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ORIGINAL ARTICLE – COLORECTAL CANCER

S100B Protein Expressions as an Independent Predictor of Early Relapse in UICC Stages II and III Colon Cancer Patients after Curative Resection

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ABSTRACT

Background. S100 calcium-binding proteins such as S100B are elevated in primary malignant melanoma and are used as tumor markers for malignant melanoma and numerous other cancers. The purpose of this study was to identify the novel predictors of early relapse in UICC stages II and III colon cancer patients and thus to identify a subgroup of patients who are at high risk for postoperative early relapse.

Methods. Clinicopathological factors and S100B expression by immunohistochemical staining were retrospectively analyzed in 357 postoperative UICC stages II and III colon cancer patients to determine the predictors of early relapse.

Results. Of 357 patients, 114 patients developed postoperative relapse during the follow-up period. Among 114 relapsed colon cancer patients, postoperative early relapse and non-early relapse were found in 56 patients (49.1%) and 58 patients (50.9%), respectively. Multivariate Cox proportional hazards analysis revealed that the presence of vascular invasion ($P = .025$; hazard ratio [HR], 5.532; 95% confidence interval [95% CI], 1.985–14.729), high postoperative CEA levels ($P = .019$; HR, 6.845; 95% CI, 2.393–15.256), and S100B overexpression ($P < .001$; HR, 26.250; 95% CI, 7.463–96.804) were demonstrated to be independent predictors of postoperative early relapse. Furthermore, postoperative relapsed colon cancer patients with S100B overexpression were demonstrated to have significantly lower overall survival rates than those without S100B overexpression ($P < .001$).

Conclusions. This study suggests that S100B protein expression is a crucial predictor of early relapse in UICC stages II and III postoperative colon cancer patients and thus could help to define patients with this tumor entity who would benefit from enhanced follow-up and therapeutic program(s).

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Colorectal cancer (CRC) is a common neoplasia in the world and a major cause of cancer-related death.[1] Although

there have been significant improvements in the treatment of patients with advanced CRC, individuals with advanced disease still have a poor prognosis.[2] Surgery remains the mainstay of therapy, but recurrence after curative surgery of CRC occurs at a constant rate according to the stage of the disease, and the more advanced the stage, the greater the recurrence rate.[3] The recurrence of CRC is for the most part a time-limited phenomenon, and 40–50% of recurrences become apparent within the first year after initial resection, and 90% within the first 4 years.[4] Recurrences in stage II and III patients show rapid increase for the first 3 years, and the cumulative appearance rates of recurrence at 1 year for stages I, II, and III in one study were 21.6, 31.0, and 44.7%, respectively.[5] Efforts have concentrated on the early detection of tumors to ensure adequate and effective treatment and to improve patient prognosis. The identification of specific colon tumor-associated proteins and new potential markers for effective early detection and disease monitoring would significantly advance the diagnosis and possibly the treatment of CRC. However, it remains a challenge for early detection and treatment of human CRC on postoperative relapse. Potential candidates that can assist diagnosis include serum carcinoembryonic antigen (CEA) level, vascular endothelial growth factor, tumor-necrosis-factor-related apoptosis-inducing ligand, oncogenes, and suppressor genes such as *c-myc*, *KRAS*, *TP53*, *CDKN1A*, and *BCL2/Bax*.[6]

The S100 family is a highly conserved group with more than 20 members of small, acidic calcium-binding proteins in vertebrates.[7] S100 proteins regulate intracellular processes such as cell growth and motility, cell-cycle regulation, transcription, and differentiation. In addition, they bind to different oligomeric forms of the tumor suppressor p53 and regulate its activity.[8] The binding of S100B to p53 disables the biological function of p53 as a tumor suppressor and probably causes cancer.[9] These activities are mediated via interactions with target proteins such as annexins, cytosolic phospholipase A2, the Ca^{2+} release channel of the sarcoplasmic reticulum, and myosin.[10] S100A protein was expressed predominantly in the cytoplasm of normal tissue; however, it was expressed in both the nuclei and cytoplasm of CRC. S100A11, a member of the S100 family, is an EF-hand type Ca^{2+}-binding protein. Lately, the correlation between S100A11 expression and tumor stage is studied by Western blotting and immunohistochemical staining, and results shows that the level of S100A11 in CRC tissue is increased following stage progression of the disease.[11] Additionally, S100B has been proved to be a prognostic marker in patients with metastatic melanoma to follow up on disease-free stages II and III in melanoma patients.[12] Egberts et al. indicated that S100B protein might be used as a highly specific and relatively sensitive marker of early distant metastasis for prospective monitoring of adjuvant treatment in high-risk melanoma patients.[13] However, to date, the role of S100B protein in the prediction of early relapse of postoperative colon cancer patients is poorly understood.

Based on our previous data of cDNA microarray by comparing colorectal tumor, CRC and adjacent normal tissues, we identified a calcium-binding protein, S100B, which was highly expressed in CRC compared with adjacent normal tissues, and was probably related to CRC progression.[14] The aims of the present study was to determine the correlation of S100B expression and clinicopathologic features and early relapse of American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) stages II and III colon cancer patients postoperatively, allow a more accurate and early diagnosis, and also represent possible targets in the clinical management of these patients.

MATERIALS AND METHODS

Patients and Sample Collection

Between January 2004 and June 2008, a total of 357 UICC stages II and III colon cancer patients who underwent a curative resection from the Kaohsiung Medical University Hospital were reviewed. Curative surgery was defined as any gross residual tumor that did not remain in the surgical bed and in which the surgical resection margin was pathologically negative for tumor invasion. The development of new postoperative recurrent or metastatic lesions was defined as postoperative relapse. Early relapse was defined as local recurrence (tumor growth restricted to the anastomosis or the region of the primary operation) or distant metastasis (distant metastasis or diffuse peritoneal seeding) within 1 year after radical resection.[15,16] We intensively followed up these enrolled patients until July 2009. Data acquisition and subsequent use were also approved by the hospital's institutional review board. High-risk stage II or III patients received adjuvant chemotherapy. Patients with risk factors for relapse (poorly differentiated, tumor perforation or obstruction, number of retrieved lymph nodes < 12, or lymphatic/vascular invasion) were considered as high-risk stage II cases. Postoperative surveillance consisted of medical history, physical examination, and laboratory studies including serum CEA levels every 3 months. Abdominal ultrasonography or computed tomography (CT) was performed every 6 months, and chest radiography and total colonoscopy were performed once a year. Patients were followed up at 3-month intervals for 2 years and 6-month intervals thereafter. The median follow-up time was 33 months (range 14–66 months). Of these patients, 114 cases (31.9%) developed postoperative relapse during the follow-up period. Of all

TABLE 1 Clinicopathologic characteristics of 357 UICC stage II and III colon cancer patients

Variables	Number (%)
Gender, male/female	193 (54.1)/164 (45.9)
Age (y/o, mean ± SD)	63.7 ± 11.9
Maximum size, <5/≥5 (cm)	191 (53.5)/166 (46.5)
Location, right-sided/left-sided	143 (40.1)/214 (59.9)
Depth of invasion, $T_2/T_3/T_4$	71 (19.9)/270 (75.6)/16 (4.5)
Lymph node metastasis, yes/no	204 (57.1)/153 (42.9)
UICC stage, II/III	153 (42.9)/204 (57.1)
Vascular invasion, yes/no	136 (38.1)/221 (61.9)
Perineural invasion, yes/no	131 (36.7)/226 (63.3)
Histology, WD/MD/PD	32 (9.0)/289 (81.0)/36 (10.1)
Postoperative CEA, <5/≥5 (ng/ml)	250 (70.0)/107 (30.0)
Postoperative relapse, yes/no	114 (31.9)/243 (68.1)
Early postoperative relapse, yes/no	56 (15.7)/301 (84.3)
Type of postoperative relapse ($n = 114$), distant/local or metachronous	90 (78.9)/24 (21.1)
S100B overexpression, yes/no	68 (19.0)/289 (91.0)
Adjuvant therapy, yes/no	232 (65.0)/125 (35.0)

UICC International Union Against Cancer, *Right-sided* cecum + ascending colon + transverse colon, *left-sided* descending colon + sigmoid colon, *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated, *CEA* carcinoembryonic antigen

these patients, there were 62 males and 52 females. Of 114 relapsed colon cancer patients, postoperative early relapse and non-early relapse was found in 56 patients (49.1%) and 58 patients (50.9%), respectively (Table 1). The study protocol was approved by the Institutional Review Board.

Immunohistochemistry

Paraffin sections (4 μm) were immunostained using Mouse/Rabbit PolyDetector HRP w/DAB system (Bio SB, Inc. Santa Barbara, CA). Five sections of each tumor tissue were stained per case. Antigen retrieval was done using microwave heating for 10 min in 10 mM citrate buffer (pH 6.0). Briefly, each section was dewaxed in xylene and rehydrated with alcohol. The slides were then incubated in PolyDetector Peroxidase Block for 5 min to block endogenous peroxidase activity. After washing in phosphate-buffered saline, the slides were incubated with diluted primary mouse monoclonal antibodies against S100B calcium binding protein (1:100; GeneTex, Inc. Irvine, CA). The horseradish peroxidase labeled second antibody was used to detect the primary antibodies. The reaction was developed with diaminobenzidine as chromogen, and the sections were subsequently counterstained with light hematoxylin. After counterstaining with hematoxylin, the slides were mounted with coverslips in xylene-based mounting medium and examined under the microscope.

To examine the possibility of false positive results, we used a nonimmune antiserum instead of the primary antibody as negative control. The percentage of positively stained tumor cells was evaluated for each tumor section. The tumors were considered positive immunoreactive if >5% of the neoplastic cells showed both cytoplasmic staining and nuclear staining. When 5% or less was stained, the results were considered negative. Semiquantitative scores were used for S100B stains according to the percentage of positively stained cells (+, 6–10%; ++, 11–25%; +++, 26–50%; ++++, >50%). Cancer tissues that expressed scores of ++ or +++ or ++++ were regarded as the S100B overexpression group, whereas those with scores of + or negative staining were regarded as the S100B non-overexpression group.

Statistical Analysis

All data were statistically analyzed using the Statistical Package for the Social Sciences, version 12.0 (SPSS Inc., Chicago, IL). A P value less than .05 was considered statistically significant. The univariate analysis of clinicopathologic features between the 2 groups (early relapse versus non-early relapse) was compared using the chi-square test. Independent predicting factors for postoperative early relapse was determined using the multivariate Cox proportional hazards regression analysis. The cumulative overall survival rates were calculated by the Kaplan–Meier method, and the differences in survival rates between S100B overexpression group and S100B non-overexpression group were analyzed by the log-rank test.

RESULTS

The average age was 63.7 years (range 29–88 years, Table 1). There were 143 tumors (40.1%) at the right-sided colon and 214 (59.9%) at the left-sided colon. With regard to the histological type of these tumors, 32 (9.0%) were well differentiated, 289 (81.0%) were moderately well differentiated, and 36 (10.1%) were poorly differentiated carcinoma. For the clinicopathologic characteristics of these 153 UICC stage II and 204 UICC stage III colon cancer patients, 136 of 357 patients (38.1%) were identified to have vascular invasion; 131 of 357 patients (36.7%) were found to have perineural invasion. Of the 357 cases, S100B overexpression was detected in 68 (19.0%) by immunohistochemical staining. All of the positive cases with score of ++, +++, and ++++ showed both brownish cytoplasmic staining and nuclear staining (Fig. 1d–f). The absence of staining was found in the negative control, where the primary antibody

was replaced with nonimmune antiserum (Fig. 1a) or negative S100B staining (Fig. 1b). Figure 1c depicts the weak positive immunoreactivity with score of +, of which was categorized into S100B non-overexpression.

Table 2 shows the correlation between clinicopathologic features and postoperative relapse pattern of 357 UICC stages II and III colon cancer patients. Using univariate analysis, we found that depth of tumor invasion ($P = .003$), presence of vascular invasion ($P < .001$), presence of perineural invasion ($P = .002$), high postoperative CEA level ($P < .001$), and S100B overexpression ($P < .001$) were significantly correlated to postoperative early relapse. Using a multivariate Cox proportional hazards regression analysis, presence of vascular invasion ($P = .025$; hazard ratio [HR], 5.532; 95% confidence interval [95% CI], 1.985–14.729), high postoperative CEA level ($P = .019$; HR, 6.845; 95% CI, 2.393–15.256), and S100B overexpression ($P < .001$; HR, 26.250; 95% CI, 7.463–96.804) were demonstrated to be independent predictors of postoperative early relapse (Table 3). Table 4 reveals that S100B overexpression was significantly associated with advanced UICC stages ($P = .001$), and S100B

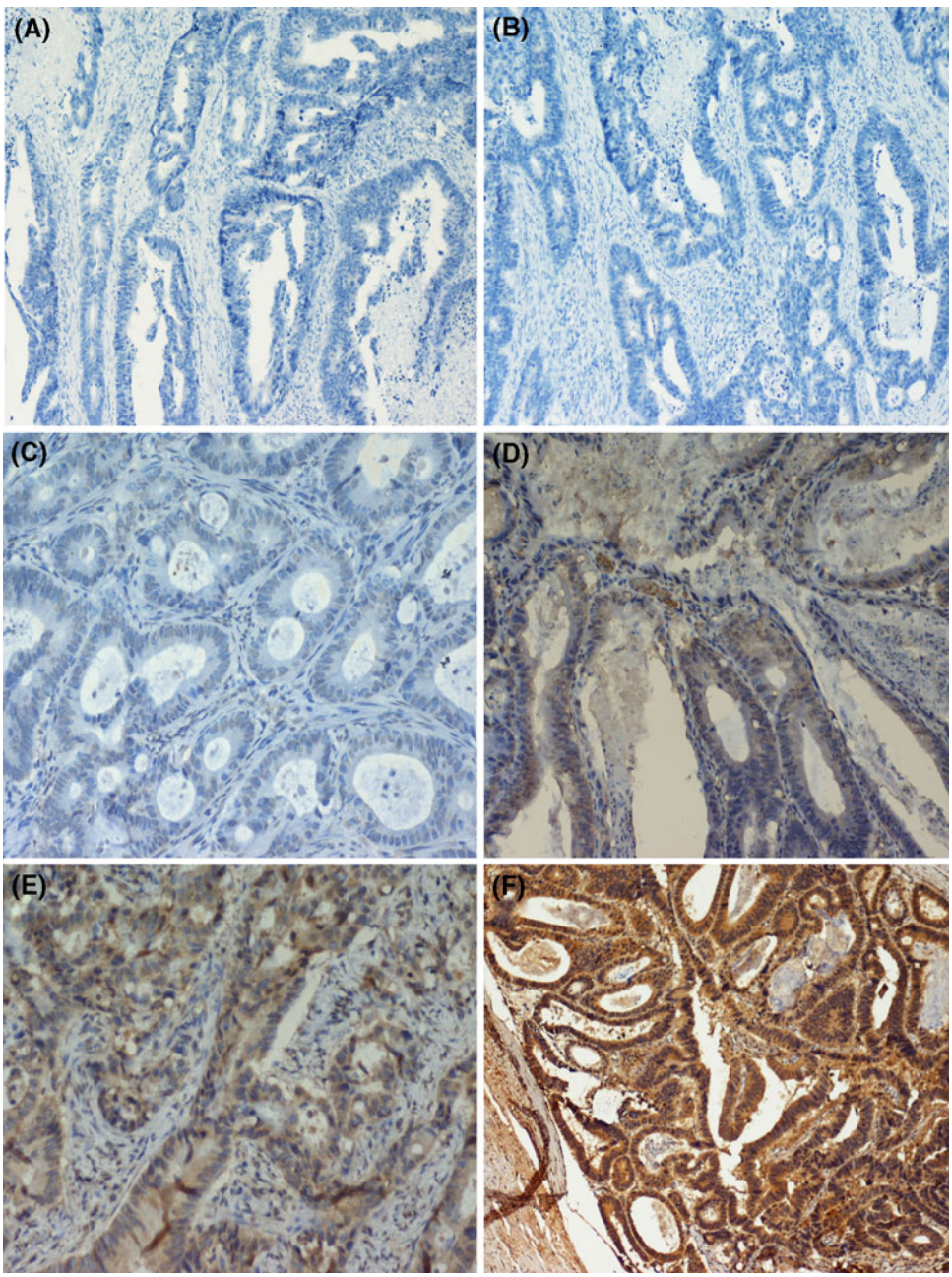

FIG. 1 Immunohistochemical staining of S100B in the colon cancer tissues. **a** staining was absent over the control section when the primary antibody was replaced with nonimmune antiserum. **b** negative S100B immunoreactivity. **c** weak S100B immunoreactivity (score +) is seen within the cytoplasm and nucleus of colorectal carcinoma tissue, of which was considered as overexpression (−) group. **d–f** S100B immunoreactivity (score ++ to ++++) was seen within the cytoplasm and nucleus of colorectal carcinoma tissue, which was considered as overexpression (+) group (original magnification: ×200)

TABLE 2 Correlation between postoperative early relapse and clinicopathologic features of 357 UICC stage II and III colon cancer patients using univariate analysis

	Early relapse ($n = 56$) No. (%)	Non-early relapse ($n = 301$) No. (%)	P value
Gender, male/female	30 (53.6)/26 (46.4)	163 (54.2)/138 (45.8)	.936
Age (y/o), <65/≥65 (years)	24 (42.9)/32 (57.1)	156 (51.8)/145 (48.2)	.218
Maximum size, <5/≥5 (cm)	26 (46.4)/30 (53.6)	165 (54.8)/136 (45.2)	.248
Location, right-sided/left-sided	23 (41.4)/33 (58.9)	120 (39.9)/181 (60.1)	.866
Depth of invasion, $T_2/T_3/T_4$	2 (3.6)/50 (89.3)/4 (7.1)	69 (22.9)/220 (73.1)/12 (4.0)	.003
Lymph node metastasis, yes/no	37 (66.1)/19 (33.9)	167 (55.5)/134 (44.5)	.141
Vascular invasion, yes/no	36 (64.3)/20 (35.7)	100 (33.2)/201 (66.8)	<.001
Perineural invasion, yes/no	31 (55.4)/25 (44.6)	100 (33.2)/201 (66.8)	.002
Histology, WD/MD/PD	3 (5.4)/45 (80.4)/8 (14.3)	29 (9.6)/244 (81.1)/28 (9.3)	.345
Postoperative CEA, <5/≥5 (ng/ml)	25 (44.6)/31 (55.4)	225 (74.8)/76 (25.2)	<.001
Adjuvant therapy, yes/no	40 (71.4)/16 (28.6)	192 (63.8)/109 (36.2)	.271
S100B overexpression, yes/no	50 (89.3)/6 (10.7)	18 (6.0)/283 (94.0)	<.001

UICC International Union Against Cancer, *Right-sided* cecum + ascending colon + transverse colon, *left-sided* descending colon + sigmoid colon, *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated, *CEA* carcinoembryonic antigen

TABLE 3 Correlation between postoperative early relapse and clinicopathologic features of 357 UICC stage II and III colon cancer patients using a Cox proportional hazards analysis

Variables	Coefficient	SE	P value	HR	95% CI
Depth of invasion (T3 + T4/T2)	1.016	0.962	.324	4.673	0.794–10.135
Vascular invasion (yes/no)	1.708	0.756	.025	5.532	1.985–14.729
Perineural invasion (yes/no)	1.428	0.715	.105	3.861	0.642–5.280
Postoperative CEA (≥5/<5)	2.776	0.738	.019	6.845	2.393–15.256
S100B overexpression (yes/no)	3.254	0.723	<.001	26.250	7.463–96.804

UICC International Union Against Cancer, *SE* standard error, *HR* hazard ratio, *CI* confidence interval, *CEA* carcinoembryonic antigen (ng/ml)

TABLE 4 Correlation between S100B overexpression and 357 UICC stage II and III colon cancer patients

UICC stages	S100B overexpression		P
	Positive ($n = 68$)	Negative ($n = 289$)	
II ($n = 153$)	17	136	.001
III ($n = 204$)	51	153	

UICC International Union Against Cancer

overexpression was more frequently observed in UICC stage III colon cancer patients than stage II colon cancer patients. Also, of 114 postoperative relapsed patients, the overall 5-year survival rate of patients with S100B overexpression was significantly lower than that of patients without S100B overexpression using a log-rank test (28.3% vs 51.0%, $P < .001$, Fig. 2).

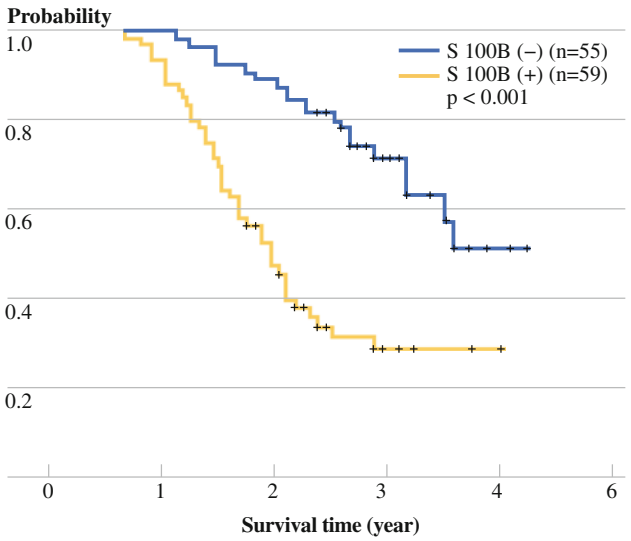

FIG. 2 Cumulative overall survival rates of 114 relapsed colon cancer patients. Colon cancer patients with S100B overexpression (+) group showed a significantly poorer overall survival rate than those with negative S100B expression overexpression (−) group (28.3% vs 51.0%, $P < 001$)

DISCUSSION

Despite recent advances of therapeutic methods, the prognosis of CRC patients with early relapse remains poor.

Early relapse cases had significantly lower overall survival rates than non-early relapse cases either in colon or rectal cancer patients.[16] Hence, it is valuable if we could detect the significant predictive factors of postoperative early relapse in these patients. To the best of our knowledge, this is the first study showing the predictive and prognostic relevance of S100B expression in early relapse and overall survival of CRC patients. The present study demonstrates that in addition to presence of vascular invasion and high postoperative CEA level, S100B protein overexpression was also an independent and more significant predictor of early relapse for UICC stages II and III colon cancer patients. In fact, S100B overexpression was significantly associated with advanced UICC stages and adverse clinical outcome. To date, 20 members of the S100 family have been discovered in humans.[7] Members of the S100 gene family are highly conserved, but individual S100 proteins show cell- and tissue-specific expression patterns. Interestingly, several S100 proteins are specifically upregulated in aggressive, advanced, metastatic tumors relative to noninvasive, nonmetastatic tumors.[17] There has been growing interest in the S100 protein family and their relationship with different cancers. Recently, increasing studies of S100A in CRC were found, while no related study regarding S100B in CRC was issued.[11,18–20] Wang et al. indicated that S100A11(a member of the S100 family) level in CRC tissue was increased from normal colorectal tissue to stage progression of the disease.[11] Likewise, enhanced S100A4 protein expression has been reported to be clinicopathologically significant to metastatic potential and p53 dysfunction in CRC.[19]

The S100 family of calcium binding proteins has been shown to be involved in a variety of physiological function, such as cell proliferation, extracellular signal transduction, intercellular adhesion, and motility as well as cancer metastasis. Elevated levels of S100B are found in malignant melanoma, renal cell tumors, malignant mature T-cells (such as doubly negative $CD4^-/CD8^-$ adult T-cells in leukemia patients), and meningiomas.[12,13,21–24] In fact, the exact mechanisms between S100B proteins expression and invasive/metastatic potential of CRC, and finally to develop early relapse needs to be clarified. S100B inhibits calcium-dependent phosphorylation of p53 by protein kinase C in vitro, which can lead to suppression of the p53 tumor suppressor mechanism, resulting in uncontrolled tumor growth.[25]

Although it is not completely clear how S100 proteins affect cell growth, S100B and several other S100 proteins (i.e., S100A1, mts1) interact with the tumor suppressor protein p53, resulting in significantly reduced p53 levels, and p53-dependent transcription activation of target genes is inhibited.[8,9,26] Similar results were obtained that S100B interacts directly with p53 and that inhibiting S100B with siRNA restores the functional p53 protein in primary malignant melanoma cancer cells.[21] Indeed, colorectal tumorigenesis arises as a result of the accumulation of genetic changes involving activation of proto-oncogenes and inactivation of tumor suppressor genes.[27] Mutations in tumor suppressor genes such as p53, can lead to the transformation from a larger adenoma to ultimately develop into a carcinoma. Consistent with the aforementioned mechanism, S100B overexpression is undoubtedly more frequently encountered in advanced stage of colon cancer patients in our investigation.

Recently, we have identified that the presence of vascular invasion, perineural invasion, and high postoperative CEA levels were demonstrated to be independent predictors of postoperative early relapse of colon cancer patients.[16] Following inclusion of S100B protein expression, the presence of perineural invasion was no longer an independent predictor of postoperative early relapse of colon cancer patients. Our current observation reveals that S100B overexpression is a more important predictor than conventional pathological variable and laboratory data (presence of vascular invasion and high postoperative CEA level) by multivariate analyses. Therefore, inclusion of S100B expression status may enhance our accuracy to predict early relapse in patients with CRC after radical resection. On the contrary, positive S100B protein expression was only in 18 of 301 of non-early relapsed colon cancer patients (6.0%); hence, it is difficult to identify the role of S100B protein expression for non-early relapse.

Furthermore, serum S100B protein concentration in stage II–III–IV melanoma is a reliable prognostic marker and has been shown to increase in a stage-dependent manner. The serum level of S100B protein is significant independent prognostic marker in respect to disease specific survival, it is a relevant marker for therapy monitoring and melanoma patient follow-up.[13,28] The means by which decreasing S100B concentrations reflect response to therapy while increasing S100B concentrations indicate tumor progression.[28] Accordingly, more prospective studies to monitor serum S100B level of adjuvant chemotherapy for high-risk stages II and stage III CRC would probably accentuate its significance of emerging as cancer biomarker or even in assessing a patient's response to treatment.

In conclusion, S100B protein expression probably plays a potential role in early relapse of UICC stages II and III colon cancer patients and eventually leads to a poor prognosis for these relapsed subjects. Certainly, there is a need for large, high-quality, well-designed, clinical trials to better define this significant predicting factor of S100B in a clinical setting for postoperative early relapse of UICC stages II and III CRC patients.

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