers or CEA significantly affected CSS. In the follow-up period, patients with high methylation levels of *TAC1* and *NELL1* measured at 6M-FU experienced a significantly higher risk for cancer-specific death ($P < .001$, $P < .05$, respectively, Table 3). At 1Y-FU, *SEPT9* and *NELL1* were significant and independent prognostic factors of CSS ($P < .01$, $P < .001$, respectively, Table 3).

The combined efficacy of *TAC1*, *SEPT9*, or *NELL1* was also studied. A sample was classified under "high methylation" if any component gene alone was highly methylated. Multivariate analyses revealed that high levels of the combination variable consisting of all 3 genes measured at 6M-FU predicted a higher risk for cancer-specific death (HR = 4.67, 95% CI = 1.81-12.04, $P = .001$, Table 3) compared to individual genes alone.

Association of Serum Methylation Levels With Disease-Free Survival

At preoperative time points, vascular embolism, perineural invasion, serum CEA, and methylation levels of *SST* were significant prognostic factors for cancer recurrence ($P = .010$-$.035$). At postoperative time points, *TAC1* at 6M-FU and *SEPT9* at 1Y-FU were significant factors. The incidence of recurrence was significantly higher in the group with high *TAC1* methylation levels at 6M-FU compared with lower level group (44.0% versus 13.3%, $P < .001$). Disease-free survival was also significantly inferior for patients with high *TAC1* methylation (Fig. 2). This was similarly observed in methylated *SEPT9* at 1Y-FU (50.0% versus 19.4%, $P < .001$). After adjustment

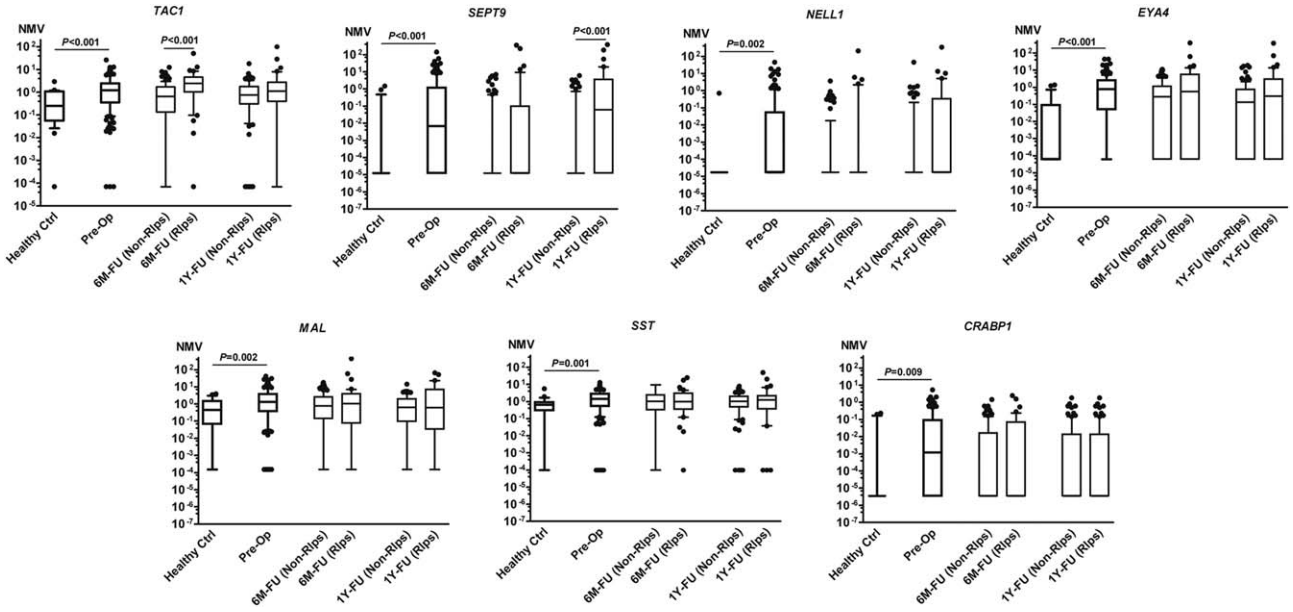

Figure 1. Serum normalized methylation values (NMVs) of 7 genes in healthy subjects ($N = 26$), and stage I-III colorectal cancer patients before surgery ($N = 150$), at 6-month follow-up (6M-FU) and 1-year follow-up (1Y-FU) stratified by recurrence status. Box plots represent 25th and 75th percentiles with midline inside representing median. Upper and lower bars represent 10th and 90th percentiles dots beyond which were outliers. Mann-Whitney U test was run to examine differences of serum methylation levels between healthy controls (reported previously[14]) and preoperative colorectal cancer patients, between subgroups with and without recurrence with blood sampling at 6M-FU or 1Y-FU. Only significantly different pairs are denoted. Pre-op, preoperative; Rlps, relapse; Non-Rlps, nonrelapse.

for other significant factors, patients with high serum methylation of *TAC1* at 6M-FU (HR = 5.72, 95% CI = 2.67-12.28, $P < .001$, see Table 4 for univariate and multivariate Cox analysis results) and high serum methylation of *SEPT9* at 1Y-FU (HR = 3.50, 95% CI = 1.67-7.32, $P = 0.001$, Table 5) had higher recurrence risk.

The combined efficacy of 3 methylation markers was also analyzed for DFS. At 6M-FU, high combined methylation of *TAC1* and/or *SEPT9*, or *TAC1* and/or *NELL1* was a significant predictor for recurrence. At 1Y-FU, impact on DFS remained significant when *SEPT9* and *NELL1* were analyzed in combination (Fig. 3).

Association of Dynamic Changes of Serum Methylation Levels With Disease-Free Survival

Dynamic changes of serum methylation levels of the 7 genes were expressed by Δ values. Three intervals defined included the first half-year interval (between preoperation and 6M-FU), second half-year interval (between 6M-FU and 1Y-FU) and 1-year interval (between preoperation and 1Y-FU). For survival analysis, we assigned "methylation increase" to the Δ variables if serum methylation levels increased during any interval, and "methylation decrease" with reduced or unchanged methylation. Multivariate Cox analyses showed that Δ*TAC1* during the first half-year interval and Δ*SEPT9* during the second half-year and 1-year intervals were independent factors for tumor recurrence (Table 6). It was observed that patients with failed transition from high preoperative to lower or undetectable methylation levels of *TAC1* at 6M-FU experienced higher risk for recurrence when compared with the other group (HR = 4.71; Table 6). It is also interesting to note that ΔCEA was only significant within the second half-year interval (Table 6).

Diagnostic Values of Serum Methylation Markers and CEA for Recurrence Detection

ROC curves were constructed based on postoperative serum levels of methylated *TAC1* and CEA at 6M-FU, as well as methylated *SEPT9* and CEA at 1Y-FU ($P \leq .001$ for all; Fig. 3). Optimal cutoff values yielded 88% specificity for *TAC1* at 6M-FU and 80% specificity for *SEPT9* at 1Y-FU. Sensitivity of *TAC1* at 6M-FU for recurrence detection was significantly higher than that of concurrent CEA concentrations (58.1% versus 32.6%, $P = .019$; Table 7). The sensitivity differences between serum *SEPT9* and CEA levels at 1Y-FU however did not reach statistical significance (Table 7).

TABLE 3. Adjusted Hazard Ratios (95% Confidence Interval) of Independent Prognostic Factors for Cancer-Specific Survival by Multivariate Cox Proportional Hazards Analysis

Factor	At 6-Month Follow-Up ($N = 144$)[a]	At 1-Year Follow-Up ($N = 137$)[b]
TNM stage (AJCC 5th edition)		
II vs I	NS	NS
III vs I	3.29 (1.01-10.72)*	6.78 (1.64-28.10)**
Perineural invasion		
Yes vs No	2.73 (1.16-6.41)*	NA
Histological type		
Mucinous carcinoma vs adenocarcinoma	NA	7.65 (1.96-29.90)**
Serum CEA		
>3.5 ng/mL vs ≤3.5 ng/mL	2.92 (1.47-5.79)**	14.17 (6.41-31.33)***
Methylation of single gene		
TAC1 (High vs Low)[c]	4.12 (1.76-9.61)***	NS
SEPT9 (High vs Low)[c]	NS	2.69 (1.26-5.73)*
NELL1 (High vs Low)[c]	2.51 (1.05-5.98)*	4.41 (1.90- 10.22)***
Combined methylation		
TAC1 and/or *SEPT9* (High vs Low)[d]	4.07 (1.69-9.82)**	NS
TAC1 and/or *NELL1* (High vs Low)[d]	4.84 (2.00-11.67)***	2.41 (1.05- 5.55)*
SEPT9 and/or *NELL1* (High vs Low)[d]	NS	3.72 (1.67- 8.26)***
TAC1 and/or *SEPT9* and/or *NELL1* (High vs Low)[d]	4.67 (1.81-12.04)***	NS

[a] At the end of follow-up (median: 59 months), 39 of these 144 cases were deceased.
[b] At the end of follow-up (median: 59 months), 34 of these 137 cases were deceased.
[c] Serum methylation markers were dichotomized by medians of methylation levels at respective follow-up time points.
[d] A sample was classified under "high methylation" if any component gene alone was highly methylated.
*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$.
NA indicates not analyzed, because statistical significance was not reached in the initial univariate survival analysis. NS indicates not significant.

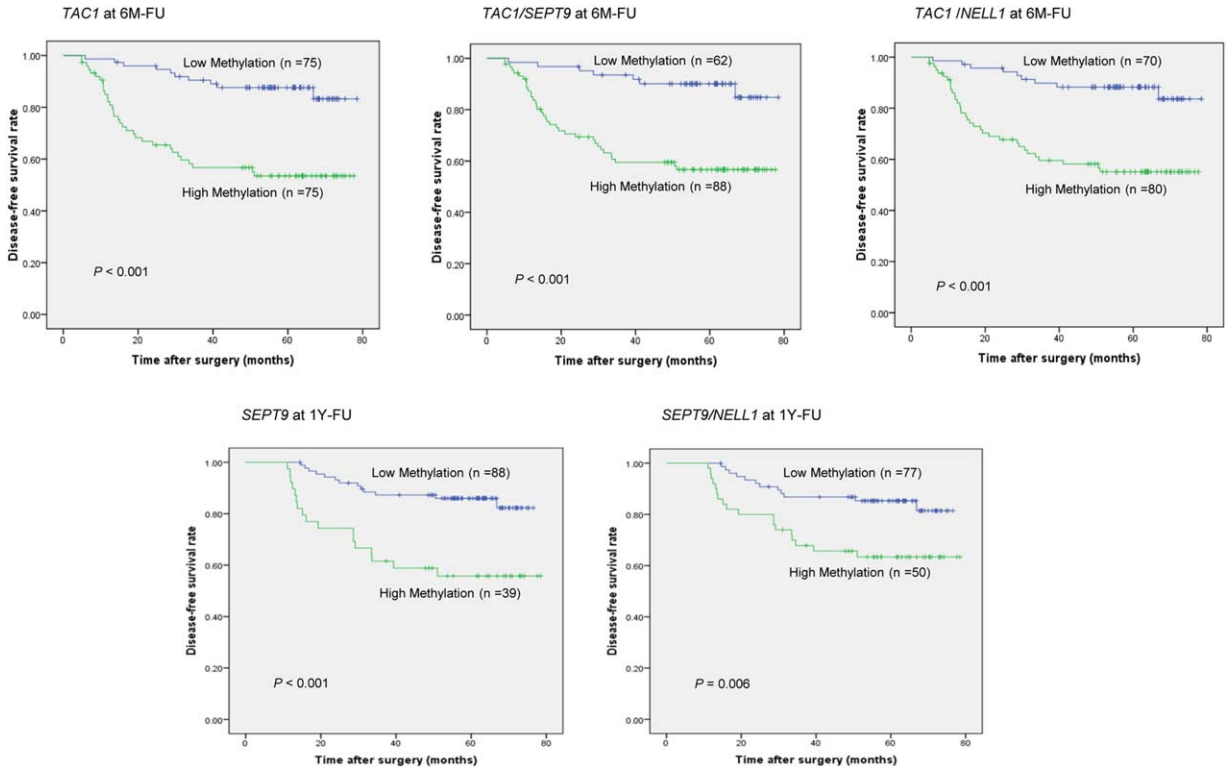

Figure 2. Disease-free survival probabilities in CRC patients stratified by serum methylation levels of *TAC1* at 6 months, and *SEPT9* at 1 year after tumor resection. Methylation levels were dichotomized according to medians at respective follow-up time-points. For assessment of gene combination, a sample was classified under "high methylation" if any component gene alone was highly methylated. 6M-FU, 6-month follow-up; 1Y-FU, 1-year follow-up. The difference in survival curves was examined by log-rank test.

TABLE 4. Cox Analysis of Disease-Free Survival (DFS) at 6-Month Follow-Up in 150 Colorectal Cancer Patients With Stage I-III Tumors

	Univariate Analysis				Multivariate Analysis	
Factor	No. of Patients	No. of Events	HR (95% CI)	P[a]	HR (95% CI)	P[a]
Age at diagnosis	150	43	1.02 (0.99, 1.05)	.138		
Sex				.669		
Male	85	26	1.0			
Female	65	17	0.88 (0.48, 1.61)			
TNM stage (AJCC 5th edition)				**.005**		**.042**
I	26	3	1.0		1.0	
II	62	15	2.43 (0.70, 8.40)		3.22 (0.92, 11.30)	
III	62	25	4.73 (1.43, 15.69)		3.79 (1.12, 12.84)	
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[b]				.982		
Right colon	19	5	1.0			
Left colon	71	20	1.00 (0.38, 2.68)			
Rectum	60	18	1.07 (0.40, 2.87)			
Perineural invasion				**.002**		**NS**
No infiltration	128	32	1.0			
Perineural infiltration	18	10	3.12 (1.53, 6.36)			
Vascular embolism				**.002**		**.003**
No embolism	120	29	1.0		1.0	
Embolism	25	13	2.76 (1.43, 5.32)		2.92 (1.45, 5.88)	
Serum methylation of NELL1[c]				.090		
Low	133	36	1.0			
High	17	7	2.01 (0.89, 4.53)			
Serum methylation of SEPT9[c]				**.035**		**NS**
Low	116	29	1.0			
High	34	14	1.99 (1.05, 3.77)			
Serum methylation of EYA4[c]				.424		
Low	75	20	1.0			
High	75	23	1.28 (0.70, 2.33)			
Serum methylation of CRABP[c]				.529		
Low	98	26	1.0			
High	52	17	1.22 (0.66, 2.24)			
Serum methylation of MAL[c]				.309		
Low	75	20	1.0			
High	75	23	1.37 (0.75, 2.49)			
Serum methylation of TAC1[c]				**<.001**		**<.001**
Low	75	10	1.0		1.0	
High	75	33	4.42 (2.18, 8.99)		5.72 (2.67, 12.28)	
Serum methylation of SST[c]				.952		
Low	75	22	1.0			
High	75	21	1.02 (0.56, 1.85)			
Combined methylation[d] of TAC1 and/or NELL1				**<.001**		**<.001**
Low	70	9	1.0		1.0	
High	80	34	4.32 (2.07, 9.01)		5.39 (2.45, 11.85)	
Combined methylation[d] of TAC1 and/or SEPT9				**<.001**		**<.001**
Low	62	7	1.0		1.0	
High	88	36	4.75 (2.11, 10.94)		6.75 (2.76, 16.94)	
Combined methylation[d] of NELL1 and/or SEPT9				.088		
Low	104	26	1.0			
High	46	17	1.70 (0.92, 3.14)			
Combined methylation[d] of TAC1 and/or NELL1 and/or SEPT9				**<.001**		**<.001**
Low	57	6	1.0		1.0	
High	93	37	4.86 (2.05, 11.54)		6.92 (2.66, 18.04)	
Serum CEA				**<.001**		**.001**
$\leq$3.5 ng/mL	121	29	1.0		1.0	
$>$3.5 ng/mL	29	14	3.18 (1.67, 2.04)		3.18 (1.62, 6.27)	

Abbreviations: CI, confidence interval; HR, hazard ratio; NS, not significant.

[a] Bold values indicate statistical significance.

[b] Right colon includes cecum through transverse colon, while left colon includes splenic flexure, descending colon and sigmoid colon.

[c] Methylation levels were dichotomized by medians at 6-month follow-up time point.

[d] A sample was classified under "high methylation" if any component gene alone was highly methylated.

TABLE 5. Cox Analysis of Disease-Free Survival (DFS) at 1-Year Follow-Up in 127 Colorectal Cancer Patients With Stage I-III Tumors

	Univariate Analysis				Multivariate Analysis	
Factor	No. of Patients	No. of Events	HR (95% CI)	P[a]	HR (95% CI)	P[a]
Age at diagnosis	127	30	1.02 (0.99, 1.06)	.171		
Sex				.120		
Male	73	21	1.0			
Female	54	9	0.54 (0.25, 1.18)			
TNM stage (AJCC 5th edition)				**.021**		**.003**
I	24	3	1.0			
II	53	9	1.51 (0.41, 5.59)		1.84 (0.50, 6.83)	
III	50	18	3.63 (1.07, 12.35)		5.29 (1.52, 18.47)	
Differentiation				.282		
Well	24	6	1.0			
Moderate	96	21	0.86 (0.35, 2.13)			
Poor	7	3	2.30 (0.57, 9.22)			
Histological type				.940		
Adenocarcinoma	123	29	1.0			
Mucinous carcinoma	4	1	1.08 (0.15, 7.92)			
Tumor site[b]				.985		
Right colon	14	3	1.0			
Left colon	61	14	1.02 (0.29, 3.56)			
Rectum	52	13	1.05 (0.31, 3.81)			
Perineural invasion				.107		
No infiltration	112	24	1.0			
Perineural infiltration	12	5	2.21 (0.84, 5.79)			
Vascular embolism				.176		
No embolism	107	23	1.0			
Embolism	16	6	1.86 (0.76, 4.58)			
Serum methylation of NELL1[c]				.110		
Low	108	23	1.0			
High	19	7	2.00 (0.86, 4.67)			
Serum methylation of SEPT9[c]				**<.001**		**.001**
Low	88	13	1.0		1.0	
High	39	17	3.62 (1.76, 7.46)		3.50 (1.67, 7.32)	
Serum methylation of EYA4[c]				.994		
Low	63	15	1.0			
High	64	15	1.00 (0.49, 2.04)			
Serum methylation of CRABP[c]				.442		
Low	90	20	1.0			
High	37	10	1.35 (0.63, 2.88)			
Serum methylation of MAL[c]				.521		
Low	65	17	1.0			
High	62	13	0.79 (0.38, 1.63)			
Serum methylation of TAC1[c]				.382		
Low	66	13	1.0			
High	61	17	1.38 (0.67, 2.84)			
Serum methylation of SST[c]				.900		
Low	65	15	1.0			
High	62	15	1.05 (0.51, 2.15)			
Combined methylation[d] of TAC1 and/or NELL1				.171		
Low	58	10	1.0			
High	69	20	1.70 (0.80, 3.64)			
Combined methylation[d] of TAC1 and/or SEPT9				.109		
Low	55	9	1.0			
High	72	21	1.90 (0.87, 4.14)			
Combined methylation[d] of NELL1 and/or SEPT9				**.009**		**.006**
Low	77	12	1.0		1.0	
High	50	18	2.66 (1.28, 5.53)		2.89 (1.37, 6.11)	
Combined methylation[d] of TAC1 and/or NELL1 and/or SEPT9				.155		
Low	49	8	1.0			
High	78	22	1.80 (0.80, 4.04)			
Serum CEA				**<.001**		**<.001**
≤3.5 ng/mL	107	18	1.0		1.0	
>3.5 ng/mL	20	12	5.86 (2.81, 12.23)		7.29 (3.33, 15.93)	

Abbreviations: CI, confidence interval; HR, hazard ratio.

[a] Bold values indicate statistical significance.

[b] Right colon includes cecum through transverse colon, whereas left colon includes splenic flexure, descending colon, and sigmoid colon.

[c] Methylation levels were dichotomized by medians at 1-year follow-up time point.

[d] A sample was classified under "high methylation" if any component gene alone was highly methylated.

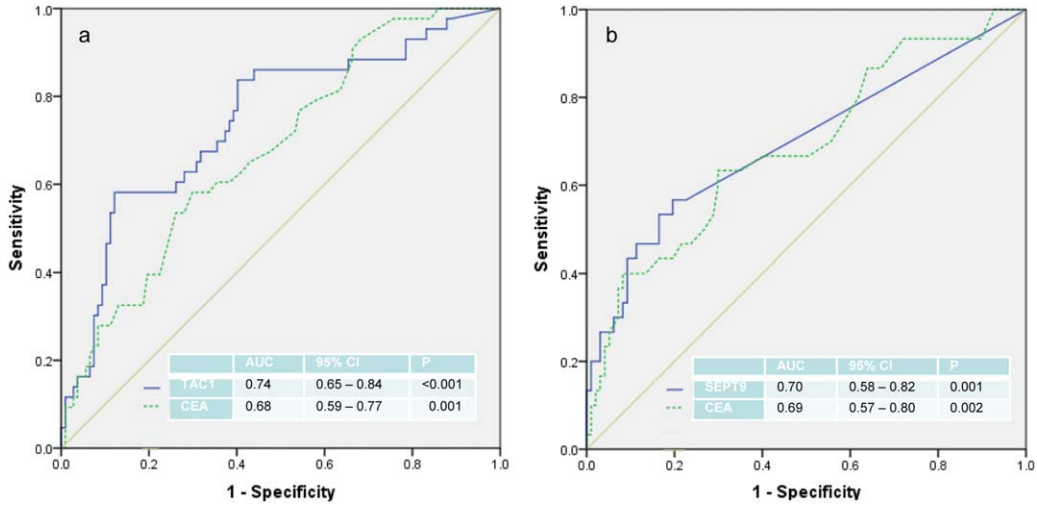

Figure 3. ROC curves of serum markers for recurrence detection. (A) *TAC1* methylation and CEA measurement at 6-month follow-up, and (B) *SEPT9* methylation and CEA measurement at 1-year follow-up. AUC, area under ROC curve; CI, confidence interval.

TABLE 6. Adjusted Hazard Ratios (95% Confidence Interval) of Independent Prognostic Factors for Disease-Free Survival by Multivariate Cox Proportional Hazards Analysis

Factor	First Half-Year Interval[a] ($N = 144$)[d]	Second Half-Year Interval[b] ($N = 127$)[e]	1-Year Interval[c] ($N = 127$)[f]
TNM stage (AJCC 5th edition)			
II vs I	NS	NS	NS
III vs I	NS	NS	3.53 (1.04-12.00)*
Perineural invasion			NA
Yes vs No	4.81 (2.14-10.78)***	NS	NA
ΔCEA (Increase vs Decrease)[g]	NS	3.14 (1.43-6.90)**	NS
Δ*TAC1* (Increase vs Decrease)[g]	4.71 (2.30-9.63)***	NS	NS
Δ*SEPT9* (Increase vs Decrease)[g]	NS	2.58 (1.23-5.41)*	3.35 (1.56-7.19)**

[a] From preoperation to 6-month follow-up; [b]From 6-month follow-up to 1-year follow-up; [c]From preoperation to 1-year follow-up.
[d,e,f]At end of follow-up (median, 59 months), recurrence diagnosed by clinical criteria was established in 42, 30, and 30 patients, respectively.
[g]Changes of serum levels within an interval with the levels at the initial time point set as the baseline levels.
*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$.
NA, not analyzed as statistical significance was not reached in the initial univariate survival analysis; NS, not significant.

The lead time (ie, period between detection of high levels of serum markers and definite recurrence established by clinical diagnostic criteria) of all serum markers in our series was non-normally distributed. The median lead time of serum *TAC1* at 6M-FU was 2.2 months earlier as compared with CEA (Table 7). Similarly, at 1Y-FU time-point, the median lead time of serum methylated *SEPT9* was 2.5 months earlier than that of serum CEA (Table 7).

Comparisons of Methylation Levels in Sera and Matched Tumors

The vast majority of tumors harbored methylation in the 3 genes of interest (100%, 99% and 95% for *TAC1*, *SEPT9*, and *NELL1* respectively, Table 8). Magnitude of methylation was more prominent in tumors than in matched sera ($P < .05$ in all tests). Correlation between methylation levels in tumor and sera was however not present ($P > .05$ for all gene pairs). On the other hand, methylated *TAC1* and *SEPT9* in sera could always be traced back to corresponding tumors that were methylated as well; Unmethylated *SEPT9* observed in only 1 tumor was also concordant with unmethylated *SEPT9* in the matched serum, demonstrating no false-positive detection of these 2 markers in sera (Table 8). One exception was observed in the analysis of *NELL1*. Of 8 unmethylated tumors, 1 was paired up with methylated serum, whereas the other 7 remained unmethylated (Table 8).

TABLE 7. Sensitivity, Specificity, and Lead Time of Postoperative Serum Markers for Detection of Recurrence

	At 6-Month Follow-Up				At 1-Year Follow-Up			
			Lead time (mo)[a]				Lead Time (mo)[a]	
	Sensitivity (%)	Specificity (%)	Median	Range	Sensitivity (%)	Specificity (%)	Median	Range
CEA (Cutoff at 3.5 ng/mL)	14/43 (32.6)	91/107 (85)	5.9	0-11.8	12/30 (40.0)	87/97 (90)	2.6	0-39.5
TAC1 (Optimal cutoff)	25/43 (58.1)*	94/107 (88)	8.1	0-46.0	NA	NA		
SEPT9 (Optimal cutoff)	NA	NA			17/30 (56.7)	78/97 (80)	5.1	0-40.0

[a] Lead time is defined as a period between detection of high levels of serum markers and definite recurrence established by clinical diagnostic criteria.
*$P = .019$ by McNemar's test between serum CEA and methylated *TAC1*.
NA, not analyzed, because statistical significance was not reached in the upstream ROC test.

TABLE 8. Methylation Levels of *TAC1*, *SEPT9*, and *NELL1* in Paired Tumor and Serum Samples

	Case No. in Tumor								
Case No. in Serum	*TAC1*			*SEPT9*			*NELL1*		
	NMV ≤ 0	NMV > 0	Total	NMV ≤ 0	NMV > 0	Total	NMV ≤ 0	NMV > 0	Total
NMV ≤ 0	0	3	3	1	71	72	7	94	101
NMV > 0	0	145	145	0	76	76	1	46	47
Total	0	148	148	1	147	148	8	140	148

NMV indicates "normalized methylation value" generated by dividing the gene/*ACTB* ratio of a sample by the gene/*ACTB* ratio of a positive control and multiplying by 1000.

DISCUSSION

The aim of CRC surveillance protocols after curative resection is early detection of recurrences. Disease recurrence is highest within the first 2 years,[1] but traditional serum markers and tools used have multiple limitations often leading to late detection thus limiting treatment options. In this study, high postoperative serum methylation levels of *TAC1* at 6M-FU, *SEPT9* at 1Y-FU were independent predictors of CRC recurrence and unfavorable cancer-specific survival ($P < .05$). Serum methylation levels of *NELL1* were significant alone for CSS at both 6M-FU and 1Y-FU but not for DFS. In further analysis, dynamic changes of *TAC1* and *SEPT9* with methylation increment were also independently predictive of cancer recurrence (Table 6). More importantly, serum methylation of *TAC1* at 6M-FU and *SEPT9* at 1Y-FU revealed an earlier lead time advantage of more than 2 months compared to concurrent serum CEA. The observed median lead time (5.1-8.1 months) of these 2 markers was also earlier than that of 2 to 4 months reported in a rectal cancer cohort by protein markers of CEA, CA 19-9 and tissue plasminogen activator,[15] and in agreement with median lead time of 7 months (range, 4-10 months) conferred by an mRNA panel consisting of hTERT, CK-19, CK-20 and CEA.[16] To our knowledge, this is the first study demonstrating superiority of circulating methylation markers in earlier detection. Greater sensitivity and earlier detection of recurrences may thus allow a repeat of curative surgical intervention or prompt initiation of more aggressive chemotherapeutic regimens with suppression of further tumor dissemination. Recurrent disease can be thus kept at low volumes with few symptoms conferring a superior quality of life for these patients. Other key advantages of serum methylation assay include that similar to serum CEA, it is minimally invasive, rapid, simple and repeatable. Certainly, further prospective and large studies to validate their full utility and survival benefits are still required.

TAC1 plays multiple biological functions in CRC pathophysiology. *TAC1* encodes a neuroendocrine gastrointestinal peptide that is a precursor for hormones including substance P and neurokinin A, affecting secretion, motility and inflammatory reactions of the gastrointestinal tract. Neurokinin A has also been reported to exert antiproliferative effects.[17] *SEPT9* is a member of the septin family involved in cytokinesis and cell cycle control. This gene is a candidate tumor suppressor gene also responsible for cancers such as ovarian cancer.

Notably, *TAC1* methylation was a significant prognostic factor at 6M-FU, but significance was replaced by methylated *SEPT9* at 1Y-FU. We further confirmed that these were not due to effects of adjuvant therapy and noted no correlation of adjuvant treatment with serum methylation levels of *TAC1* at 1Y-FU, nor associated with changes during the second half-year interval ($P > .05$). It has been described that *TAC1* methylation intensity is significantly higher in early-stage tumors.[18] *SEPT9* methylation in contrast is more frequent in tumors with advanced stage III-IV (64% versus 20% stage I-II).[19] The reasons are not clear at this point in time but indicate the heterogeneity in tumorigenesis. We thus hypothesize that disease recurrence similar to tumorigenesis, may occur and develop via various pathways. Different methylation markers become apparent at different time frames. The use of single methylation markers may thus be not suitable and a combination panel for surveillance is required.

In this study, there were no false-positive methylation of either *TAC1* or *SEPT9* observed in serum, but methylation of tumor DNA was not always accompanied by parallel detection in serum DNA (Table 8). One possibility is the profound dilution effect of the circulatory system on tumor DNA resulting in very low and undetectable levels with current technologies. Furthermore, there may be insufficient amounts, or shedding of detectable neoplastic DNA may not have occurred during blood sampling. The low abundance of methylation variants present in excess amount of background DNA also confers technical challenges to qPCR further compromising assay sensitivity.

One of the limitations in this study was the relative long intervals between blood sampling. In contrast to the current clinical surveillance protocol of 3-monthly intervals, the study protocol of 6-monthly intervals unfortunately led to 10 cases developing recurrences that preceded the scheduled date for 1Y-FU evaluation. There were thus fewer cases available for DFS analysis and computation of lead time pertinent to *SEPT9* methylation measured at 1Y-FU. Furthermore, if the objectives are for earlier detection, a more frequent sampling, for example, at 2-month intervals may be required. We await further data of our ongoing validation study in a larger and independent cohort.

In summary, we have identified 2 novel prognostic markers that may be beneficial for surveillance after curative resection. We have demonstrated their concordance in detecting tumor recurrence as well as a lead time advantage over serum CEA. Additional validation studies are required to fully define the utility of these markers in recurrence surveillance and to assess whether subsequent modification of treatments will result in improved survival.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

REFERENCES

1. Sargent D, Sobrero A, Grothey A, et al. Evidence for cure by adjuvant therapy in colon cancer: Observations based on individual patient data from 20,898 patients on 18 randomized trials. *J Clin Oncol*. 2009;27:872-877.
2. Baca B, Beart RW, Etzioni DA. Surveillance after colorectal cancer resection: a systematic review. *Dis Colon Rectum*. 2011;54:1036-1048.
3. Rozen M, Chan L, Beart RW, et al. Follow-up of colorectal cancer: a meta-analysis. *Dis Colon Rectum*. 1998;41:1116-1126.
4. Nicolini A, Ferrari P, Duffy MJ, et al. Intensive risk-adjusted follow-up with the CEA, TPA, CA 19.9, and CA72.4 tumor marker panel and abdominal ultrasonography to diagnose operable colorectal cancer recurrence: effect on survival. *Arch Surg*. 2010;145:1177-1183.
5. Rockall TA, McDonald PJ. Carcinoembryonic antigen: its value in the follow-up of patients with colorectal cancer. *Int J Colorectal Dis*. 1999;14:73-77.
6. Duffy MJ. Carcinoembryonic antigen as a marker for colorectal cancer: Is it clinically useful? *Clin Chem*. 2001;47:624-630.
7. Peach G, Kim C, Zacharakis E, et al. Prognostic significance of circulating tumour cells following surgical resection of colorectal cancers: a systematic review. *Br J Cancer*. 2010;102:1327-1734.
8. Ytting H, Christensen IJ, Thiel S, et al. Pre- and post-operative levels in serum of mannan-binding lectin associated serine protease-2–a prognostic marker in colorectal cancer. *Hum Immunol*. 2008;69:414-420.
9. Stein U, Burock S, Herrmann P, et al. Diagnostic and prognostic value of metastasis inducer S100A4 transcripts in plasma of colon, rectal, and gastric cancer patients. *J Mol Diagn*. 2011;13:189-198.
10. National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology, Colon Cancer. Version 2.2014. http://www.nccn.org/professionals/physician_gls/f_guidelines.asp#site. Accessed November 16, 2013.
11. Wallner M, Herbst A, Behrens A, et al. Methylation of serum DNA is an independent prognostic marker in colorectal cancer. *Clin Cancer Res*. 2006;12:7347-7352.
12. Herbst A, Wallner M, Rahmig K, et al. Methylation of helicase-like transcription factor in serum of patients colorectal cancer is an independent predictor of disease recurrence. *Eur J Gastroenterol Hepatol*. 2009;21:565-569.
13. Phillip AB, Stiber P, Nagel D, et al. Prognostic role of methylated free circulating DNA in colorectal cancer. *Int J Cancer*. 2012;131:2308-2319.
14. Liu Y, Tham CK, Ong SY, et al. Serum methylation levels of TAC1, SEPT9 and EYA4 as diagnostic markers for early colorectal cancers: a pilot study. *Biomarkers*. 2013;8:399-405.
15. Barillari P, Bolognese A, Chirletti P, et al. Role of CEA, TPA, and CA19-9 in the early detection of localized and diffuse recurrent rectal cancer. *Dis Colon Rectum*. 1992;35:471-476.
16. Uen YH, Lin SR, Wu DC, et al. Prognostic significance of multiple molecular markers for patients with stage II colorectal cancer undergoing curative resection. *Ann Surg*. 2007;246:1040-1046.
17. Rameshwar P, Gascon P. Induction of negative hematopoietic regulators by neurokinin-A in bone marrow stroma. *Blood*. 1996;88:98-106.
18. Mori Y, Cai K, Cheng Y, et al. A genome-wide search identifies epigenetic silencing of somatostatin, tachykinin-1, and 5 other genes in colon cancer. *Gastroenterology*. 2006;131:797-808.
19. Herbst A, Rahmig K, Stieber P, et al. Methylation of NEUROG1 in serum is a sensitive marker for the detection of early colorectal cancer. *Am J Gastroenterol*. 2011;106:1110-1118.