

S100P, a potential novel prognostic marker in colorectal cancer

QIANG WANG^{1*}, YUE-NING ZHANG^{1,4*}, GUO-LE LIN², HUI-ZHONG QIU², BIN WU²,
HAI-YAN WU¹, YU ZHAO³, YUAN-JIA CHEN¹ and CHONG-MEI LU¹

Departments of ¹Gastroenterology, ²Surgery, and ³Pathology, Peking Union Medical College Hospital,
Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100730, P.R. China

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Abstract. Previous studies have shown that S100P contributes to the development of a number of tumors. However, its prognostic significance in colorectal cancer (CRC) has not been demonstrated. This study aimed to confirm the expression of S100P in colorectal cancer as well as the epigenetic mechanism underlying its gene expression, and to demonstrate whether S100P could be used to predict prognosis as a biomarker. We tested the expression of S100P in 96 CRCs and their paired tissue controls, as well as 13 colon cancer cell lines by RT-PCR and western blotting. Expression of the S100P protein and mRNA was significantly higher in cancerous regions compared to that in paired non-cancerous tissues ($P=4.59\times 10^{-17}$, 0.005 respectively). The expression was significantly correlated with the hypomethylation of the S100P promoter ($P=4.92\times 10^{-5}$), which was detected by bisulphite sequencing PCR (BSP) and quantitative methylation-specific real-time PCR (QMSP). In stages I to III, the patients with positive expression of S100P protein showed poorer overall survival compared to those with S100P negative expression, $P=0.031$. We also measured the preoperative serum S100P levels by ELISA. The patients with normal serum levels of S100P showed favorable prognosis compared with patients with elevated S100P levels ($P=0.008$). These data suggest that S100P protein may be a potential novel prognostic biomarker in CRC patients.

Introduction

Colorectal cancer is one of the most common types of cancers worldwide and in recent years, the incidence of CRC has increased significantly in Asian countries including China (1). The incidence of CRC in China ranks fourth and it is the 4th leading cause of tumor-related mortality (2). Despite several advances in understanding and clinical management of this cancer, the outcome of CRC patients has not improved profoundly. In some developing countries including China, the 10-year survival rates for men diagnosed with colorectal cancer range from 28 to 42% (3). Recently, several studies have shown a number of molecular biomarkers with promising prognostic values in CRC (4-9), however, the molecular markers should be further validated (9) and, some biomarker could be used in a specific stage, such as stage III (10). Moreover, there are much less reliable prognostic biomarkers which can be tested in peripheral blood though preoperative serum levels of carcinoembryonic antigen (CEA) could be a prognostic predictor (9) and has been a biomarker for recognizing CRC recurrence in recent guidelines (11). Therefore, more prognostic biomarkers are needed for clinical care in CRC patients, especially those markers that can be tested in serum/plasma. In this study, we demonstrate a novel prognostic biomarker, which can be tested in CRC tissues as well as in the patient serum.

S100 calcium-binding protein P (S100P), a member of S100 protein family, was first isolated from human placenta (12,13). The expression of S100P has been found in a number of tumors including pancreatic cancer cell lines and tissues (14,15), lung cancer, and it was correlated with immortalization of human breast epithelial cells *in vitro* and progression of breast cancer *in vivo* (16,17). S100P expression was associated with the grade of tumors, and it promoted tumor invasion and metastasis (14,17,18). Expression of S100P was shown in CRC tissues compared with much less expression in normal mucosa (19-21), and it stimulated cancer cell growth and migration *in vitro* (20,21). It is conceivable to speculate that S100P might be correlated with the development and the progression of CRC as well as the prognosis of patients with CRC. Therefore, the purpose of the study was to confirm the expression of S100P in CRC as well as the epigenetic mechanism underlying the gene expression, and more importantly, to demonstrate whether the S100P could be used to predict the prognosis as a biomarker. We tested the gene expression, promoter methylation status in CRC tissues and their paired control tissues, examined the

Correspondence to: Dr Chong-Mei Lu or Dr Yuan-Jia Chen, Department of Gastroenterology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100730, P.R. China
E-mail: pekingunion@yahoo.cn
E-mail: yuanjchen@gmail.com

Present address: ⁴Center of Hepatology and Gastroenterology, Beijing Youan Hospital, Capital Medical University, Beijing 100069, P.R. China

*Contributed equally

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serum levels of *S100P* in CRC patients and healthy controls, and correlated the experimental data with clinicopathological characteristics of the patients.

Materials and methods

Cancer cell lines and colorectal cancer tissues. Thirteen human colorectal cancer cell lines were used in the study: SW480, SW620, LoVo, HCT116, HT29, T84, Colo205, CL187, HCT8, RKO, HCT15, SW837 and DLD1. All of them were American Type Culture Collection (ATCC) cell lines. Ninety-six CRC tissue samples and their paired colorectal tissues (2 cm away from the tumor) were obtained after resection in Peking Union Medical College Hospital from July 2003 to July 2006. The tissue samples were snap-frozen in liquid nitrogen immediately after resection and stored at -80°C. All tissues were examined morphologically, and the diagnosis was confirmed by experienced pathologists. Peripheral serum samples were taken from the patients with CRC and healthy controls. The study protocol was approved by the Scientific Research/Ethics Committee of the Peking Union Medical College Hospital and patients provided informed written consent to participate in this study. The clinicopathological characteristics were prospectively collected and summarized in Table I. All the 96 patients with CRC were followed up.

Western blotting. Antibodies against the *S100P* (BD Biosciences, Franklin Lakes, NJ, USA), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat against mouse Ig G conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used in western blotting at 1:100, 1:100 and 1:900 dilution, respectively. Protein extracted from fresh frozen CRC tissues and their paired colorectal tissues as well as CRC cell lines, was tested. β-actin was used as a internal control. The signal was detected by the ECL detection system (Amersham, UK) protocol.

Quantitative assessment of *S100P* by real-time RT-PCR. Total RNA was extracted from the tissues and cultured CRC cells using RNAqueous Kit (Ambion, USA) according to the manufacturer's protocol. cDNA was synthesized following the instructions of SuperScript II kit (Gibco-BRL, Gaithersburg, MD, USA). Quantitative real-time PCR of the template cDNA with gene-specific priming was done with SYBR® Premix Ex Taq™ (Takara, Dalian, China) using a PRISM 7500 Real-Time PCR System (ABI, Vernon, CA, USA). PCR was initiated with one cycle of 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, and 60°C for 34 sec, and then 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec for melting analysis to visualize non-specific PCR products. Primers designed for human *S100P* were: forward 5'-AGGTGCTGATGGAGAAGGA-3' and reverse, 5'-GTGATTGCAGCCACGAAC-3'; primers for human GAPDH, which was used as an internal reference gene for the real-time PCR reactions, were: forward, 5'-ACTTCAAC AGCGACACCCACT-3' and reverse, 5'-GCCAAATTGTT GTCATACCAAG-3'. Each primer set used in the present study produced a single melting peak and a single prominent band of the expected size on agarose gel electrophoresis. Each experiment was repeated at least three times. CRC cell line CL187 was taken as a calibrator.

Table I. The clinicopathological characteristics in CRC patients.

Characteristics	N ^a
No. of patients	96
Gender	
Male	61
Female	35
Age at surgery (years), mean ± SE (range)	62.4±1.2 (28-92)
Male	62.6±1.6 (28-92)
Female	62.2±1.8 (37-78)
Family history of malignant tumor (%) ^b	5 (5.2)
Presurgical chemo- and/or -radiotherapy	3 (3.1)
TNM staging (%)	
Stage I	12 (12.5)
Stage II	37 (38.5)
Stage III	36 (37.5)
Stage IV	11 (11.5)
Primary tumor location (%) ^c	
Right semicolon	29 (30.2)
Left semicolon	17 (17.7)
Rectum	50 (52.1)
Tumor size (%) ^d (cm)	
<5	47 (49.0)
≥5	49 (51.0)
Histological type (%)	
Adenocarcinoma	77 (80.2)
Mucoid adenocarcinoma	18 (18.8)
Signet-ring carcinoma	1 (1.0)
Differentiation grade (%)	
Well	18 (18.8)
Moderate	60 (62.5)
Poor	18 (18.8)

SD, standard error. ^aData are N unless otherwise stated. ^bFamily history of cancer was restricted to the first- and second-degree relatives. ^cRight semicolon, caecum to splenic flexure and left semicolon, splenic flexure to sigmoid colon; ^dmeasured by maximum diameter.

Bisulphite sequencing of crucial region within the promoter. Genomic DNA from both frozen tissues and cultured CRC cells were prepared using the QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Genomic DNA was bisulphite modified as previously described (22), and then was purified by Wizard DNA Clean-Up System (Promega, Madison, WI, USA). To investigate the CpG sites within *S100P* promoter that were crucial for gene regulation, we performed the bisulphited sequencing of a relative CpG-rich region of 969 bp (from -739 to +229) including the upstream of transcriptional start site of *S100P* gene and part of the first exon. The bisulphited DNA was amplified by PCR with 4 primer pairs located in the region. The primers for BSP were

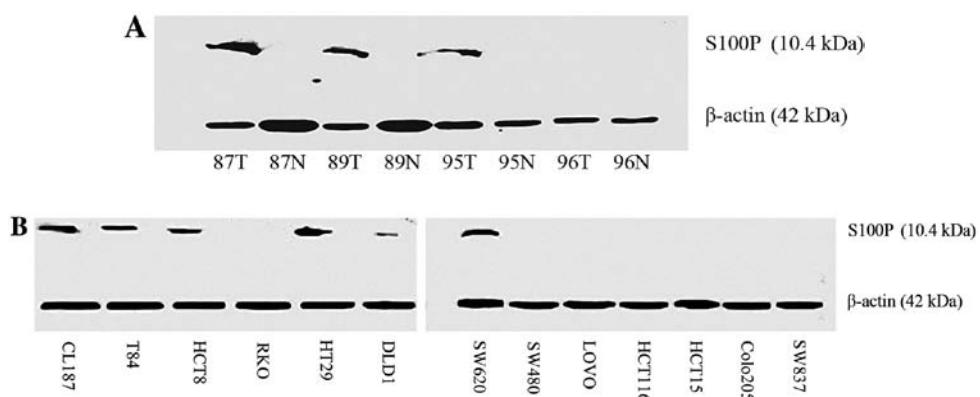


Figure 1. *S100P* protein expression in human CRCs and cell lines. (A) The results of western blot analysis for 4 tumor samples (T) and their paired tissues (N), the expression of *S100P* was found in tumor #87, #89 and #95 but not in #96. (B) Six of the 13 CRC cell lines (CL187, T84, HT29, HCT8, DLD1 and SW620) show the expression of the protein. β-actin was used as internal control.

as follows: i) forward, 5'-GGTTTTAGGGAATTGATTAA TAGGT-3' and reverse, 5'-TCTATATCCTCAAAAACCC AACTT-3'; ii) forward, 5'-GGGAGTTTTGTTGGTT TTATAG-3' and reverse, 5'-CACAATAACATCCTCATTCC TAAC-3'; iii) forward, 5'-GAATGAGGATGTTATTGTGG TTTAGT-3' and reverse, 5'-CACCTTCCTCCTAAAAACTA ACAAA-3', iv) forward, 5'-GGTGGGTTGAATTAGT ATTATGA-3' and reverse, 5'-CAAAAAACCTAATAACTC CTTCTCC-3'. PCR was initiated with one cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 45 sec, 54°C for 45 sec, and 72°C for 45 sec, and one cycle of 72°C for 10 min. The PCR products were cloned into a pEASY-T3 vector (Trans, Beijing, China) and sequenced.

Quantitative analysis of methylation. SYBR-Green quantitative methylation-specific real-time PCR (QMSP) of bisulphite modified DNA of both tissues and cultured CRC cells was performed. The primers of both methylated and unmethylated covered the CpG sites which were related with the *S100P* gene expression according to the sequencing results. The primers for QMSP were as follows: i) methylated primers, forward, 5'-TATAGAGTGTAAAGAAAGGGACGA-3' and reverse, 5'-TACTAACACCCAACGTACTAACACG-3'; ii) unmethylated primers, forward, 5'-TATAGAGTGTAAAGAAAG GGATGA-3' and reverse, 5'-CTAACACCCAACATACTAA CACAAA-3'; iii) internal reference (β-actin) primers, forward, 5'-GGGAGGGTTAGGAATGAGGA-3' and reverse, 5'-CCC AACCAAACCCAAAATA-3'. The primers for β-actin were designed to amplify a region without CpG sites so that PCR amplification would occur irrespective of methylation status (23,24). QMSP was done with SYBR® Premix Ex Taq™ (Takara) using the PRISM 7500 Real-Time PCR System (ABI). PCR was initiated with one cycle of 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, 58°C for 20 sec, and 72°C for 34 sec, and then 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec for melting analysis. Each primer set used in the study produced a single melting peak and a single prominent band of the expected size on electrophoresis. Each experiment was repeated at least three times.

ELISA for *S100P* in serum samples. The preoperative serum levels of *S100P* in CRC patients were measured using an *S100P*

ELISA kit (CycLex Co., Ltd., Nagano, Japan) according to the manufacturer's protocol. The serum levels of *S100P* in 57 healthy people were measured as normal control.

Statistical analysis. Significance was calculated using the Student's t-test, χ^2 test, the Fisher exact test, the Spearman rank correlation test and the Mann-Whitney U test. Logistic regression analysis was used for multivariate analysis. Survival data were evaluated by Kaplan-Meier curves and Cox regression analysis. Two-tailed test was used in all of the analysis. $P \leq 0.05$ was considered significant.

Results

Expression of *S100P* in CRC tissue and cell lines. The expression of *S100P* protein was found in 63 of 96 CRC (65.6%), significantly higher than that in 7 of 96 matched colonrectal mucosa (7.3%), $P=4.59 \times 10^{-17}$, (Fig. 1A). *S100P* protein was detected in 6 of 13 human CRC cell lines (46%), (Fig. 1B). Measured by quantitative real-time RT-PCR, the expression of *S100P* mRNA was significantly higher in 62 CRC tissues than that in matched tissues (range 0.034-42.447, median 1.540, vs. range 0.005-0.489, median 0.143, $P=0.005$). The expression for *S100P* mRNA in CRC tissues was significantly correlated with the protein expression ($rs=0.451$, $P=2.38 \times 10^{-4}$). The expression for mRNA in 13 CRC cell lines was also detected, and it was significantly correlated with the protein expression ($rs=0.825$, $P=0.001$).

The promoter methylation of *S100P* gene and the crucial region. Bisulphite sequencing revealed the methylation status of 24 CpG sites within the region across the promoter (from -739) and a part of exon 1 (to +229) in 10 CRC cell lines (Fig. 2). The methylation status of 19 CpG sites was significantly associated with the expression level of *S100P* mRNA ($rs=-0.885$, $P=0.002$).

Demethylation of *S100P* gene in tumors with gene expression. The expression for *S100P* mRNA detected by quantitative real-time RT-PCR significantly correlated with the demethylation levels of the crucial CpG sites tested by QMSP in 13 CRC cell lines ($rs=0.879$, $P=7.5 \times 10^{-5}$). The ratio of demethylation/

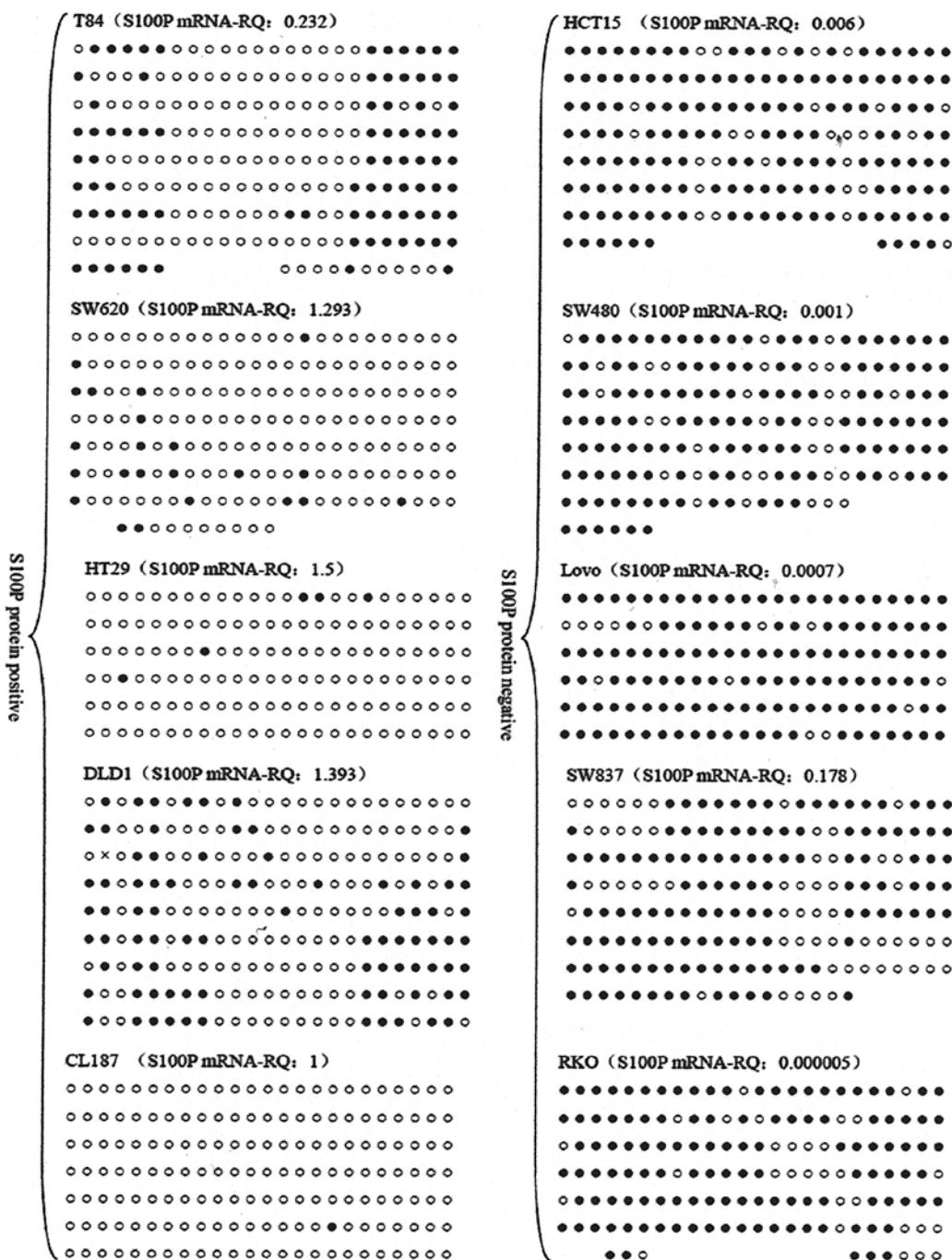


Figure 2. Methylation status of CpG sites detected by BSP in 10 CRC cell lines. ●, methylated; ○, unmethylated. Each line designates one clone, and each row designates one CpG site. RQ mean value of relative quantification by real-time RT-PCR.

methylation (U/M) of the crucial CpG sites was also significantly associated with the *S100P* mRNA expression ($rs=0.791$, $P=0.001$). These data of QMSP were consistent with the results of bisulphite sequencing in the cell lines.

Similar demethylation status of *S100P* promoter was found in 78 tumor and their matched tissues of 78 CRC cases by QMSP. The demethylation levels of *S100P* promoter in tumors were significantly higher than that in matched tissues (0.850 ± 0.080 vs. 0.608 ± 0.024 , $P=0.002$), and the ratio of U/M levels was 1.589 ± 0.092 and 0.780 ± 0.027 , in tumors and their paired tissues, respectively, $P=1.7\times10^{-13}$. The demethylation levels of *S100P* promoter as well as the ratio of U/M levels

significantly correlated with the expression of *S100P* mRNA ($rs=0.432$, $P=3.99\times10^{-5}$ and $rs=0.475$, $P=4.98\times10^{-6}$, respectively). These data indicated that the demethylation of *S100P* promoter led to strong expression of *S100P* mRNA in CRC cell lines as well as in the primary tumors.

Expression of S100P and clinicopathological characteristics of CRC cases. Logistic analysis revealed that the expression of *S100P* protein was only associated with the location of the primary CRC tumor ($\text{Wald}=9.570$, $P=0.008$), independent of age, gender, clinical stage and the size or differentiation of the tumor.

Table II. Comparison of clinicopathological characteristics of *S100P* expression in tumor tissues and serum of CRC patients.

Characteristics	<i>S100P</i> protein in tumors			<i>S100P</i> level in serum		
	Positive	Negative	P-value	Elevated	Normal	P-value
Number of patients (%)	63 (65.6)	33 (34.4)		29 (37.7)	48 (62.3)	
Male	41 (67.2)	20 (32.8)	0.665	21 (40.4)	31 (59.6)	0.477
Female	22 (62.9)	13 (37.1)		8 (32.0)	17 (68.0)	
TNM staging (%)						
Stage I	10 (83.3)	2 (16.7)	0.458	3 (23.1)	10 (76.9)	0.312
Stage II	25 (67.6)	12 (32.4)		8 (30.8)	18 (69.2)	
Stage III	21 (58.3)	15 (41.7)		12 (44.4)	15 (55.6)	
Stage IV	7 (63.6)	4 (36.4)		6 (54.5)	5 (45.5)	
Primary tumor location (%) ^a						
Right semicolon	14 (48.3)	15 (51.7)	0.022	9 (42.9)	12 (57.1)	0.832
Left semicolon	10 (58.8)	7 (41.2)		4 (33.3)	8 (66.7)	
Rectum	39 (78.0)	11 (22.0)		16 (36.4)	28 (63.6)	
Tumor size (%) ^b (cm)						
<5	31 (66.0)	16 (34.0)	0.946	13 (35.1)	24 (64.9)	0.660
≥5	32 (65.3)	17 (34.7)		16 (40.0)	24 (60.0)	
Histological type (%)						
Adenocarcinoma	51 (66.2)	26 (33.8)	0.705	22 (32.8)	45 (67.2)	0.067
Mucoid adenocarcinoma	11 (61.1)	7 (38.9)		6 (75.0)	2 (25.0)	
Signet-ring carcinoma	1	0		1	1	
Differentiation grade (%)						
Well	14 (77.8)	4 (22.2)	0.368	7 (46.7)	8 (53.3)	0.119
Moderate	39 (65.0)	21 (35.0)		14 (29.2)	34 (70.8)	
Poor	10 (55.6)	8 (44.4)		8 (57.1)	6 (42.9)	

^aRight semicolon, caecum to splenic flexure and left semicolon, splenic flexure to sigmoid colon; ^bmeasured by maximum diameter.

We correlated the *S100P* protein expression with the clinicopathological features (Table II). The location of primary CRC tumor was significantly associated with *S100P* protein expression, from right to left, the rate of protein expression gradually increased (i.e. 48.3% in the right semicolon, 58.8% in the left semicolon, and 78.0% in the rectum, P=0.022). *S100P* protein was more frequently expressed in rectal cancer than in colon cancer (78.0 vs. 52.2%, P=0.008). The ratio of U/M of *S100P* gene promoter was also associated with the primary location of CRC (P=0.027) as analyzed by multiple linear regression. Moreover, the demethylation of *S100P* promoter in rectal cancer was significantly higher than that in colon cancer ($t=2.795$, P=0.007), which was consistent with the protein expression.

Prognostic significance of *S100P* protein expression in tumor tissues and serum *S100P* levels. The average time of follow-up in 96 patients was 52 months, ranging from 32 to 79 months. Kaplan-Meier analysis showed that *S100P* protein expression in tumor tissues was associated with the overall survival time of 96 CRC patients, but the P-value did not reach significance (P=0.185, Fig. 3A). However, in the

85 patients with stages I-III, we found the overall survival time of 56 CRC patients with expression of *S100P* protein in tumors was significantly shorter than the 29 CRC patients without expression of *S100P* protein in tumors (Kaplan-Meier analysis, P=0.031, Fig. 3B). Moreover, in patients with stage I-III, those without expression of *S100P* protein in tumors had a 3-year and 5-year 100% survival rate (29/29), whereas in those patients with positive expression of *S100P* in tumors, the 3-year and 5-year survival rate was 89% (50/56) and 82% (47/56), respectively. Cox regression analysis revealed that, in all of the 85 patients with stage I-III, the survival time of CRC patients was associated with the clinical stage of the tumor (P=0.023) and *S100P* protein expression in tissues (P=0.031), not associated with patients age, gender, primary tumor location, size and tumor differentiation. Thus, the expression of *S100P* protein in tumor tissues may be an independent factor for prognosis of CRC patients in stages I-III.

The preoperative serum *S100P* level of CRC patients was measured by ELISA, and was obtained in 77 CRC patients. The serum *S100P* level of patients (median 9.233 ng/ml, range, 0.829-70.296 ng/ml) was significantly higher than that in healthy controls (median 2.998 ng/ml, range, 0.532-

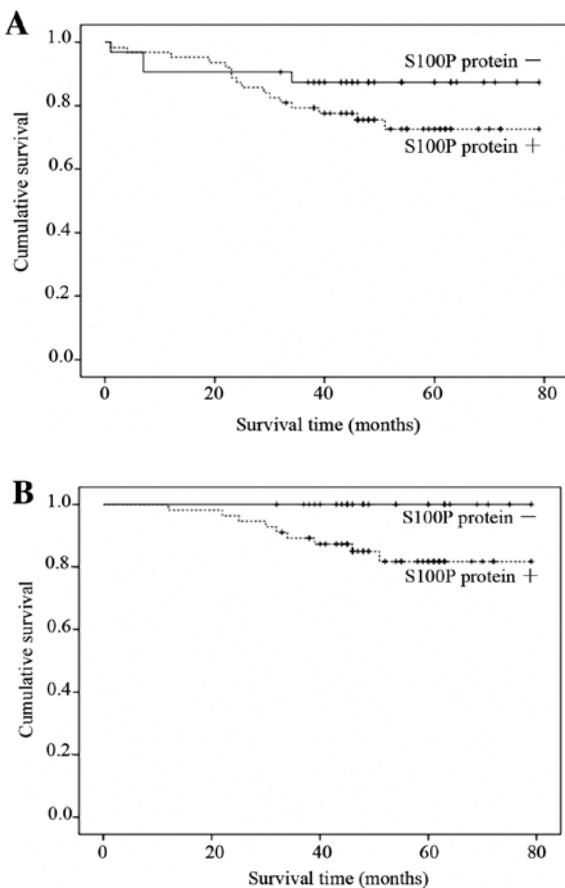


Figure 3. (A) Survival curve of *S100P* protein expression in tumor tissues of stage I-IV CRC patients (Kaplan-Meier analysis, P=0.185). (B) Survival curve of *S100P* protein expression in tumor tissues of stage I-III CRC patients (Kaplan-Meier analysis, P=0.031).

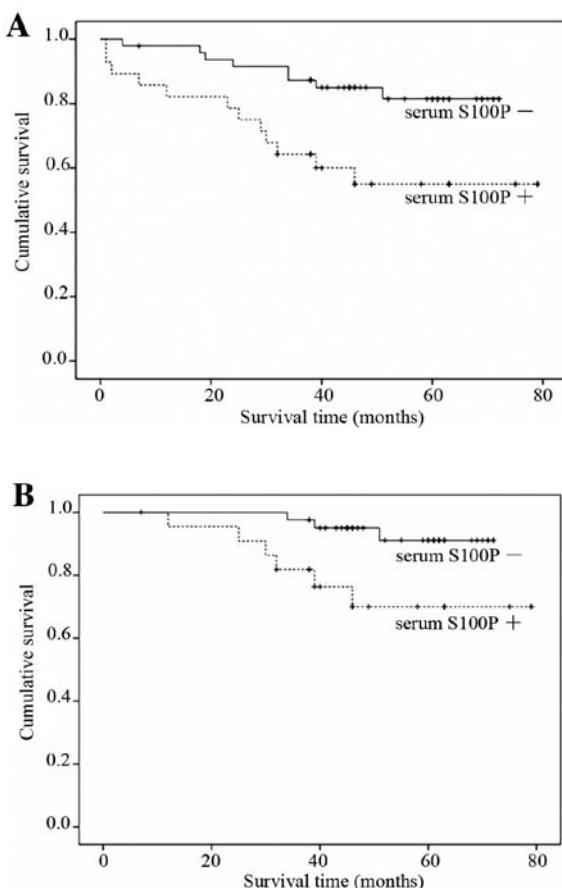


Figure 4. (A) Survival curve of pre-operative serum *S100P* level in stage I-IV CRC patients (Kaplan-Meier analysis, P=0.008). (B) Survival curve of pre-operative serum *S100P* level in stage I-III CRC patients (Kaplan-Meier analysis, P=0.017).

17.737 ng/ml, Mann-Whitney U test, P=2.5x10⁻¹⁰). We defined the cut-off value of serum *S100P* level as 12.352 ng/ml (P₉₅ of normal control level). Kaplan-Meier analysis showed that the survival times of CRC patients with serum *S100P* levels <12.352 ng/ml were longer than those with elevated *S100P* levels in 77 patients (P=0.008, Fig. 4A), and in 66 patients with stages I-III (P=0.017, Fig. 4B). The 5-year survival rate of CRC patients with normal preoperative serum *S100P* level was 81.4%, while in those with elevated serum *S100P* it was 55%. Cox regression analysis revealed that the survival time of CRC patients was significantly associated with the preoperative serum *S100P* levels (P=0.004), but not associated with age, gender, tumor size, differentiation and primary tumor location (Table II). These results also suggested that serum levels of *S100P* could be an independent factor for prognosis of CRC patients.

Discussion

Although a great improvement has been achieved over the past decade, most prognostic biomarkers are not used in clinical care (9). Novel and reliable biomarkers are required to predict prognosis of CRC. *S100P* has been detected in a variety of tissues (19) and proven to play an important role in regulation of cell functions since its discovery in 1992 (12,13). It has

become of special interest because of its ability to promote tumor invasion and metastasis (14,17,18).

We found that *S100P* protein and mRNA was expressed in 2/3 CRC tissues while only a few paired control tissues were positive for *S100P*, this was consistent with previous studies (19-21). In our group of CRC patients, all the 11 cases with stage IV died within 3 years after resection, thus, we analyzed CRC patients of stages I to III, the data showed that patients with *S100P*-positive tumors had unfavorable prognosis. Moreover, the patients with elevated serum levels of *S100P* also had shorter overall survival times compared with patients with normal *S100P* levels (P=0.008), this result is consistent with the expression of *S100P* protein in CRC tissues. These results indicated that expression of *S100P* protein in tumors and higher levels of *S100P* protein in serum were associated with unfavorable prognosis of the CRC patients in stages I-III. Detecting a biomarker in the serum is a non-invasive procedure and would have a much higher patient acceptability. The serum samples can be obtained during follow-up, thus, the easy accessibility of samples makes it easier to monitor the disease closely. To the best of our knowledge, this is the first demonstration that the serum *S100P* levels as well as *S100P* expression in cancer tissues could be used as a potential prognostic biomarker for CRC patients.

In a study of 303 breast cancer patients followed for up to 20 years, survival of patients with *S100P*-positive cancer was significantly worse than those with *S100P*-negative cancer (25). It was reported that the *S100P* expression was associated with metastasis and poor prognosis in early stage non-small cell lung cancer (26) and ovarian cancer patients (27). These previous findings and our data suggested that *S100P* protein could be a potential biomarker for prognosis in a variety of cancers.

S100P could promote the proliferation, migration, and invasion of pancreatic cancer cell via a receptor for activated glycation end products (28), it also stimulated colon cancer cell proliferation and migration *in vitro* (20,21), and lung cancer cells in an autocrine manner (26,29). These findings may, at least partially, provide molecular insight into the relationship of *S100P* and prognosis in cancers. Nevertheless, further studies focusing on the molecular mechanisms of *S100P* functions in CRC should be conducted.

The expression of *S100P* was associated with the stage of tumor in pancreatic cancer (14), prostate cancer (18), and lung cancer (30), but we did not find that *S100P* expression was associated with CRC stage. Our findings showed that the rate of expression of *S100P* protein increased from the proximal colon to the distal colon and the rectum (48, 59 and 78%, respectively).

Although there is no CpG island in the promoter and the first exon in *S100P* gene, it is of interest that demethylation of *S100P* promoter was significantly correlated with mRNA/protein expression of *S100P* in CRC tissues and cell lines. These findings are identical to previous studies on *S100P* expression and hypomethylation in pancreatic cancer (31), prostate cancer (32) and lung cancer (33). Furthermore, we identified 19 crucial CpG sites within *S100P* promoter that were significantly associated with the expression level of *S100P* mRNA for the first time. Based on these findings, we detected the methylation levels of *S100P* by QMSP. The results of QMSP were consistent with the data of BSP, confirming that our results were reliable.

In conclusion, our findings demonstrated that expression of *S100P* protein in CRC tissues and high serum levels of *S100P* could be a potential novel prognostic biomarker for CRC. *S100P* expression was significantly correlated with the demethylation or hypomethylation of the *S100P* promoter.

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