Identification of SATB1 as a Specific Biomarker for Lymph Node Metastasis in Colorectal Cancer

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Abstract. *Background/Aim: The integration of gene expression analysis and genomic profiling represents an efficient approach to the discovery of cancer-related genes. Lymph node metastasis (LNM) is a significant prognostic factor in colorectal cancer (CRC). Detection and analysis of factors related to LNM will help develop new diagnostic methods or therapies. In this study, we aimed to identify genes that are significantly related to LNM in CRC through integrated copy number analysis (CNA) and gene expression analysis. Materials and Methods: Genes showing both up-regulated expression and copy number gains in cases involving CRC with LNM were extracted as candidate biomarkers. Expression of the mRNA of the final candidate was validated in 124 patients using quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. Expression of the protein encoded by this candidate gene was assessed using immunohistochemical (IHC) staining of tissueσ from 328 patients. The association between protein expression and clinicopathological features was also examined. Results: Special AT-rich sequence-binding protein 1 (SATB1) was extracted from integrated microarray analysis. SATB1 mRNA expression in cancer tissue was significantly higher in patients with LNM than without LNM. SATB1 protein overexpression was significantly associated with LNM. Moreover, overexpression of SATB1 was an independent poor prognostic factor in stage I-III, especially in stage II CRC. Conclusion: SATB1 may play an important role in LNM of CRC. SATB1 may be a biomarker of LNM and of recurrence after surgery for CRC.*

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Colorectal cancer (CRC) is the third most common cancer and the second most common cause of cancer-related mortality in the world (1). In Japan, the incidence of CRC has doubled over the past 20 years and it is the second most common cause of death among neoplastic diseases (2). The tumor-node-metastasis (TNM) classification system of the International Union against Cancer (UICC) remains the most reliable indicator of prognosis for CRC patients and provides the basis for therapeutic decision-making. Overall survival (OS) in patients with lymph node metastasis (LNM) is significantly shorter than in patients without LNM (3). LNM is a poor prognostic factor and precise diagnosis of LNM contributes to more selective therapy for CRC. Thus, identification of genes that correlate with LNM should be useful in the diagnosis and therapy of CRC.

Microarray analysis is a useful tool for identifying potential biomarker genes for use in cancer prognosis and investigating several thousand cancer-related or cancer-specific genes at once (4, 5).

Recently, single nucleotide polymorphism microarray (SNP array) analysis has become a useful tool for examining copy number aberrations (CNAs). CNAs, including gains at chromosomes 7, 8, 13 and 20, as well as losses at chromosomes 1, 8, 17 and 18, are often observed in CRC and are related to the metastasis of CRC (6-9). Several studies have suggested the utility of integrating gene expression analysis with genomic profiling for the discovery of cancer-related genes (10, 11). Genes that show a significant positive association with expression and CNAs may play an important role in cancer progression. We, therefore, aimed to identify genes that are significantly related to LNM in CRC through integrated copy number and gene expression analysis. We considered that genes that are overexpressed and have an amplified copy number in LNM

of CRC must have the potential to serve as useful therapeutic targets or clinical biomarkers.

We identified several such genes through integrated analysis and showed that one of those genes, Special AT-rich sequence-binding protein 1 (*SATB1*), is up-regulated in tumors with LNM. *SATB1* has been found to be overexpressed in several kinds of malignant tumors, which suggests a crucial role for *SATB1* in promoting tumor invasion and metastasis (12-20). This is the first study to demonstrate a significant correlation of *SATB1* with LNM in CRC.

Materials and Methods

Patients. Primary tumors from 328 patients who underwent curative surgery for CRC between 2002 and 2011 at the Tokyo Medical and Dental University Hospital (Tokyo, Japan) were analyzed in this study. According to pathological examination, LNM were present in 144 patients and absent in 184 patients (median follow-up time=62 months). All patients provided written informed consent to participate in this study that was approved by the Institutional Review Board (approval number: 831).

Among the 328 patients enrolled, a total of 156 patients were assigned to the integrated analyses for extraction of candidate genes. The immunohistochemical (IHC) studies were performed using formalin-fixed paraffin-embedded (FFPE) samples from 328 patients.

DNA extraction and copy number analysis. After resection, cancer tissues were immediately embedded in Tissue-Tek OCT compound medium (Sakura Finetek Japan, Tokyo, Japan). Laser capture microdissection (LCM) was performed using an Application Solutions LCM System (Leica Microsystems, Tokyo, Japan). Tumor DNA was extracted and purified using a QIAamp DNA micro kit (Qiagen, Hilden, Germany). Copy number analysis (CNA) was performed using a Gene Chip Human Mapping 250K Sty array (Affymetrix, Santa Clara, CA, USA). Genomic DNA was digested using the enzyme Sty I and a Sty1 adaptor was used prior to the polymerase chain reaction (PCR) reaction. After hybridization, the microarrays were transferred to a totally automated Gene Chip Fluidics Station 450 (Affymetrix). After fluorescence staining, microarray images were scanned using the Gene Chip Scanner 3000 7G (Affymetrix). The microarray data were analyzed using the Chromosome CNA Tool (Affymetrix).

RNA extraction and gene expression analysis. For quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis, total RNA was extracted from bulk samples of cancer and adjacent non-neoplastic tissues. Total RNA was extracted and purified using the RNeasy mini kit (Qiagen) with on-column DNase digestion. Gene expression was analyzed using the Gene Chip Human Genome U133 Plus 2.0 Array (Affymetrix).

Extraction of candidate genes. The gene expression profiles of cancer cells and the tumor CNAs were each compared between the patients with LNM (n=58) and without LNM (n=98). Subsequently, integrated analyses were performed to identify candidate genes related to LNM. Data regarding genes that were significantly up-regulated in patients with LNM were extracted using the Wilcoxon exact rank sum test ($p<0.01$). Data regarding genes that showed a copy number gain were extracted using Fisher's exact test ($p<0.01$). Among the genes that were common to both groups, those that were overexpressed (fold change >2.0, $p<0.05$) in patients with LNM were selected as candidates for further analysis.

Quantitative RT-PCR. Quantitative RT-PCR analyses were performed using frozen tissues from 124 patients. Total RNA (10 μg) that was extracted from bulk samples of cancer and adjacent non-neoplastic tissues was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). TaqMan gene expression assays (*SATB1*, Hs00161515_m1; β-actin, Hs99999903_m1; Applied Biosystems) were used to determine the expression of SATB1. β-Actin was used as an internal control. PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). All calculated concentrations of target genes were normalized to the amount of the endogenous reference by the comparative Ct method for relative quantification (ΔΔCt method) using Relative Quantification Study Software (7300 Sequence Detection System version 1.2.1; Applied Biosystems). Each assay was performed in duplicate.

Immunohistochemistry. IHC studies of SATB1 expression were conducted using a FFPE tissue block from each 328 patients.

After deparaffinization through a series of incubations in decreasing concentrations of ethanol, antigen was retrieved by microwaving (Microwave-MI 77; Azumaya Corporation, Tokyo, Japan) for 25 min in pH 9 buffer (code 415211; Nichirei, Biosciences, Tokyo, Japan). Endogenous peroxidase activity was quenched by 15 min of incubation in a mixture of 3% hydrogen peroxide solution in 100% methanol. After washing with phosphate-buffered saline (PBS), the specimens were incubated with a rabbit monoclonal anti-SATB1 antibody (EPR3895, ab92307; Abcam, Cambridge, UK) at a 1:100 dilution for 15 min at room temperature and, then, overnight at 4˚C. The sections were washed three times with 0.1% Tween 20/PBS and incubated with peroxidase-labeled polymer (Histofine Simple Stain Max PO (MULTI); Nichirei Bioscience) for 30 min at room temperature. Peroxidase activity was detected *via* incubation with 0.02% 3,3'-diaminobenzi-dine tetrahydrochloride (Nichirei Bioscience) for 30 min. The sections were counterstained with 1% Mayer's hematoxylin, dehydrated and finally coverslipped.

SATB1 expression was graded by two independent observers. The staining patterns were evaluated using the immunoreactive score (IRS) proposed by Remmele and Stegner (21) in which IRS=staining intensity (SI) × percentage of positive cells (PP). SI was determined 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 1, 0-9%; 2, 10-50%; and 3, >50% positive cells. The final intensity of staining was defined as 'low expression group' and 'high expression group', corresponding to IRS values of ≤1 and >1, respectively.

Statistical analysis. Statistical analyses were conducted using SPSS (version 17.0, SPSS Inc., Chicago, IL, USA) for Windows software. Differences between groups were estimated using the Mann-Whitney *U*-test, the Wilcoxon signed-rank test and the χ^2 test. Correlation was estimated using Spearman's rank correlation coefficient. Survival curves were estimated using the Kaplan-Meier method, whereas comparisons between curves were made using the log-rank test. Prognostic factors were estimated using univariate and multivariate analyses (Cox proportional hazards model). $p<0.05$ denoted statistical significance.

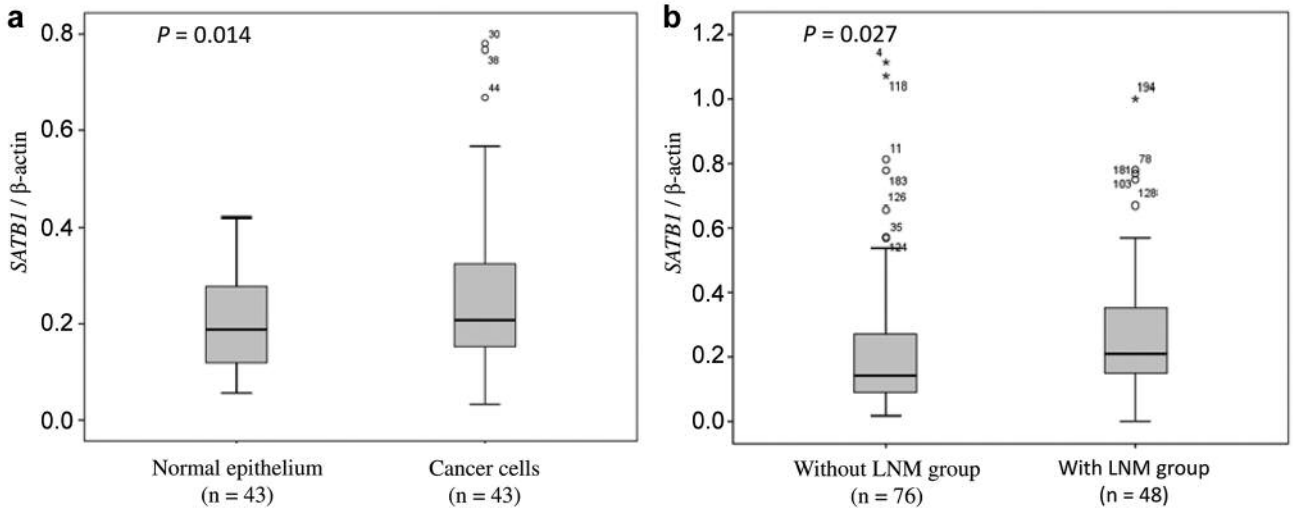

Figure 1. *SATB1 mRNA expression by qRT-PCR analysis. (a) Cancer tissues showed higher SATB1 expression than normal epithelium in 43 patients with LNM (p=0.014). (b) SATB1 expression was higher in patients with LNM (n=48) than in patients without LNM (n=76; p=0.027).*

Results

Gene expression and copy number analyses. Using comprehensive analysis of gene expression and copy number, we found that the expression of 24,910 genes was elevated in tissues of CRC patients with LNM, with a false discovery rate (FDR) of <0.25. *SATB1* was the only one of these genes whose expression was up-regulated with a fold change >2.0.

Expression of SATB1 mRNA. Quantitative RT-PCR analysis of *SATB1* mRNA expression in 43 patients with LNM showed that the expression of *SATB1* was significantly higher in cancerous tissue than in paired neighboring non-neoplastic tissue. The expression of *SATB1* in cancer tissues was also significantly higher in patients with LNM than in those without LNM (Figure 1).

SATB1 protein expression. Following antibody optimization and staining, the cellular localization of the SATB1 protein was investigated using cancer tissue from 328 patients. IHC staining indicated that SATB1 was localized in the nucleus of CRC cells. Staining for SATB1 in normal epithelial cells, adjacent to the cancer cells, was negative or weak (Figure 2).

There was a correlation between *SATB1* mRNA expression and SATB1 protein expression as assessed by RT-PCR and IHC, respectively (correlation coefficient r=0.424, $p<0.001$) (Figure 3).

Correlations of clinicopathological features and SATB1 protein expression. For statistical evaluation purposes, the 328 samples subjected to IHC analysis were divided into two groups: SATB1 high-expressing (IRS>1, n=147) and low-expressing (IRS≤1, n=181) groups.

The relationship between the SATB1 expression level and various clinicopathological factors of the patients is shown in Table I. Venous invasion, recurrence after curative surgery and LNM were significantly associated with overexpression of SATB1.

In addition, there was a significant correlation between SATB1 expression of the primary tumor and its metastatic lymph node.

Relationship between SATB1 expression and survival. Kaplan-Meier analysis of OS and relapse-free survival (RFS) rates of stage I-III CRC were both significantly lower ($p<0.001$ for both) in SATB1 high expression group (Figure 4).

Multivariate analysis indicated that T4 tumor and overexpression of SATB1 were significant prognostic factors of OS for patients with CRCs.

Regarding RFS, multivariate analysis indicated that location, elevated serum carcinoembryonic antigen (CEA), tumor depth, LNM and SATB1 protein overexpression were independent risk factors for the prognosis of patients with CRC (Table II). Moreover, overexpression of SATB1 was also found to be an independent prognostic factor of OS and RFS in stage II patients.

Discussion

SATB1, localized at human chromosome 3p23, encodes for a nuclear protein initially identified in thymocytes (22). The function of *SATB1* has been mainly studied in immune cells

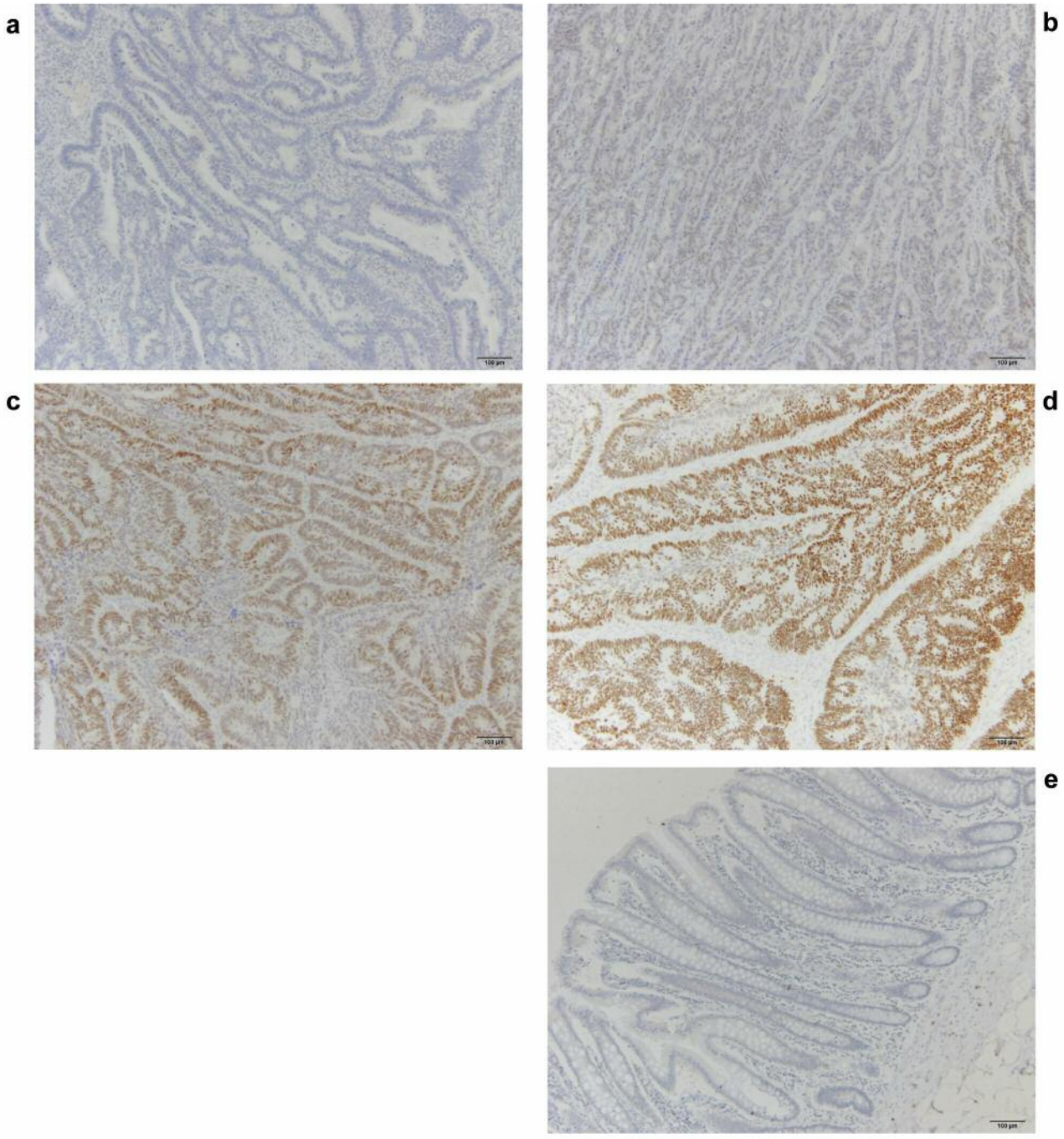

Figure 2. *Representative immunostaining for SATB1 in colorectal cancer and normal colorectal epithelium: (a) 0, no staining; (b) 1+, weak staining; (c) 2+, moderate staining; (d) 3+, strong staining; (e) staining of normal epithelium (magnification 100×).*

and it is known to play a vital role in T-cell development. *SATB1* functions as a genome organizer that binds to base-unpairing regions (BURs) characterized by the ATC sequence context (12). It functions as a nuclear architectural platform for anchoring several chromatin remodeling/modifying enzymes and for loci that bind to specialized genomic BURs. Phosphorylation of *SATB1* serves as a molecular switch that determines whether it acts as a transcriptional activator or repressor (13, 23). Thus, *SATB1* reprograms the cell's gene expression profile by folding

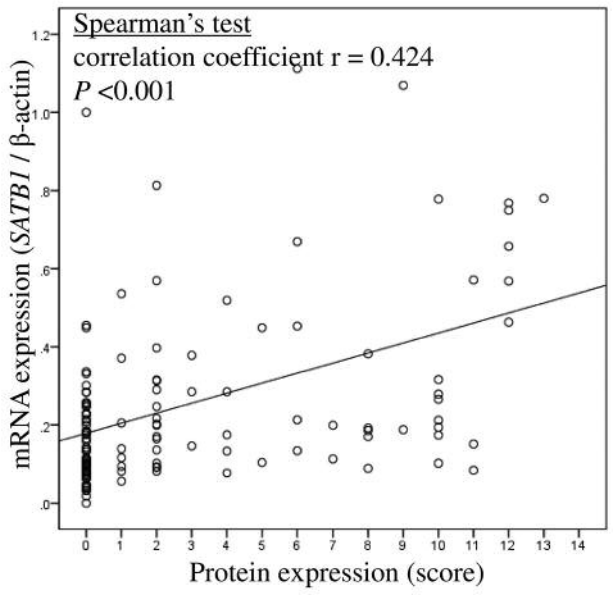

Figure 3. *Correlation between SATB1 mRNA and protein expression. SATB1 mRNA expression was correlated with protein expression in 124 patients as assessed by RT-PCR analysis and immunohistochemistry, respectively (correlation coefficient r=0.424, p<0.001).*

Table I. *Correlations between clinicopathological features and SATB1 protein expression in stage I-III CRCs.*

Variables	SATB1 expression		*p*-Value
	Low (n=181)	High (n=147)	
Age, years (median=66)			
≤66	90	80	0.397
>66	91	67	
Gender			
Male	112	97	0.442
Female	69	50	
Location			
Colon	114	83	0.23
Rectum	67	64	
Histology			
Well	75	47	0.078
Others	106	100	
CEA (ng/ml) (missing 4 patients)			
<5	115	88	0.511
≥5	64	57	
Depth			
T1/T2/T3	124	91	0.211
T4	57	56	
Lymphatic invasion			
Absent	62	37	0.075
Present	119	110	
Venous invasion			
Absent	33	13	0.015*
Present	148	134	
Lymph node metastasis			
Absent	121	64	<0.001**
Present	60	83	
Recurrence			
Absent	156	93	<0.001**
Present	25	54	
SATB1 expression in metastatic lymph node			
Low	9	14	0.011*
High	4	32	

SATB1, Special AT-rich sequence-binding protein 1; CRC, colorectal cancer; CEA, carcinoembryonic antigen; *p<0.05.

chromatin into loops and allows genes to rapidly change phenotypes. The genes regulated by *SATB1* vary among cell types and cell functions (13, 24).

Regarding its role in tumor progression, Han *et al*. (12) showed that *SATB1* is overexpressed in human aggressive breast cancer and likely functions to reprogram chromatin organization and transcriptional profiles to promote growth and metastasis. Through unsupervised clustering of genes differentially expressed between control shRNA and *SATB1* shRNA transfected cells, they showed that the overexpressed *SATB1* in these breast cancer cells up-regulates 456 genes and down-regulates 409 genes. *SATB1* was suggested to function as an independent prognostic factor by directly up-regulating metastasis-associated genes, such as *VEGF, MMP3, ERRB, LMNA* and others. These genes have many biological activities, such as cell proliferation, cell cycle, apoptosis, adhesion, differentiation and proliferation (12, 13). More recent studies have shown that *SATB1* contributes to tumor metastasis, invasion, proliferation and migration both *in vitro* and *in vivo* in CRC (20, 25).

Although the specific mechanism by which *SATB1* affects the biological behavior of CRC is unknown, previous studies have shown that *SATB1* plays a crucial role in the molecular regulation of cancer behavior. Thus, *SATB1* might promote LNM through regulation of cancer-related genes, such as *VEGF*.

There have been some studies that analyzed the expression of SATB1 in CRC. Both Meng *et al*. and Zhang *et al*. reported that SATB1 overexpression was significantly associated with tumor depth and pTNM stage (18, 20). In a large study that included 529 CRC patients, Nodin *et al*. showed that, although high SATB1 expression was related with poor prognosis, it was not an independent prognostic factor by multivariate analysis (19). In contrast, our study showed that the overexpression of SATB1 was not only significantly correlated with LNM but was also an independent prognostic factor for CRC.

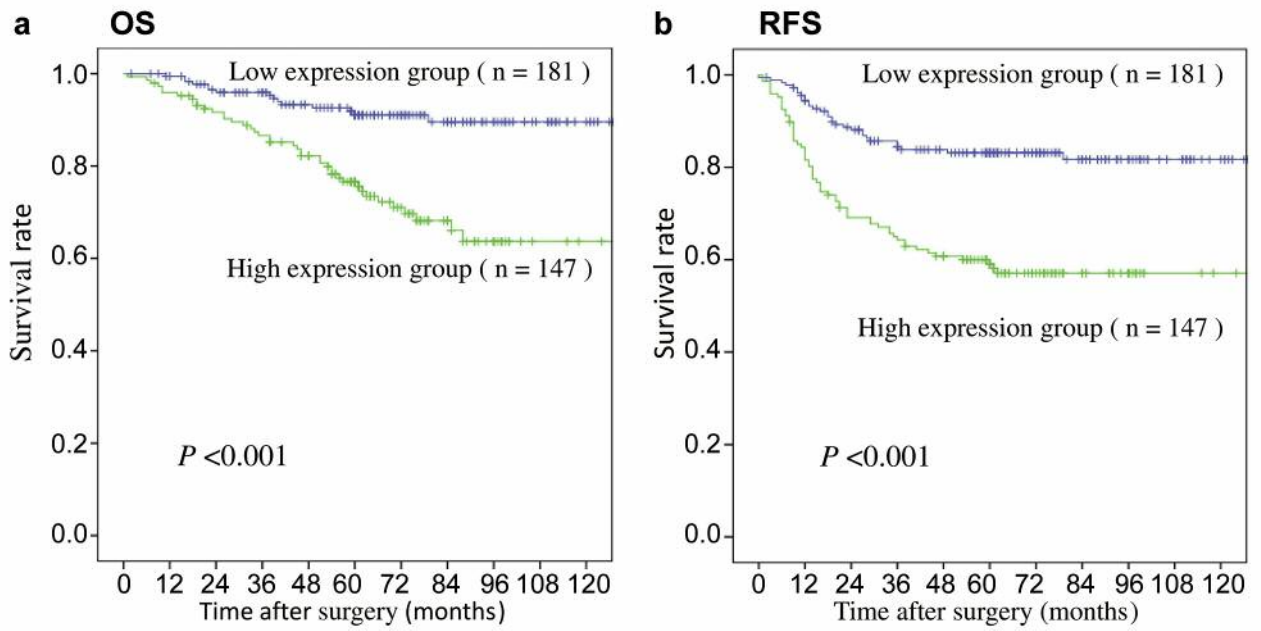

Figure 4. *Kaplan-Meier curves showing overall survival (OS) (a) and relapse-free survival (RFS) (b) of 328 Stage I-III CRC patients by SATB1 protein expression level.*

Regarding treatment for CRC, surgery remains the most effective treatment for localized cancer. However, approximately 40% of patients with such localized CRC will eventually relapse after curative surgery (26). Various adjuvant chemotherapies have been shown to contribute to the improvement of prognosis after surgery (27, 28). Despite the use of these effective chemotherapies, CRC can recur in some patients and some of these patients die of CRC. Furthermore, these chemotherapies are costly and present the risk of severe adverse effects (29). Identification of molecular markers that can predict a high risk of metastasis or recurrence is important for the decision between whether to offer adjuvant therapy or more aggressive treatment.

For stage II disease, major Western guidelines recommend adjuvant chemotherapy when patients have risk factors, even though the efficacy of adjuvant chemotherapy for stage II CRC has not been well-established and remains controversial (32).

Our study showed that overexpression of SATB1 was an independent prognostic factor of OS and RFS in stage II, suggesting that stage II patients with high STAB1 expression might be candidates for adjuvant chemotherapy as patients at high-risk of recurrence.

Endoscopic mucosal resection is standard treatment of Tis or T1 CRC without LNM. However, if there is a high risk of LNM, additional colectomy with LN dissection after endoscopic resection is recommended for T1-CRC in various guidelines. Each guideline recommends an additional curative surgery for T1-CRC with histological findings (30-32). According to the criteria of the Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines, the incidence of LNM for T1-CRC has been reported to be approximately 10% (30). This finding suggests that about 90% of patients with T1-CRC do not have LNM and suffer overtreatment. Precise diagnosis of LNM is, therefore, important and a new biomarker of LNM might contribute to less invasive personalized therapy. SATB1 expression might be a marker for the decision of additional resection in patients with T1-CRC. However, since this study included only a small number of samples of T1-CRC patients (n=35), further investigation using a large number of T1-CRC might be needed to confirm our findings.

Conclusion

This study showed that overexpression of *SATB1* was significantly correlated with LNM and that it was an independent factor of poor prognosis in CRCs. *SATB1* may become a useful biomarker and a target of treatment for patients with CRC.

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Table II. *Univariate and multivariate analysis of clinicopathological features affecting relapse-free survival (RFS) in (a) Stage I-III and in (b) Stage II.*

Variables	(a) Stage I-III					(b) Stage II				
	UV		MV			UV		MV		
	n	*p*-Value	RR	95%CI	*p*-Value	n	*p*-Value	RR	95%CI	*p*-Value
Age, years (median=66)										
≤66	170	0.161				73	0.511			
>66	158					76				
Gender										
Male	209	0.169				67	0.700			
Female	119					82				
Location										
Colon	197	0.003*	1	1.31-3.03	0.001**	101	0.024*	1	0.86-3.91	0.114
Rectum	131		1.99			48		1.84		
Histology										
Well	122	0.018*	1	0.80-2.07	0.302	64	0.622			
Others	206		1.28			85				
CEA (ng/ml)										
<5	203	0.003*	1	1.00-2.35	0.049*	95	0.538			
≥5	121		1.53			53				
Depth										
T1/T2/T3	215	<0.001**	1	1.23-2.98	0.004*	106	0.135			
T4	113		1.91			43				
Lymphatic invasion										
Absent	99	0.005*	1	0.65-2.11	0.589	60	0.865			
Present	229		1.18			89				
Venous invasion										
Absent	46	0.018*	1	0.56-3.21	0.515	20	0.720			
Present	282		1.34			129				
Lymph node metastasis										
Absent	185	<0.001**	1	1.08-2.83	0.024*	149				
Present	143		1.74			0				
SATB1 expression										
Low	181	<0.001**	1	1.50-3.65	<0.001**	96	0.001**	1	1.38-6.67	0.006*
High	147		2.34			53		3.04		

RFS, Relapse-free survival; UV, univariate; MV, multivariate; SATB1, special AT-rich sequence-binding protein 1; CRC, colorectal cancer; CEA, carcinoembryonic antigen; RR, relative risk. *$p<0.05$ was considered statistically significant.

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