

Overexpression of the S100A2 protein as a prognostic marker for patients with stage II and III colorectal cancer

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Received October 18, 2015; Accepted December 17, 2015

DOI: 10.3892/ijo.2016.3329

Abstract. We aimed to identify a novel prognostic biomarker related to recurrence in stage II and III colorectal cancer (CRC) patients. Stage II and III CRC tissue mRNA expression was profiled using an Affymetrix Gene Chip, and copy number profiles of 125 patients were generated using an Affymetrix 250K Sty array. Genes showing both upregulated expression and copy number gains in cases involving recurrence were extracted as candidate biomarkers. The protein expression of the candidate gene was assessed using immunohistochemical staining of tissue from 161 patients. The relationship between protein expression and clinicopathological features was also examined. We identified 9 candidate genes related to recurrence of stage II and III CRC, whose mRNA expression was significantly higher in CRC than in normal tissue. Of these proteins, the S100 calcium-binding protein A2 (S100A2) has been observed in several human cancers. S100A2 protein overexpression in CRC cells was associated with significantly worse overall survival and relapse-free survival, indicating that S100A2 is an independent risk factor for stage II and III CRC recurrence. S100A2 overexpression in cancer cells could be a biomarker of poor prognosis in stage II and III CRC recurrence and a target for treatment of this disease.

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers in the world (1). It is the second leading cause of cancer-related mortality worldwide. In Japan, the incidence of CRC has doubled over the past 20 years such that CRC is now the second most deadly neoplastic disease (2,3).

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Key words: S100A2, colorectal cancer, biomarker, microarray, gene expression analysis, DNA copy number analysis

Surgery is still the most effective treatment for CRC. Among the patients that undergo curative surgery, some develop local recurrence or distant metastases that lead to shorter survival times (4). Distant metastasis has a critical influence on the prognosis of CRC. Clinicopathological indicators such as the TNM classification proposed by the International Union Against Cancer (UICC) remain the indicator of prognosis and provide the basis for therapeutic decision making. However, the current TNM classification system is limited in that it cannot predict prognosis for individual patients (5). In order to develop personalized therapeutic regimens, it is therefore critical that novel genes involved in distant metastasis are identified that can serve as prognostic biomarkers (6). Microarray is a particularly powerful tool for identifying potential biomarker genes for use in cancer prognosis (7-9). Using microarray analysis it is now possible to investigate several thousand cancer-related or cancer-specific genes at once.

Chromosomal structural alterations play an important role in cancer development. In CRC, copy number aberrations (CNAs), including gains on chromosomes 7, 8, 13 and 20, and losses on chromosomes 1p, 8p, 17p and 18, are frequently observed (10-13). Some of these CNAs are related to metastasis of CRC and can thus be used in prognosis. The single nucleotide polymorphism microarray (SNP array) analysis has become a useful tool for examining CNAs, permitting highly accurate exploration of thousands of genetic markers in a single study (14).

Studies of the relationship between chromosomal aberrations and gene expression in cancers, including CRC, have shown that CNAs directly influence gene expression (15-19). Several groups have therefore suggested that integrating gene expression analysis with genomic profiling represents an efficient approach for the discovery of cancer-related genes (20-22). Genes that show a strong positive correlation between expression and copy number may play an important role in cancer progression. In this study, we therefore integrated gene expression and copy number analyses to identify novel genes associated with the distant metastasis of CRC. We focused on the genes that are overexpressed and have an amplified copy number in cases of distant metastasis because such characteristics indicate that these genes have the potential to serve as useful therapeutic targets or clinical biomarkers.

Using the aforementioned comprehensive analysis, we identified S100 calcium-binding protein A2 (S100A2) as a gene involved in the distant metastasis of stage II and III CRC. It has been suggested that S100A2 plays an important role in cell cycle progression, and overexpression of S100A2 has been reported in several cancers (23-28). This is the first study to demonstrate the prognostic significance of S100A2 expression in CRC, using integrated copy number and gene expression analyses of clinical tissue samples.

Patients and methods

Patients. Primary tumors from 278 patients who underwent curative surgery for stage II and III CRC between 2002 and 2009 at the Tokyo Medical and Dental University Hospital (Tokyo, Japan) were studied. Written informed consent was obtained from all the patients, and the study was approved by the ethics committee of Tokyo Medical and Dental University, and all the following procedures were performed strictly in accordance with the ethical standards established by this committee. Clinical data were obtained from the medical records of each patient, and histopathological evaluations were assessed by reference to the criteria of the TNM-system of the UICC, 7th edition. A total of 125 patients were assigned to the comprehensive analyses for extraction of candidate genes. All of the patients were assigned to the gene expression and the CNA study, including 66 patients with stage II and 59 patients with stage III disease. The median follow-up time for these patients was 62 months (range, 1-76 months). Quantitative reverse transcription polymerase chain reaction (RT-PCR) assays were performed for validation using samples from 50 stage II and III CRC patients, including 24 patients with stage II and 26 patients with stage III disease. The median follow-up time for these patients was 61 months (range, 7-96 months). Furthermore, 161 patients, including patients subjected to RT-PCR validation, were analyzed using immunohistochemistry (IHC). The IHC study included 80 patients with stage II and 81 patients with stage III disease. The median follow-up time for these patients was 86 months (range, 1-96 months). The patients enrolled in the comprehensive analyses were excluded from these validation studies.

DNA extraction. After resection, cancer tissues were immediately embedded in Tissue-Tek OCT compound medium (Sakura Finetek Japan, Tokyo, Japan). Serial frozen sections of 9-mm in thickness were mounted onto a 90 FOIL-SL25 foil-coated glass slide (Leica Microsystems, Wetzlar, Germany). Laser capture microdissection (LCM) was performed using an Application Solutions LCM System (Leica Microsystems). Tumor DNA was extracted and purified using a QIAamp DNA micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Non-neoplastic tissues were homogenized in microtubes, and DNA was extracted and purified from these tissues using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

CNA analysis. Copy number analysis was performed using a GeneChip® Human Mapping 250K Sty array (Affymetrix, Santa Clara, CA, USA) in strict adherence to the assay manual. Genomic DNA was digested using the enzyme *StyI*, and a *StyI*

adaptor was used prior to the PCR reaction. Amplicons were fragmented after purification and then labeled. After hybridization, the microarrays were transferred to a totally automated GeneChip® Fluidics Station 450 (Affymetrix) for the washing and staining steps. After fluorescence staining, microarray images were scanned using a GeneChip® Scanner 3000 7G (Affymetrix). The microarray data from the scanner were used for copy number analysis with the Chromosome Copy Number Analysis Tool (Affymetrix). Data were analyzed using R statistical software (version 2.12.1; <http://www.r-project.org/>).

RNA extraction. Cancer cells were microdissected using LCM. Total RNA was extracted from cancer cells and purified using an RNeasy micro kit (Qiagen) with on-column DNase digestion, according to the manufacturer's instructions. Total RNA collected from bulk samples of cancer tissues and adjacent non-neoplastic tissues was extracted and purified using an RNeasy mini kit (Qiagen) with on-column DNase digestion, according to the manufacturer's instructions. The integrity of the total RNA was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Samples with an RNA integrity number >5.0 were used for the rest of the experiments.

Gene expression analysis. Complementary RNA was prepared from total RNA using two-cycle target labeling and a control reagents kit (Affymetrix). The experiment was performed using the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix), according to the manufacturer's instructions. Statistical analyses of microarray data were normalized using the robust multi-array average method with R statistical software (version 2.12.1; <http://www.r-project.org/>) together with the BioConductor package (<http://www.bioconductor.org/>).

Extraction of candidate genes. We defined patients with metastatic recurrence of stage II and III CRC as the recurrence group, and patients without any recurrence as the non-recurrence group. Local recurrence and lymph node recurrence were excluded from the recurrence group. The CNA data and the gene expression data were analyzed and compared between the 2 groups to identify genes involved in the metastatic recurrence of stage II and III CRC.

Data regarding genes which showed a copy number gain in the recurrence group were extracted using Fisher's exact test ($P < 0.01$). Data regarding genes that were significantly upregulated in the recurrence group were extracted using the Wilcoxon exact rank sum test ($P < 0.01$). Among the genes that were common to both groups, those that were overexpressed (fold change >1.5) in the recurrence group were selected as candidates for further analysis.

The gene expression data and DNA copy number data were deposited in the Gene Expression Omnibus (GEO) under accession IDs GSE64256, GSE64257 and GSE64258.

Quantitative RT-PCR. Total RNA collected from bulk samples of cancer tissues and adjacent non-neoplastic tissues was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A TaqMan® gene expression assay (Applied Biosystems; S100A2;

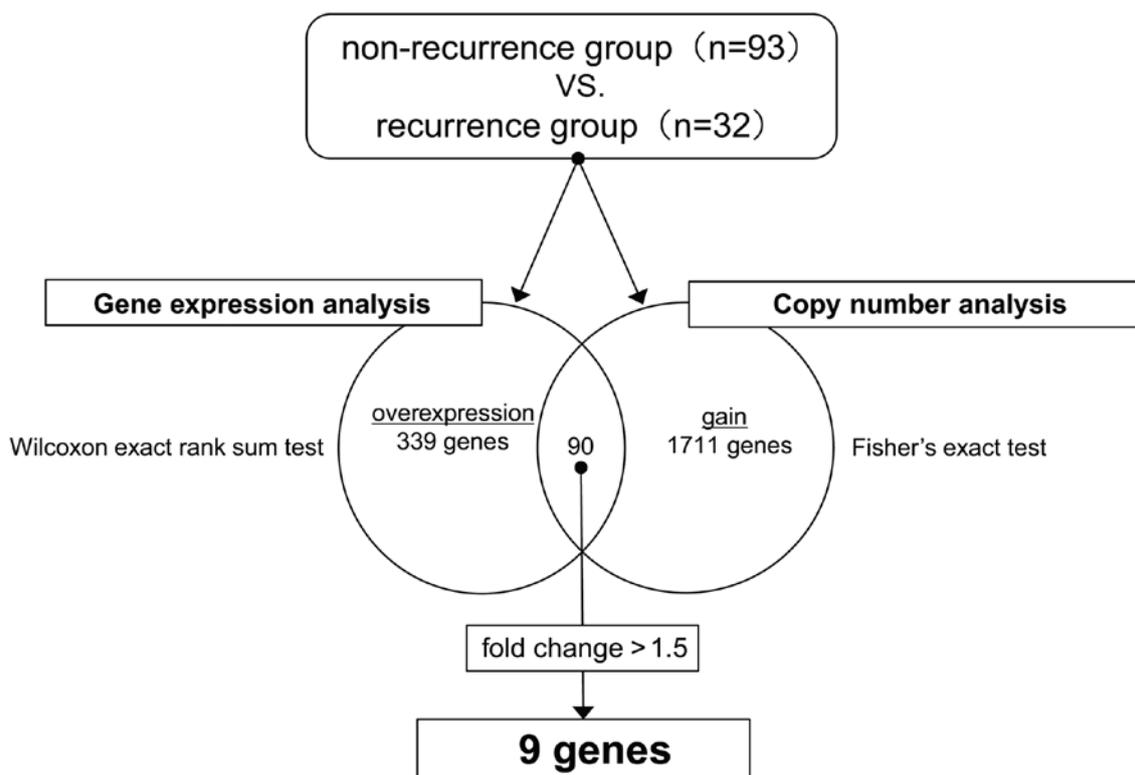


Figure 1. Identification of candidate genes. The scheme for identification of candidate genes related to recurrence in patients with stage II and III colorectal cancer is shown. A total of 339 genes were extracted from the gene expression analysis, and 1,711 genes were extracted from the CNA analysis. Among the 90 genes extracted from both analyses, 9 genes with a fold change in expression of >1.5 were selected as candidates for further analysis.

Hs00195582_m1, β -actin; Hs99999903_m1) was used to investigate the expression of S100A2, and β -actin was used as an internal control. The PCR reaction was carried out using TaqMan® Universal PCR Master Mix (Applied Biosystems). The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. All calculated concentrations of target genes were normalized by the amount of the endogenous reference using the comparative Ct method for relative quantification with Relative Quantification Study Software (7300 Sequence Detection System version 1.2.1, Applied Biosystems).

Immunohistochemistry. IHC analyses of S100A2 were conducted on formalin-fixed paraffin-embedded tissue blocks from each patient. For S100A2 staining, antigen retrieval by autoclave treatment was carried out for 15 min in 1X TE (1X Tris-EDTA, pH 8.0) at 121°C after deparaffinization in xylene and rehydration through a series of incubations in graded concentrations of ethanol. The slides were then incubated in a solution of 3% hydrogen peroxide in 100% methanol for 15 min at room temperature in order to quench endogenous peroxidase activity. Subsequently, the slides were incubated with mouse monoclonal antibody against S100A2 (Sigma-Aldrich), at a 1:50 dilution, for 30 min at room temperature. The slides were then incubated with peroxidase-labeled antibody [Histofine Simple Stain Max PO (MULTI; Nichirei Bioscience)] for 30 min at room temperature. Peroxidase activity was detected with DAB Solution (Histofine Simple

Stain DAB Solution; Nichirei BioScience). Finally, the slides were counterstained with 1% Mayer's hematoxylin.

All the sections were divided into four stages (negative, weak, moderate, or strong) by staining intensity. Expression was graded by two independent observers who were blinded to the patient information.

Statistical analysis of S100A2 expression. Statistical analyses of S100A2 expression were carried out using SPSS (version 17.0, SPSS Inc, Chicago, IL, USA) software for Windows. To estimate the significance of differences between groups, Wilcoxon signed-rank, Mann-Whitney U, and C^2 tests were used where appropriate. Survival curves were estimated using the Kaplan-Meier method, and curves were compared using the log-rank test. Survival times were determined from the date of surgery. Prognostic factors were examined with univariate and multivariate analyses using the Cox proportional hazards model. A P-value <0.05 was considered statistically significant.

Results

Gene expression and copy number analyses. In the copy number and gene expression analyses, 9 genes (S100A2, PROX1, TCN1, PROM1, CHRM3, ZNF678, CREB5, PPARGC1A, and ATF6) were identified that fulfilled the specified criteria. Of the 9 genes with both elevated copy number and expression, the expression was upregulated with a fold change >1.5 (Fig. 1). Only S100A2 and TCN1 of these genes have been shown to be associated with cancer. Overexpression of S100A2 has been

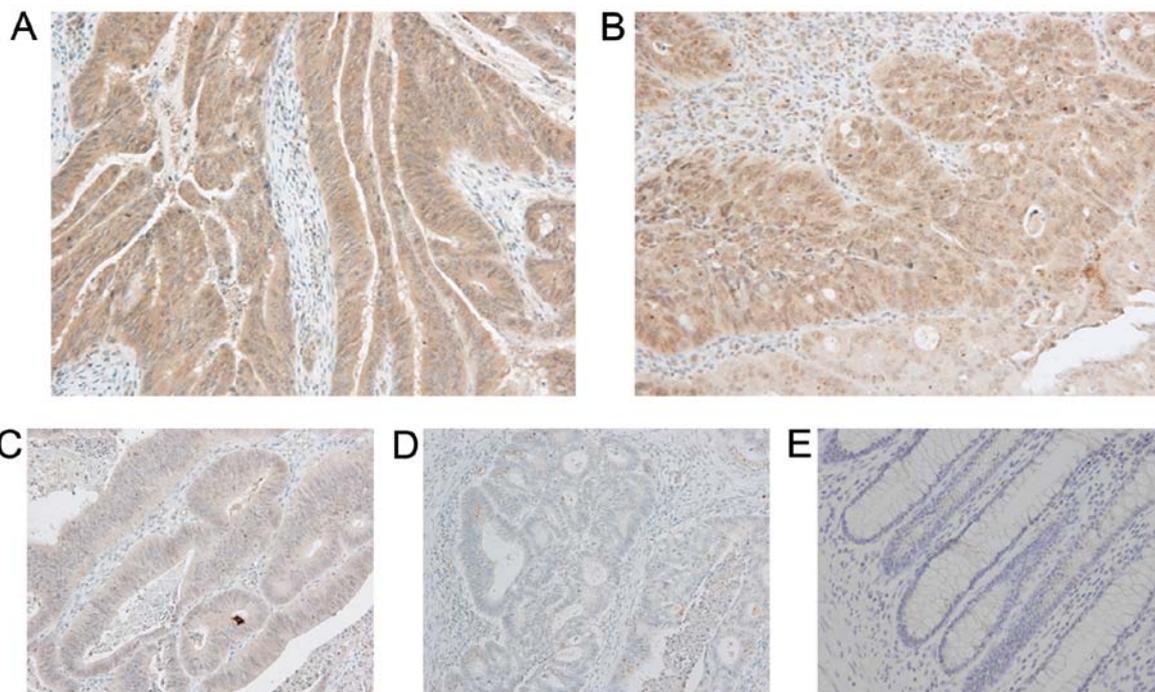


Figure 2. Immunohistochemical staining of S100A2 in colorectal carcinoma tissue. Representative images of the invasive tumor front of colorectal carcinoma tissue showing strong (A), moderate (B), weak (C), negative (D) normal tissue (E) immunohistochemical staining for S100A2.

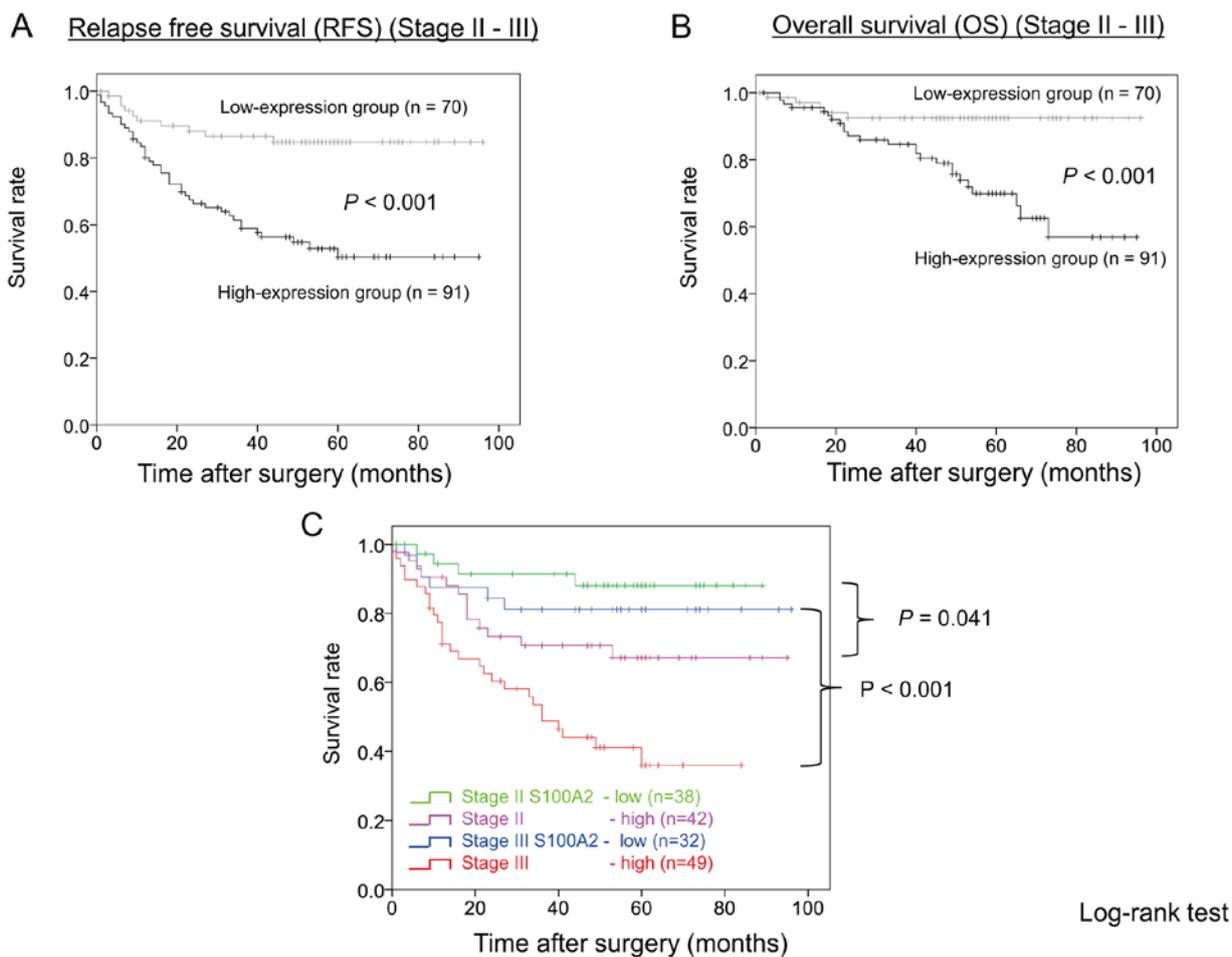


Figure 3. Association of S100A2 expression levels with survival of stage II and III colorectal cancer patients (n=161) were divided into S100A2 high- and low-expressing groups that were analyzed for (A) relapse-free survival (RFS) and (B) overall survival (OS), using Kaplan-Meier curves. (C) Kaplan-Meier curves show RFS stratified by TNM-7th stage and S100A2 expression group. RFS curves were significantly separated by S100A2 expression group in both stage II and III patients.

reported to occur in several cancers, although it has not been reported in relation to prognosis in CRC (23-29). We therefore focused on S100A2 in the subsequent analyses.

S100A2 mRNA and protein expression. Quantitative RT-PCR analysis of stage II and III CRC tissue from 50 patients showed that expression of S100A2 mRNA is significantly higher in cancerous tissue than in neighboring non-neoplastic tissue (data not shown). The cellular localization of the S100A2 protein was investigated using cancer tissue from the same 50 CRC patients that were analyzed using RT-PCR. IHC indicated that the S100A2 protein is localized in the cytoplasm of CRC cells, whereas staining for the S100A2 protein in normal epithelial cells adjacent to the cancer cells was negative or weak. There was no difference in staining intensity of the invasive tumor front and the marginal tissue of the tumor. As a result of these data, we then estimated the extent and intensity of S100A2 staining in sections containing the area of infiltration in stage II and III CRC tissue samples from 161 patients. As shown in Fig. 2, in the cytoplasm of cancer cells, staining was observed in three patterns, strong (Fig. 2A), moderate (Fig. 2B) and weak (Fig. 2C).

Relationship between the expression of S100A2 and patient characteristics. For statistical evaluation purposes, the 161 samples that underwent IHC were divided into 2 groups: a high-expression group (strong staining spread of >10% or moderate staining spread of >70%, n=91) and a low-expression group (the others, n=70).

The correlation between S100A2 expression and various clinicopathological factors in 161 patients with stage II or III CRC is shown in Table I. Location (rectum; P=0.001), venous invasion (positive; P=0.011), and the presence or absence of recurrence (recurrence group; P<0.001) were significantly associated with overexpression of S100A2.

S100A2 expression and prognosis of stage II and III patients. The relapse-free survival (RFS) rate was significantly lower (P<0.001) in the S100A2 high-expression group than in the low-expression group (Fig. 3A). Univariate analysis indicated that gender (P=0.024), histology (P=0.022), location (P=0.003), lymphatic invasion (P=0.024), lymph node metastasis (P=0.007), CEA level (5.0 ng/ml or higher; P=0.003), and S100A2 expression (P<0.001) were significantly associated with RFS. Multivariate analysis indicated that overexpression of S100A2 is a significant prognostic factor of RFS for Stage II or III CRC patients (P=0.007; relative risk (RR) = 2.726; 95% confidence interval, 1.318-5.638) (Table II).

Likewise, the overall survival (OS) rate was significantly lower (P<0.001) in the S100A2 high-expression group than in the low-expression group (Fig. 3B). Univariate analysis indicated that gender (P=0.007), histology (P=0.005), location (P=0.009), tumor depth (P=0.007), lymphatic invasion (P=0.013), lymph node metastasis (P=0.024), CEA level (P=0.004), and S100A2 expression (P<0.001) were significantly associated with OS. Multivariate analysis indicated that S100A2 overexpression is an independent and significant prognostic factor of OS for patients with stage II or III CRC (P=0.008; RR=3.941; 95% confidence interval, 1.434-10.830) (Table III).

Table I. Relationship between clinicopathologic variables and S100A2 expression in patients with stage II-III colorectal cancer.

Variables	S100A2 expression		P-value
	Low (n=70)	High (n=91)	
Age (median), years	31-92 (67)	20-86 (68)	0.63
Gender			0.555
Male	43	60	
Female	27	31	
Histology			0.301
Well	27	28	
Moderate, poor and others	43	63	
Location			<u>0.001</u>
Colon	53	46	
Rectum	17	45	
Depth			0.933
T1/T2/T3	42	54	
T4	28	37	
Lymphatic invasion			0.139
Negative	20	17	
Positive	50	74	
Venous invasion			<u>0.011</u>
Negative	10	3	
Positive	60	88	
Lymph node metastasis			0.306
Negative	38	42	
Positive	32	49	
CEA (ng/ml)			0.201
<5	46	50	
≥5	20	38	
Recurrence			<u><0.001</u>
Non-rec	61	56	
Rec	9	35	

P-values less than 0.05 are underlined.

Fig. 3C shows RFS curves stratified by TNM-7th stage and S100A2 expression level group. RFS curves were significantly separated by S100A2 expression level group in both stage II and III patients. RFS was significantly worse in the S100A2 high expression group than in the S100A2 low expression group in stage II (P=0.041) as well as in stage III (P<0.001) patients.

Discussion

This study is the first to demonstrate the prognostic significance of intratumoral S100A2 expression in clinical tissue samples of CRC. S100A2 was identified as a recurrence-related

Table II. Univariate and multivariate analysis of clinicopathologic factors affecting relapse-free survival in patients with stage II-III colorectal cancer.

Variables	No. of patients	Univariate analysis P-value	Multivariate analysis	
			Relative risk (95% confidence interval)	P-value
Age		0.705		
≤65	81			
>65	80			
Gender		0.024		
Male	103			
Female	58			
Histology		0.022		0.149
Well	55			
Moderate, poor and others	106			
Location		0.003		0.078
Colon	99			
Rectum	62			
Depth		0.091		
T1/T2/T3	96			
T4	65			
Lymphatic invasion		0.024		0.332
Negative	37			
Positive	124			
Venous invasion		0.446		
Negative	13			
Positive	148			
Lymph node metastasis		0.007		0.195
Negative	80			
Positive	81			
CEA (ng/ml)		0.003		0.335
<5	96			
≥5	58			
S100A2 expression		<0.001	2.726	<u>0.007</u>
Low	70		(1.318-5.683)	
High	91			

P-value less than 0.05 in multivariate analysis is underlined.

Table III. Univariate and multivariate analysis of clinicopathologic factors affecting overall survival in patients with stage II-III colorectal cancer.

Variables	No. of patients	Univariate analysis P-value	Multivariate analysis	
			Relative risk (95% confidence interval)	P-value
Age		0.061		
≤65	81			
>65	80			
Gender		0.007		0.058
Male	103			
Female	58			
Histology		0.005		0.115
Well	55			
Moderate, poor and others	106			
Location		0.009		0.111
Colon	99			
Rectum	62			
Depth		0.007		0.07
T1/T2/T3	96			
T4	65			
Lymphatic invasion		0.013		0.363
Negative	37			
Positive	124			
Venous invasion		0.257		
Negative	13			
Positive	148			
Lymph node metastasis		0.024		0.613
Negative	80			
Positive	81			
CEA (ng/ml)		0.004		0.533
<5	96			
≥5	58			
S100A2 expression		<0.001	3.941	<u>0.008</u>
Low	70		(1.434-10.830)	
High	91			

P-value less than 0.05 in multivariate analysis is underlined.

gene in the combined analysis of gene expression and copy number, and high S100A2 expression was an independent and

significant prognostic factor of distant recurrence after curative surgery for stage II or III CRC.

The S100A2 gene is located on chromosome 1q.21. The human S100A2 is a member of the S100 family. It has been suggested that this family promotes tumor progression and metastasis by regulating the cell cycle, motility, and invasion in many human neoplasms (30-34). S100A2 is an EF-hand calcium-binding protein that regulates protein phosphorylation, cytoskeletal components, and calcium homeostasis both inside and outside of cells (30,35). The S100A2 protein, which is found in the cytoplasm and nucleus of epithelial cells including those in the esophagus and colon, is involved in TGF- β signaling (36,37). It has also been suggested to play a role in the cell cycle regulation of p53. S100A2 protein overexpression has been reported in lung cancer, brain cancer, and several types of gastroenterological cancers such as pancreatic and esophageal cancers (35). The S100A2 protein is reported to be involved in the chemotactic activity of tumor cells in colon cancer (37,38). In addition, S100A2 knock-down has been reported to reduce TGF- β -induced cellular chemotaxis (37). In the present study, high expression of S100A2 was significantly related with recurrence with distant metastasis. Our results support the idea that S100A2 might play an important role in distant metastasis of CRC. Further studies are warranted to investigate the roles and functions of S100A2 in CRC.

At present, recurrence risk and prognosis are predicted largely based on pathological tumor staging (TNM classification). The usefulness of postoperative adjuvant chemotherapy for stage II CRC has not been established yet, and it is recommended that determination of whether or not to use adjuvant chemotherapy should be based on the recurrence risk predicted for each patient. Major western guidelines, such as the National Comprehensive Cancer Network of Clinical Practice Guidelines in Oncology, recommend adjuvant chemotherapy when patients have risk factors including T4 lesions, less than 12 lymph nodes examined, perforation, poorly differentiated histology, and lymphovascular involvement. Our results suggested that stage II CRC patients with high S100A2 expression might be candidates for adjuvant chemotherapy as high-risk patients of distant recurrence. For stage III CRC, postoperative chemotherapy is recommended without exception. However, when stage III is further divided into IIIA, IIIB, and IIIC according to the TNM-7th classification and each sub-stage is considered separately, it has been reported that an additive effect of oxaliplatin cannot be anticipated in stage IIIA patients, and that stratification of the recurrence risk is important for stage III colon cancer as is the case for stage II CRC (39,40). Our study suggests that stage III CRC patients with high S100A2 expression may require strong adjuvant chemotherapy. Validation of the usefulness of risk-guided treatment is important in postoperative chemotherapy for stage II and III CRC and further clinical studies need to be conducted.

In conclusion, this study demonstrated that S100A was expressed at a significantly increased level in the CRC recurrence group. In our screen, we focused on highly expressed genes, because we intended to use the identified gene, S100A2, as a blood biomarker in actual clinical practice. The results of our study suggest the potential of the S100A protein as a recurrence-predicting factor and a target for molecular-targeted drugs for CRC.

Acknowledgements

The authors thank Y. Takagi and M. Itoda for excellent technical assistance.

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