Expression status of S100A14 and S100A4 correlates with metastatic potential and clinical outcome in colorectal cancer after surgery

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Abstract. To investigate whether S100A14 and S100A4 expression correlates with metastatic potential and prognosis in colorectal cancer (CRC), we firstly used RT-PCR analysis to detect mRNA expression of S100A14 and S100A4 in 40 pairs of fresh tumor samples matched with adjacent normal tissues. We then evaluated the clinical significance of our findings with immunohistochemistry on 115 samples of formalin-fixed and paraffin-embedded tumors on tissue micro-arrays. Typically, we identified decreased S100A14 mRNA levels (52.5%, 21/40), and increased S100A4 mRNA levels (70.0%, 28/40) in primary CRC samples. In addition, down-regulated or absent S100A14 expression was detected in 56.5% of samples (65/115) and was correlated with poor differentiation (P=0.010). In contrast, overexpressed S100A4 was detected in 57.4% of samples (66/115) and was associated with lymph node metastasis (P=0.001). Simultaneous S100A14 low-expression and S100A4 high-expression was correlated with high CRC metastatic potential (P<0.001). Taken together, the signature derived from the combined expression status of S100A14 and S100A4 could be a valuable prognostic indicator in CRC.

Introduction

The S100 proteins are a family of 21 small, acidic proteins with calcium binding motifs. The S100 family of calcium binding proteins comprises at least 25 members, forming the largest group of EF-hand signaling proteins in humans (1).

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Although structurally similar, the S100 proteins have been shown to be involved in a variety of different physiological functions in human tissues, such as calcium homeostasis, protein phosphorylation, cell proliferation and differentiation, the cytoskeleton, extracellular signal transduction, inter-cellular adhesion and motility as well as cancer metastasis (2-6). Currently, the S100 family is of great interest because some members show deregulated expression in human diseases, especially in cancer.

Among the S100 family members, S100A4, a known metastasis-associated gene, was isolated by subtractive hybridization of non-metastatic cells from metastatic mouse mammary adenocarcinoma cells (8). It is implicated in metas-tasis because of its overexpression in many human solid cancers including gastric, colorectal and breast cancers (9-14). Additionally, the expression of S100A4 in breast cancer, esophageal squamous cell cancer, gall bladder cancer, non-small cell lung cancer, gastric cancer and colorectal cancer (CRC) has been associated with poor survival (14-19).

The S100 proteins are expressed in many tissues; however, in general, each protein is preferentially expressed in one particular tissue. For instance, S100A14 was first cloned and characterized by analyzing a human lung cancer cell line subtraction cDNA library, and was mapped to human chromosome 1q21 (20). S100A14 mRNA was initially shown to be expressed in several normal human tissues of epithelial origin, and most abundantly in normal colorectal mucosa. Significantly, down-regulated levels of *S100A14* mRNA were detected in colorectal tumor tissues (20). S100A1, S100A2, S100A11, S100A14 and S100Z have been postulated to have a tumor suppressor function because of their decreased expression in many types of human cancer (21-24).

Although overexpression of S100A4 in CRC has been reported, little is known about the expression levels of S100A14 in matched samples of non-cancerous and cancerous parts of colorectal tumors. There is no available data on co-expression of S100A14 and S100A4 in the development and progression of CRC. In the present study, we evaluated the expression levels of S100A14 and S100A4 mRNA in five CRC cell lines and in 40 matched sample pairs of primary CRC and adjacent normal colorectal mucosa. We

also performed parallel immunohistochemical staining of S100A14 and S100A4 to analyze their association with clinicopathological characteristics in 115 CRCs, and tried to clarify the distinct roles of S100A14 and S100A4 in the progression of human CRC.

Materials and methods

Patients and tissues. Specimens were obtained from 115 patients with primary CRC diagnosed and treated in the Department of Surgery, School of Oncology, Peking University, from 1999 to 2001. Fresh tissue samples were transferred immediately to liquid nitrogen and stored at -80˚C for later RT-PCR. Tissue microarray blocks containing formalin-fixed and paraffin-embedded human tissues were constructed in our laboratory as described previously (25). Informed consent was obtained from all patients. All patients underwent surgery and none of the patients had received chemotherapy or radiation therapy. Histopathological analyses were performed independently by two pathologists. Tumor stage was classified according to the American Joint Committee on Cancer (AJCC) TNM classification (26). In addition, the patients were divided into proximal and distal tumor groups according to the anatomic location of their tumors, as described previously (27).

Cell lines and cell culture. CRC cell lines RKO, SW480, SW620, LoVo and HT29 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA), supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were maintained at 37˚C in 5% CO_2.

Extraction of total RNA and RT-PCR. Total RNAs were isolated from fresh tissues using TRIzol reagent (Qiagen, USA). The prepared RNA (5 μg) was mixed with oligo-dT primer and was reverse-transcribed with MMLV reverse transcriptase (Promega, USA) for 60 min at 37˚C, followed by PCR amplification with specific primers for the *S10014* gene (F, 5'-ACT CTC ACC AAA GGA CCA GAC AC-3', R, 5'-CAG GTG CAG GCT AGG GTA CAG-3') or the *S100A4* gene (F, 5'-CCT GGA TGT GAT GGT GTC-3', R, 5'-TCT TCC TGG GCT GCT TAT-3'). PCR amplification was performed in 20 μl using a thermocycler (Biometra, Germany) with the following PCR program: predenaturation for 3 min at 94˚C, denaturation for 45 sec at 94˚C, annealing for 45 sec at 55˚C, extension for 45 sec at 72˚C and a final elongation at 72˚C for 10 min. ß-actin served as an internal positive control. PCR products were analyzed by 1.5% agarose gel electrophoresis and band intensity was measured directly on an Alphaimager 2200 analysis system (Alpha Innotech, San Leandro, CA). The amount of each mRNA was expressed as a ratio between S100A14 or S100A4 and ß-actin.

Tissue microarray immunohistochemistry. Formalin-fixed and paraffin-embedded tissue array sections were cut into 5-μm-thick sections (average area 2.0 cm^2), deparaffinized in xylene and rehydrated. Antigen retrieval was performed in a 650 W microwave for 10 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Immuno-

Table I. Distribution of clinicopathological features, S100A14 and S100A4 in patients with colorectal cancer.

Clinicopathological features	No. of cases	(%) of patients
All patients	115	
Sex		
Male	63	54.8
Female	52	45.2
Age at diagnosis		
<60	55	47.8
≥60	60	52.2
Tumor site		
Proximal colon	49	42.6
Distal colon	28	24.3
Rectum	38	33.1
Degree of differentiation		
Well	20	17.4
Moderate	52	45.2
Poor	43	37.4
T stage		
T1	3	2.6
T2	19	16.5
T3	62	53.9
T4	31	27.0
N stage		
N0	55	47.8
N1	38	33.1
N2	22	19.1
M stage		
M0	97	84.3
M1	18	15.7
S100A14		
Positive	50	43.5
Negative	65	56.5
S100A4		
Positive	66	57.4
Negative	49	42.6

histochemistry was performed using Power Vision™ Two-Step Histostaining (ImmunoVision, USA). A monoclonal antibody specific to human S100A14 (LA-5) was produced in our laboratory. LA-5 (diluted 1:100) or a rabbit anti-S100A4 polyclonal antibody (diluted 1:200; NeoMarkers, USA) were incubated on the sections overnight at 4˚C. Antibody binding was visualized using a 3,3-diaminobenzidine (DAB) Kit (Zhongshan Jinqiao, China) according to the manufacturer's instructions. For general negative controls, the primary antibodies were replaced by PBS.

All sections were examined microscopically and scored by two independent pathologists who were blinded to the clinical information pertaining to the subjects. Evaluation of S100A14 and S100A4 immunohistochemical staining was performed according to the percentage of positively
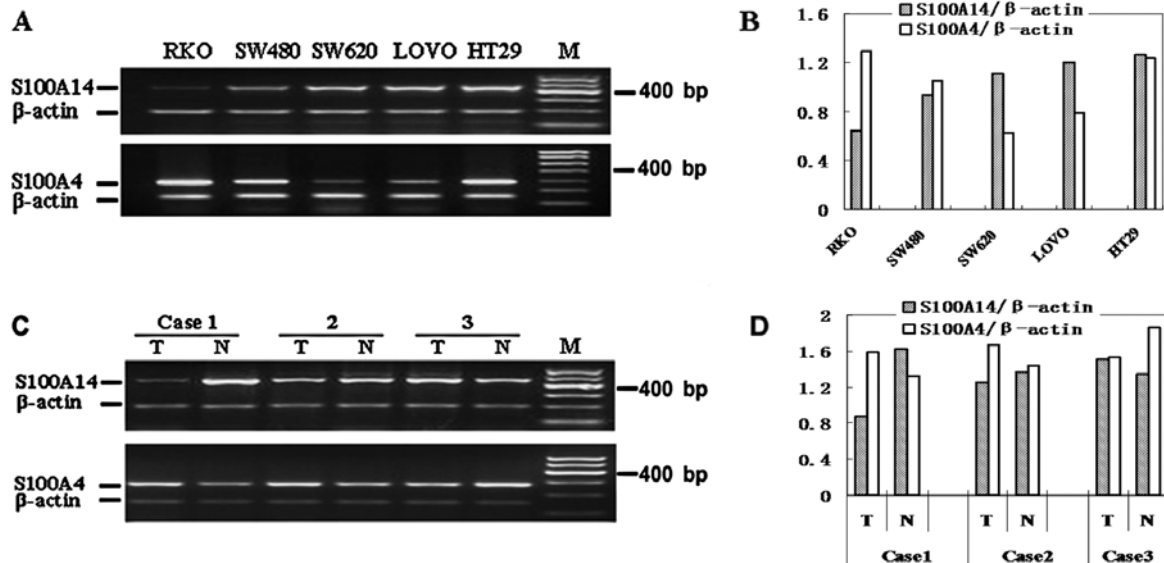
Figure 1. Detection of *S100A14/S100A4* mRNA in colorectal cancer cell lines and tissues. (A and B) RT-PCR demonstrated *S100A14/S100A4* mRNA expression in five colorectal cancer cell lines: RKO, SW480, SW620, LoVo and HT29. (C and D) Differential expression of *S100A14* and *S100A4* mRNA was detected in colorectal tumors and matched normal tissues by RT-PCR. ß-actin served as an internal control.

stained tumor cells. When 30% or more of the tumor cells were stained for S100A14 on the cell membrane, the tumors were defined as increased (positive) S100A14 expression. In contrast, the cases were evaluated as decreased (negative) S100A14 expression when S100A14 immunoreactivity was found in <30% of the tumor cells. For S100A4, cases in which at least 20% of the tumor cells were immunoreactive were scored as positive.

Statistical analysis. Statistical analysis was performed using the SPSS software package (version 13.0, SPSS Inc., USA). The χ^2 test was used to evaluate relationships between the clinicopathological variables and S100A14 and S100A4 expression. Kaplan-Meier survival analysis with the log-rank test was used to evaluate the prognosis of the patients according to their levels of S100A14 and S100A4 expression. Multivariate analysis was performed with the Cox pro-portional hazards regression model to assess the effects of different variables on patient survival. Differences were considered significant at $P<0.05$.

Results

Clinicopathological characteristics at the time of diagnosis in patients with CRC. Sixty-three males and 52 females were included in the study. The mean age of the patients at surgery was 59 years (range, 26-72). There were 49 right-sided tumors, 28 left-sided tumors and 38 rectal tumors (Table I). Clinical follow-up data were available for all patients. The median follow-up was 62 months (range 4-76); 50 of 115 patients (43.5%) died of cancer metastasis or local recurrence after surgery.

S100A14 and S100A4 mRNA expression in CRC cell lines. To examine whether S100A14 is expressed in CRC, we firstly

examined the expression of S100A14 mRNA in human CRC cell lines (RKO, SW480, SW620, LoVo and HT29). As shown in Fig. 1A, SW480, SW620, LoVo and HT29 cells expressed a substantial amount of S100A14 mRNA, whereas RKO cells only a slight amount. S100A4 mRNA was also detected in these cell lines. RKO, SW480 and HT29 expressed high levels of S100A4 mRNA; lower levels of S100A4 mRNA were found in SW620 and LoVo cells (Fig. 1A). Fig. 1B shows the ratios of S100A14 or S100A4 levels to ß-actin in these cell lines.

Among these cell lines, reciprocal expression of S100A14 and S100A4 mRNA was found in four cell lines (RKO, SW480, SW620 and LoVo). Interestingly, our previous study demonstrated that RKO cells had higher hepatic metastasis potential (28).

S100A14 and S100A4 mRNA expression in primary CRC. Next, we examined by RT-PCR the expression levels of S100A14 and S100A4 mRNA in matched samples of pri-mary CRC and adjacent normal mucosa. Of the 40 cases analyzed, we found that S100A14 mRNA was down-regulated in 52.5% (21/40) and up-regulated in 47.5% (19/40) of CRC specimens. In contrast, S100A4 mRNA demonstrated high expression levels in 70.0% (28/40) and low expression levels in 30.0% (12/40) of samples. S100A14 and S100A4 mRNA expression showed an inverse pattern in primary CRC. Representative examples of the expression level of S100A14 and S100A4 mRNA are shown in Fig. 1C and D.

Association of the immunohistochemical expression status of S100A14 and S100A4 with clinicopathological features. In normal colorectal mucosa, most specimens showed a moderate to strong uniform immunopositivity for S100A14, but the expression of S100A4 was mainly faint or negative. In the present study, negative or absent expression of S100A14
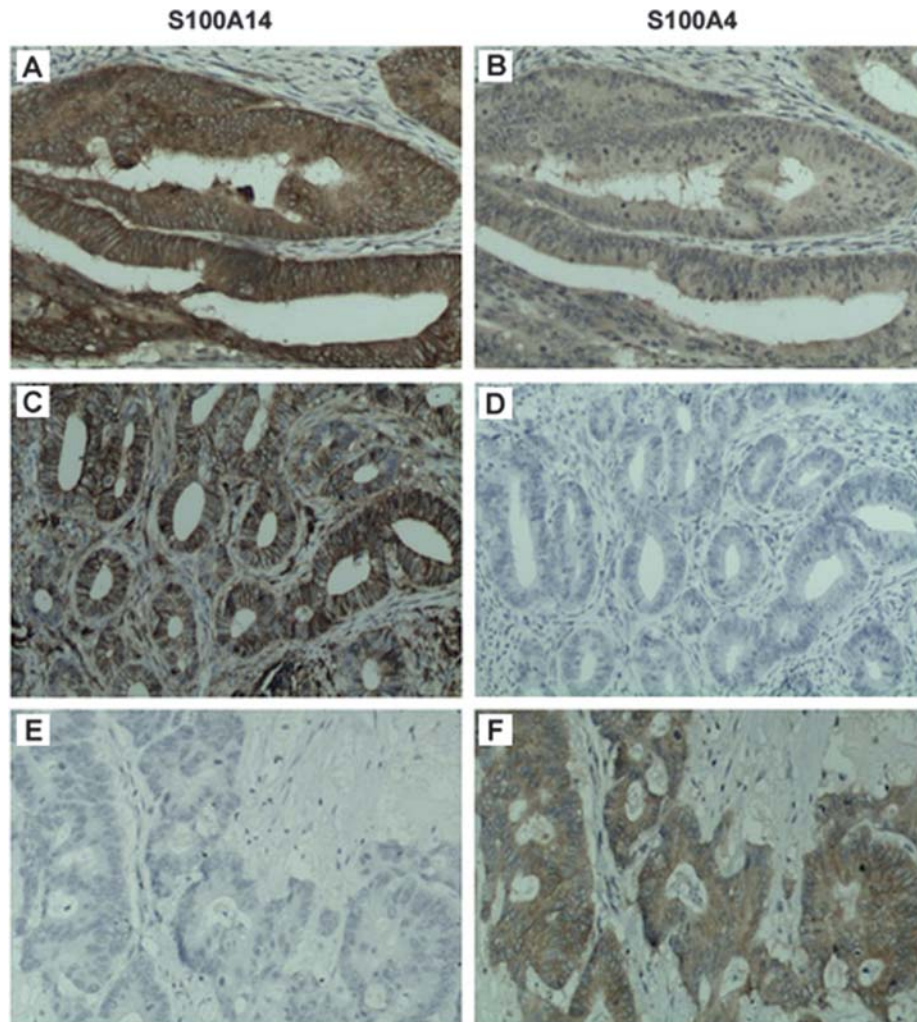
Figure 2. Representative immunohistochemical staining of S100A14 and S100A4 protein in the same tumor. S100A14$^+$/S100A4$^+$ tumor (A and B). S100A14$^+$/S100A4$^-$ tumor (C and D). S100A14$^-$/S100A4$^+$ tumor (E and F).

was found in 56.5% (65/115) of primary CRC specimens, whereas positive S100A14 expression was detected in 43.5% (50/115). S100A14 expression was mainly found on the cell membrane of tumor cells, and well-differentiated cancer tended to show uniform S100A14 expression (Fig. 2A and C). Overexpression of S100A4 was observed in 66 (57.4%) cancers. The subcellular location of S100A4 protein was mainly in the cytoplasm (Fig. 2B and F). There was a statistically inverse trend between positive expression of S100A14 and S100A4 (P=0.075).

Further analysis of the clinicopathological characteristics of the 115 CRC specimens revealed that there was a significant correlation of S100A14 expression with tumor differentiation and tumor distant metastasis (P=0.010 and P=0.048, respectively). As reported previously, there was also a strong correlation of S100A4-positive expression with lymph node involvement and tumor distant metastasis (P=0.001 and P=0.003, respectively). These results are summarized in Tables II and III.

To our knowledge, this is the first study in which S100A14 and S100A4 expression have been immunohistochemically assessed in CRC and related to clinicopathological findings and prognosis. The CRCs were subdivided into four groups

according to the expression status of S100A14 and S100A4 as follows: S100A14$^-$/S100A4$^+$, S100A14$^-$/S100A4$^-$, S100A14$^+$/S100A4$^+$ and S100A14$^+$/S100A4$^-$, where + indicates high expression and - indicates low expression. The number of tumors in each group was 42 (36%), 23 (20%), 24 (21%) and 26 (23%), respectively. Representative immunohistochemical staining of S100A14 and S100A4 protein in the four subgroups is shown in Fig. 2. S100A14$^-$/S100A4$^+$ tumors showed a strong correlation with lymph node involvement and tumor distant metastasis (P=0.006 and P<0.001, respectively) (Table IV). These data suggest that combined analysis of S100A14 and S100A4 expression provides useful information and may enhance accuracy in identifying patients at high risk of regional lymph node or distant metastasis.

Survival analyses of patients according to S100A14 and S100A4 expression status after surgery. Among the 115 CRC patients, the 5-year survival rate was 56.5%. Patients with S100A14$^-$ tumors had significantly reduced overall survival compared with those with S100A14$^+$ tumors (P<0.001, Fig. 3A). In contrast, patients with S100A4$^+$ tumors had a significantly poorer prognosis than those with S100A4$^-$ tumors

Table II. S100A14 status in relation to clinicopathological features in patients with colorectal cancer [n (%)].

| Variables | No. of cases | S100A14 | | p-value |
		Positive (%)	Negative (%)	
Sex				
Male	63	27 (42.9)	36 (57.1)	
Female	52	23 (44.2)	29 (55.8)	NS
Age at diagnosis				
<60	55	27 (49.1)	28 (50.9)	
≥60	60	23 (38.3)	37 (61.7)	NS
Tumor site				
Proximal colon	49	20 (40.8)	29 (59.2)	
Distal colorectum	66	30 (45.5)	36 (54.5)	NS
Degree of differentiation				
Well/moderate	72	38 (52.8)	34 (47.2)	
Poor	43	12 (27.9)	31 (72.1)	0.010
Tumor depth				
T1-T2	22	11 (50.0)	11 (50.0)	
T3-T4	93	39 (41.9)	54 (58.1)	NS
Lymph node status				
N0	55	28 (50.9)	27 (49.1)	
N1-2	60	22 (36.7)	38 (63.3)	NS
Distant metastasis				
M0	97	46 (47.4)	51 (52.6)	
M1	18	4 (22.2)	14 (77.8)	0.048

NS, not significant.

Table III. S100A4 status in relation to clinicopathological features in patients with colorectal cancer [n (%)].

| Variables | No. of cases | S100A4 | | p-value |
		Positive (%)	Negative (%)	
Sex				
Male	63	33 (52.4)	30 (47.6)	
Female	52	33 (63.5)	19 (36.5)	NS
Age at diagnosis				
<60	55	30 (54.5)	25 (45.5)	
≥60	60	36 (60.0)	24 (40.0)	NS
Tumor site				
Proximal colon	49	25 (51.0)	24 (49.0)	
Distal colorectum	66	41 (62.1)	25 (37.9)	NS
Degree of differentiation				
Well/moderate	72	39 (54.2)	33 (45.8)	
Poor	43	27 (62.8)	16 (37.2)	NS
Tumor depth				
T1-T2	22	12 (54.6)	10 (45.4)	
T3-T4	93	54 (58.1)	39 (41.9)	NS
Lymph node status				
N0	55	23 (41.8)	32 (58.2)	
N1-2	60	43 (71.7)	17 (28.3)	0.001
Distant metastasis				
M0	97	50 (51.5)	47 (48.5)	
M1	18	16 (88.9)	2 (11.1)	0.003

NS, not significant.

Table IV. S100A14 and S100A4 status in relation to clinicopathological features in patients with colorectal cancer [n (%)].

| Variables | No. of cases | S100A14 and S100A4 expression | | | p-value |
		A14⁻/A4⁺	A14⁻/A4⁻ or A14⁺/A4⁺	A14⁺/A4⁻	
Sex					
Male	63	20 (31.7)	29 (46.0)	14 (22.2)	
Female	52	22 (42.3)	18 (34.6)	12 (23.1)	NS
Age at diagnosis					
<60	55	16 (29.1)	23 (41.8)	16 (29.1)	
≥60	60	26 (43.3)	24 (40.0)	10 (16.7)	NS
Tumor site					
Proximal colon	49	26 (53.1)	8 (16.3)	15 (30.6)	
Distal colorectum	66	16 (24.2)	39 (59.1)	11 (16.7)	NS
Degree of differentiation					
Well/moderate	72	19 (26.4)	30 (41.7)	23 (31.9)	
Poor	43	23 (53.5)	17 (39.5)	3 (7.0)	NS
Tumor depth					
T1-T2	22	7 (31.8)	9 (40.9)	6 (27.3)	
T3-T4	93	35 (37.6)	38 (40.9)	20 (21.5)	NS
Lymph node status					
N0	55	12 (21.8)	26 (47.3)	17 (30.9)	
N1-2	60	30 (50.0)	21 (35.0)	9 (15.0)	0.006
Distant metastasis					
M0	97	29 (29.9)	43 (44.3)	25 (25.8)	
M1	18	13 (72.2)	4 (22.2)	1 (5.6)	<0.001

NS, not significant.

Table V. Multivariate analysis of prognostic factors in patients with colorectal cancer.

Variables	Indicator of poor prognosis	RR	95% CI	p-value
Distant metastasis	M1	5.25	2.94-9.38	<0.001
S100A14/S100A4 status	S100A14⁻/S100A4⁺	3.11	1.46-6.61	0.006
S100A14 expression	Negative	2.54	1.27-5.12	0.030

RR, relative risk; CI, confidence interval.

(P=0.009, Fig. 3B). Subsequently, we analyzed the survival curves of the four groups according to the expression status of S100A14 and S100A4. Patients with S100A14⁺/S100A4⁻ tumors had the longest survival among these groups. The S100A14⁻/S100A4⁻ group and the S100A14⁺/S100A4⁺ group showed similar survival curves. The S100A14⁻/S100A4⁺ group had the poorest survival (P<0.001, Fig. 3C).

     Furthermore, we analyzed our data with a Cox proportional hazards model using tumor differentiation, lymph node involvement, tumor distant metastasis and S100A14/S100A4 expression status as covariates. Tumor distant metastasis was the strongest prognostic factor, S100A14⁻ expression and S100A14⁻/S100A4⁺ expression status were independent prognosis factors (Table V).

Discussion

To our knowledge, this is the first study showing the status of S100A14 expression in a large series of CRC patients. S100A14 was identified as being a useful marker, adding prognostic information to the clinical course of patients with CRC. Interestingly, our results revealed that reduced expression of S100A14 tended to co-occur with increased expression of S100A4 in CRC cell lines and tissue specimens, as demonstrated by RT-PCR analysis and immunohistochemistry.

     In the present study, we evaluated the expression of S100A14, and correlated this with the clinical features of patients with CRC. The expression and subcellular location
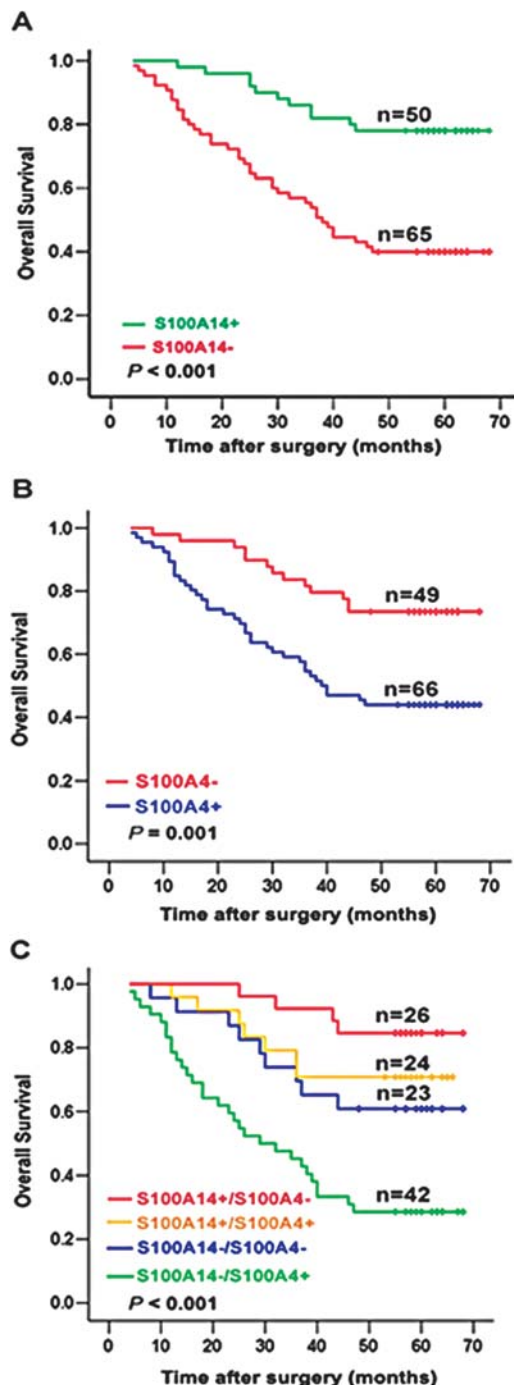
Figure 3. Kaplan-Meier overall survival analysis of colorectal cancer patients according to the expression status of S100A14 (A), S100A4 (B) and the combined expression of S100A14 and S100A4 (C).

of S100A14 protein was studied by immunohistochemistry in 115 CRCs and matched normal colorectal mucosal specimens. These results revealed that S100A14 was mainly expressed in the cell membrane of the epithelial cells; most of the normal colorectal mucosal specimens showed homogeneous immunopositivity. In CRC, positive S100A14 expression was detected in 43.5% of cancers, whereas negative or absent S100A14 expression was found in 56.5%. Consistent with these results, *S100A14* mRNA was differentially expressed in CRC cell lines and matched samples of primary CRC

and adjacent normal mucosa. Among 40 cases analyzed, we found that *S100A14* mRNA was up-regulated in 47.5% and down-regulated in 52.5%. Previous studies have provided evidence for differential expression of S100A14 in ovarian, breast, kidney and uterine tumors, suggesting a potentially important function for this gene in malignant transformation (20).

Based on our immunohistochemical assessment, we further analyzed the association between S100A14 expression and clinicopathologic variables. Strong correlations were found between S100A14 expression and the degree of tumor differentiation. A higher incidence of S100A14 expression was observed in well- and moderately-differentiated tumors, which indicated that this protein might function in cell differentiation. Our results agree with a previous report that some members of the S100 protein family are involved in cell differentiation (29).

S100A4 was believed to be a metastasis-inducing protein and associated with cancer invasion and metastasis because S100A4 protein secreted from tumor cells can increase endothelial cell motility and induce angiogenesis (30). Immunohistochemical expression of S100A4 has been proven to be a highly significant, independent prognostic marker in a large series of CRC patients (18). In the present study, overexpression of S100A4 was found in 66 (57.4%) of 115 CRC specimens and was closely associated with metastatic potential and poor prognosis of CRC. Flatmark *et al* (13) reported that overexpression of S100A4 was more frequently found in liver metastases than in primary tumors. These data further support the significant correlation between over-expression of S100A4 and progression of CRC.

Currently, other than the stage of the tumor at the time of the resection, few molecular markers are accepted as indicators of prognosis in CRC. This is mainly due to the conflicting results reported in the literature. Several studies have indicated that altered expression of S100 members such as S100A2, S100A4, S100A6, S100A11, S100C and S100P might be informative markers for genesis, progression and prognosis in some human tumors including gastric cancer, CRC, esophageal cancer and gall bladder cancer (31-35). A recent study proposed that stratification of patients into groups according to the expression of S100A4 and S100P might facilitate more accurate prediction of survival in breast cancer patients (36). Our studies showed that S100A14⁻/S100A4⁺ CRC had a highly invasive and metastatic potential, compared with S100A14⁺/S100A4⁻ tumors. Furthermore, S100A14⁻/S100A4⁺ status was specifically correlated with lymph node and distant metastasis. The Cox proportional hazard model demonstrated that S100A14⁻/S100A4⁺ CRC had a 3-fold higher relative risk of death than S100A14⁺/S100A4⁻ CRC. These results suggest that a combined analysis of S100A14 and S100A4 expression status may enhance our accuracy in identifying patients at high risk of regional lymph node or distant metastasis, and hence provide useful information for clinical management.

In conclusion, our study demonstrated that S100A14 and S100A4 expression plays a role in the progression and metastasis of CRC. The simultaneous immunohistochemical detection of S100A14 and S100A4 expression may be useful to evaluate the prognosis of patients with CRC.

Acknowledgements

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