Prognostic Significance of Survivin Expression and Combined Analysis with Cancer Stem Cell and Epithelial–Mesenchymal Transition-related Markers in Patients with Rectal Cancer Undergoing Preoperative Chemoradiotherapy

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Abstract. *Aim:* To identify the candidate marker predicting treatment response and survival outcome in rectal cancer patients who received preoperative chemoradiotherapy (CRT). *Patients and Methods:* Between 2000 and 2015, 159 patients with histologically-confirmed rectal adenocarcinoma underwent preoperative CRT followed by surgery. Among them, 70 patients were enrolled and the expression of survivin, cancer stem cell markers (CD44 and CD133) and epithelial–mesenchymal transition markers (E-cadherin and TWIST1) in pretreatment biopsy specimens were evaluated by immunohistochemistry. Associations between the expression of markers and clinical outcomes were evaluated. *Results:* The median follow-up period of all patients was 71 (range=15-203) months. Five-year overall (OS), disease-free (DFS), locoregional recurrence-free (LRRFS) and distant metastasis-free (DMFS) survival were 80.5%, 60.2% 90.1%

and 76.5%, respectively. A significant association between survivin overexpression and worse treatment outcome was shown on univariate analyses for OS, DFS and DMFS ($p=0.022$, 0.002 , and 0.005 , respectively). On multivariate analysis, survivin overexpression was an adverse prognosticator for DFS and DMFS ($p=0.007$ and 0.015 , respectively), with a borderline significant trend towards a shorter OS ($p=0.069$). Four other single biomarkers were not associated with survival outcomes. However, overexpression of both survivin and CD44 was significantly associated with worse OS on multivariate analysis ($p=0.003$). *Conclusion:* Survivin combined with CD44 might be a candidate biomarker for the prediction of recurrence and survival in patients who received preoperative CRT for rectal cancer. Further research with a larger population is needed to validate these results.

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The traditional management of locally advanced rectal cancer was surgery followed by postoperative chemoradiotherapy (CRT) (1). In comparison with postoperative CRT, however, preoperative CRT has the advantage of lower toxicity, a higher sphincter preservation rate, and a lower locoregional recurrence rate. These benefits were demonstrated in a randomized trial, therefore multimodality treatment including preoperative CRT and surgical resection with or without adjuvant chemotherapy has been established as a standard of care for locally advanced rectal cancer (2-4). However, this treatment strategy is not equally effective for all patients. In spite of multimodality therapy, 30-40% of patients experience relapse (2, 3). Treatment failure and heterogeneity of prognosis is probably due to biological properties of the tumor (5). Hence, a number of studies have evaluated

potential biomarkers based on the hallmarks of cancer, and several proteins functioning in apoptosis, proliferation, angiogenesis, DNA repair, hypoxia, metabolism, and pluripotency have been suggested as possible prognostic biomarkers for rectal cancer in patients who received preoperative CRT (5-8).

Among these biomarkers, survivin has been found to be one of the most intriguing. Survivin, a member of the inhibitor of apoptosis protein family, plays an important role in suppressing caspase-mediated apoptosis (9). Recently, a meta-analysis revealed that survivin overexpression was significantly associated with poorer overall survival (OS) and a metastatic capacity in patients with colorectal cancer (10, 11). In addition, much evidence indicates that expression of cancer stem cell (CSC)/epithelial-mesenchymal transition (EMT) markers were correlated with aggressive behavior of carcinoma and a poor prognosis (12, 13). However, the relationship between CSC/EMT marker expression and prognosis remains controversial in rectal cancer. In recent years, co-expression of promising markers has demonstrated an enhanced role in predicting prognosis (14, 15). However, no data are presently available concerning the co-expression of survivin and CSC/EMT markers in rectal cancer. We assumed that combined use of these markers may better predict treatment response and prognosis.

The purpose of this study was to investigate survivin, CD44, CD133, E-cadherin and TWIST1 expression in patients who underwent preoperative CRT for rectal cancer, and identify their relations to treatment response and prognosis. In addition, the prognostic significance of combined expression of these markers was evaluated.

Patients and Methods

Patients. Between 2000 and 2015, 159 patients with locally advanced rectal cancer underwent preoperative CRT with 50.4 Gy of pelvic irradiation followed by surgery. Patients whose biopsies were performed at outside institutions, who refused operation or with follow-up loss were excluded. As a result, 70 pretreatment tumor biopsy specimens were available. Medical records were reviewed to obtain clinicopathological parameters such as sex, age, comorbidity, date of first diagnosis, hematological profile, carcinoembryonic antigen (CEA), preoperative CRT data, surgery type, tumor size, tumor differentiation, presence of lymphovascular invasion, presence of perineural invasion, circumferential resection margin status and TNM stage according to the American Joint Committee on Cancer seventh edition (16). Information on locoregional recurrence, distant metastasis, and survival were also retrieved. The histological features from all biopsies and resected specimens were reassessed by a specialized pathologist blinded to clinical information. This study was approved by the Institutional Review Board (approval number; 2016-03-058).

Immunohistochemical staining. Immunohistochemical staining for survivin (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD44 (dilution 1:100; Leica Biosystems, Novocastra,

Newcastle-upon-Tyne, UK), CD133 (dilution 1:100; Novus Biologicals, Littleton, Colorado, USA), E-cadherin (dilution 1:100; Leica Biosystems, Novocastra) and TWIST1 (dilution 1:200; Abcam, Cambridge, MA, USA) was carried out using a Bond-max autoimmunostaining system (Leica Biosystem, Melbourne, Australia) with Bond™ Polymer refine detection, DS9800 kit (Leica Biosystems, Melbourne, Australia). Four-micrometer-thick sections from formalin-fixed, paraffin embedded pretreatment tumor biopsy specimens were transferred to adhesive slides and dried at 62°C for 30 min. The slides were then deparaffinized and endogenous peroxidase was quenched by incubating the tissue with 3% hydrogen peroxide for 10 min. Antigen retrieval was performed using Bond Epitope Retrieval solution for 20 min at 9°C. Sections were incubated with primary antibodies for 15 min, the post-primary antibody for 10 min, and polymer for 30 min, followed by 3,3'-diaminobenzidine and counterstaining with hematoxylin.

Immunohistochemistry analysis. Survivin, CD133, and TWIST1 were expressed in nuclei and both the intensity (0, none; 1, weak; 2, moderate; 3, strong staining) and proportion of positively stained cells (0, <5%; 1, 5-24%; 2, 25-49%; 3, 50-74%; 4, ≥75%) were assessed. For survivin, the staining intensity and percentage of positively stained tumor cells were multiplied to produce a weighted score for each case and dichotomized, according to Sinicrope *et al.* (17). Tumors with a weighted score of 0 to 4 were considered to have low expression, while those with scores of 5 to 12 were considered to have high expression. For evaluation of CD133 and TWIST1 expression, the sum of the intensity score and proportion score were calculated, according to Zhang *et al.* (18) and Zhu *et al.* (19). Scores of 0 to 3 were defined as low expression, while scores of 4 to 7 were defined as high expression. E-Cadherin and CD44 exhibited membranous staining. E-Cadherin was assessed by intensity (0, normal; 1, mild loss; 2, moderate loss; 3, complete loss). It was subsequently categorized into intact (scores of 0 and 1) or loss (scores of 2 and 3) of expression. CD44 was also assessed by intensity (0, negative; 1, weak; 2, moderate; 3, strong) and was categorized into low expression (scores of 0 and 1) or high expression (scores of 2 and 3).

Assessment of treatment response. Response to preoperative CRT was evaluated in the resected specimens. According to grading system described by Dworak, tumor regression grade (TRG) was evaluated as following: TRG0, No regression (no evidence of change); TRG1, minimal regression (dominant tumor mass with obvious change); TRG2, moderate regression (dominant change with residual tumor); TRG3, near total regression (microscopic residual tumor in fibrotic tissue); TRG4, total regression (no residual tumor cells, only fibrotic mass). Patients with TRG0 and 1 were grouped as non-responders, while those with TRG2, -3 and -4 were grouped as responders. Pathological complete remission was defined as no detection of any residual cancer cells in the resection specimen. Tumor down-staging was characterized by evidence of reduction in T or N categories.

Statistical analysis. Pearson chi-square tests or Fisher's exact tests were used to identify statistically significant differences between categorical variables. Correlations between expressions of molecular markers were analyzed using Pearson correlation coefficients. Kaplan-Meier analysis was used to estimate the rates of overall (OS), disease-free (DFS), locoregional recurrence-free (LRF) and distant metastasis-free (DMFS) survival. A log-rank test was

performed to test for differences in the survival curves. All time-to-event endpoints were calculated from the date of biopsy and recurrences were determined by imaging. The Cox proportional hazard regression model was used for univariate and multivariate analyses. Prognostic factors with a *p*-value of less than 0.2 on the univariate analysis were included in the multivariate model using forward selection. Statistical tests were performed with SPSS software version 18.0.0 (SPSS, Chicago, IL, USA), and *p*-values of less than 0.05 were considered significant. Cut-off values for CSC/EMT markers have varied in previous studies and there were no commonly used criteria. Hence, the cut-off values in this study were determined by a maximally selected rank statistics using the R statistical software package version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics and immunohistochemical expression of markers. The patient characteristics are summarized in Table I. The median age was 57 (range=26-82) years. Forty-four patients (62.9%) were males and 26 patients were females (37.1%). The majority of patients had clinical stage T3/T4 (67 patients, 95.7%) and clinically positive lymph nodes were noted in 52 patients (74.3%).

Figure 1 shows representative cases of immunohistochemical staining. For survivin, 22 patients (31.4%) were included in the high-survivin expression group. Forty-four out of 70 patients (62.9%) were categorized as having high CD44 expression, and 35 patients (50.0%) were categorized as high CD133 expression. Fifty-eight patients (82.9%) were categorized with E-cadherin loss, and 40 patients (57.1%) were categorized into the high TWIST1 expression group. There was no significant correlation between the expression of markers and clinicopathological factors.

Correlation between expression of markers and treatment response. Pathological complete remission was observed in 10 patients (14.3%), TRG3 in 21 (30.0%), TRG2 in 22 (31.4%), TRG1 in 16 (22.9%), and no regression in one (1.4%). T down-staging was observed in 38 patients (54.3%) and N down-staging was observed in 31 (44.3%). Among five markers, only survivin expression was negatively associated with TRG (*p*=0.031). Expression of molecular markers was not related to down-staging (Table II).

Correlation between expression of markers and survival. The median follow-up period of patients overall was 71 (range=15-203) months. During the follow-up period, cancer relapse occurred in 20 patients (28.6%): isolated distant metastasis occurred in 14 patients (20.0%), isolated locoregional recurrence in five (7.1%) and one patient (1.4%) experienced both local and distant progression. Five-year OS, DFS, LRFS and DMFS were 80.5%, 60.2%, 90.1% and 76.5%, respectively. Univariate analysis indicated that high expression of survivin (*p*=0.022), CEA>5 ng/ml (*p*=0.031), ypT3/4 disease (*p*=0.024),

Table I. Patient characteristics.

Variable	No.	%
Gender		
Male	44	62.9
Female	26	37.1
Age		
≤50 Years	14	20
>50 Years	56	80
CEA		
≤5 ng/ml	39	55.7
>5 ng/ml	31	44.3
Clinical T category		
T1-2	3	4.3
T3-4	67	95.7
Clinical N category		
Negative	18	25.7
Positive	52	74.3
Chemotherapy regimen		
FL	61	87.1
Capecitabine	6	8.6
FOLFOX	3	4.3
Surgery		
Transanal excision	5	7.1
Low anterior resection	47	67.1
Abdominoperineal resection	18	25.7
Differentiation ^a		
WD, MD	53	75.7
PD	7	10.0
Lymphatic invasion ^a		
Absent	45	64.3
Present	15	21.4
Perineural invasion ^a		
Absent	48	68.6
Present	12	17.1
Circumferential resection margin ^a		
Negative	58	82.9
Positive	2	2.9
Pathological T category		
T0	10	14.3
T1	6	8.6
T2	20	28.6
T3	33	47.1
T4	1	1.4
Pathological N category ^b		
N0	43	61.4
N1	14	20.0
N2	6	8.6
Adjuvant chemotherapy		
No	12	17.1
Yes	58	82.9

CEA, Carcinoembryonic antigen; FL, 5-fluorouracil plus leucovorin; FOLFOX, 5-fluorouracil plus oxaliplatin; WD, well-differentiated; MD, moderately differentiated; P/D, poorly differentiated. ^aEvaluations were performed in patients without pathological complete response. ^bLymph node evaluation was performed in 63 patients (90.0%).

poor differentiation (*p*=0.029), TRG0-1 (*p*=0.002), no down-staging (*p*=0.037), and positivity of the circumferential resection margin (*p*<0.001) were associated with a shorter OS.

Figure 1. Representative immunohistochemical staining of markers. Survivin: Weak intensity (A), moderate intensity (B) ($\times 200$). CD44: Negative (C), strong intensity (D) ($\times 200$). CD133: Weak intensity (E), strong intensity (F) ($\times 200$). E-Cadherin: Preserved expression (G) ($\times 100$), complete loss of intensity (H) ($\times 100$). TWIST1: Weak intensity (I), strong intensity (J) ($\times 200$).

Table II. Correlation between expression of markers and treatment response.

Marker	Expression	TRG, n (%)			T Down-staging, n (%)			N Down-staging, n (%) ^a		
		0-1 (n=17)	2-4 (n=53)	p-Value	No (n=32)	Yes (n=38)	p-Value	No (n=32)	Yes (n=31)	p-Value
Survivin	Low	8 (47.1%)	40 (75.5%)	0.031	20 (62.5%)	28 (73.7%)	0.228	23 (71.9%)	20 (64.5%)	0.361
	High	9 (52.9%)	13 (24.5%)		12 (37.5%)	10 (26.3%)		9 (28.1%)	11 (35.5%)	
CD44	Low	6 (35.3%)	20 (37.7%)	0.548	16 (46.9%)	11 (28.9%)	0.097	13 (40.6%)	9 (29.0%)	0.242
	High	11 (64.7%)	33 (62.3%)		17 (53.1%)	27 (71.1%)		19 (59.4%)	22 (71.0%)	
CD133	Low	7 (41.2%)	28 (52.8%)	0.289	13 (40.6%)	22 (57.9%)	0.115	17 (53.1%)	16 (51.6%)	0.552
	High	10 (58.8%)	25 (47.2%)		19 (59.4%)	16 (42.1%)		15 (46.9%)	15 (48.4%)	
E-Cadherin	Mild loss	3 (17.6%)	9 (17.0%)	0.604	7 (21.9%)	5 (13.2%)	0.259	9 (28.1%)	3 (9.7%)	0.060
	Complete loss	14 (82.4%)	44 (83.0%)		25 (78.1%)	33 (86.8%)		23 (71.9%)	28 (90.3%)	
TWIST1	Low	5 (29.4%)	25 (47.2%)	0.157	11 (34.4%)	19 (50.0%)	0.141	14 (43.8%)	14 (45.2%)	0.556
	High	12 (70.6%)	28 (52.8%)		21 (65.6%)	19 (50.0%)		18 (56.3%)	17 (54.8%)	

TRG, Tumor regression grade; ^aLymph node evaluation was performed in 63 patients (90.0%). Bold values indicate significant difference.

Survivin overexpression ($p=0.002$), TRG0-1 ($p=0.038$), and circumferential resection margin positivity ($p=0.002$) were also associated with a shorter DFS. Survivin overexpression was the only factor associated with a shorter DMFS ($p=0.005$; Table III) whereas it was not associated with LRFS ($p=0.258$). Figure 2 shows the Kaplan–Meier curves for OS, DFS, LRFS, and DMFS according to survivin expression. On multivariate analysis, TRG0-1 ($p=0.026$) and circumferential resection margin positivity ($p=0.002$) were independent predictors for OS. High expression of survivin was an independent prognostic factor for DFS and DMFS ($p=0.007$ and 0.015 , respectively) and had a borderline significant trend towards a shorter OS ($p=0.069$; Table III). The four other single biomarkers were not associated with survival outcomes (Table IV).

Prognostic value of combined marker analysis. Although CD44, CD133, E-cadherin and TWIST1 expression status did not predict OS ($p=0.279$, 0.569 , 0.346 , and 0.374 respectively),

the combined analysis of CSC/EMT markers with survivin showed significant association with OS (Table IV). Most significant prognostic biomarker combinations included co-expression of both survivin and CD44. On univariate analysis, phenotype for positivity for survivin and CD44 was a significant predictor for OS and DFS ($p=0.001$ and 0.010 , respectively; Figure 3). In addition, multivariate Cox proportional hazard model demonstrated that this phenotype remained an independent predictor for OS (hazard ratio=6.45; 95% confidence interval, 1.87 to 22.25, $p=0.003$; Table V).

Discussion

In the present study, we examined the expression of survivin, CSC (CD44 and CD133) and EMT (E-cadherin and TWIST1) markers in pretreatment biopsy specimens in patients who received preoperative CRT for rectal cancer. Survivin overexpression was an independent prognostic

Table III. Univariate and multivariate analyses for overall (OS), disease-free (DFS) and distant metastasis-free (DMFS) survival.

Variable	OS				DFS				DMFS			
	UVA		MVA		UVA		MVA		UVA		MVA	
	<i>p</i> -Value	HR	95% CI	<i>p</i> -Value	<i>p</i> -Value	HR	95% CI	<i>p</i> -Value	<i>p</i> -Value	HR	95% CI	<i>p</i> -Value
Survivin												
High vs. low	0.022	2.96	0.92-9.52	0.069	0.002	3.30	1.40-7.79	0.007	0.005	3.74	1.30-10.79	0.015
Gender												
Male vs. female	0.392				0.566				0.471			
Age												
>50 vs. ≤50 Years	0.140	1.91	0.36-10.11	0.447	0.449				0.551			
CEA												
>5 ng/ml vs. ≤5 ng/ml	0.031	2.88	0.88-9.42	0.080	0.339				0.762			
Pathological T category												
T3-4 vs. T0-2	0.024	0.53	0.11-2.63	0.438	0.169	0.62	0.23-1.71	0.356	0.251			
Pathological N category												
N1-2 vs. N0	0.705				0.972				0.835			
Differentiation												
PD vs. WD/MD	0.029	2.66	0.62-11.38	0.188	0.112	1.97	0.57-6.82	0.283	0.645			
Lymphatic invasion												
Present vs. absent	0.960				0.799				0.656			
Perineural invasion												
Present vs. absent	0.101	0.47	0.09-2.39	0.472	0.264				0.580			
Circumferential resection margin												
Positive vs. negative	<0.001	40.49	3.74-437.85	0.002	0.002	3.20	0.44-23.21	0.250	0.082	4.13	0.48-34.76	0.192
TRG												
2-4 vs. 0-1	0.002	0.18	0.04-0.81	0.026	0.038	0.47	0.15-1.40	0.173	0.975			
T Down-staging												
Yes vs. no	0.037	0.69	0.17-2.85	0.603	0.252				0.440			
N Down-staging												
Yes vs. no	0.211				0.849				0.500			
Adjuvant chemotherapy												
No vs. yes	0.213	1.26	0.25-6.28	0.778	0.206				0.221			

UVA, Univariate analysis; MVA, multivariate analysis; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; PD, poorly differentiated; WD, well differentiated; MD, moderately differentiated; TRG, tumor regression grade. Bold values indicate significant difference.

factor for DFS and DMFS. In addition, survivin combined with CD44 might be a candidate biomarker for the prediction of recurrence and survival in these patients.

Early studies on survivin expression were generally performed in patients undergoing surgery alone without preoperative treatment. Kawasaki *et al.* observed that survivin expression was strongly associated with bcl-2 expression and inversely correlated with apoptotic index (20). Survivin became an important predictive parameter of worse outcome, and several studies investigated this small novel apoptosis inhibitor. Almost all studies demonstrated significantly worse survival outcomes of patients with survivin-overexpressing tumors (21), except for one study (22). Recently, the need to find biomarkers that can predict radiosensitivity and treatment response before treatment has increased, since preoperative CRT followed by surgery has become the standard treatment for locally advanced rectal cancer (2). Several studies

investigated survivin expression in pretreatment biopsy specimens and demonstrated there was no association between survivin expression and treatment response to preoperative CRT or prognosis (23, 24). In contrast, Kim *et al.* reported that survivin overexpression was significantly associated with poor down-staging (25), and Takasu *et al.* also recognized significant inverse correlation between preoperative CRT response and survivin expression (26). In the present study, a significant association was found between survivin overexpression and TRG. In addition, survivin overexpression was related to poor prognosis.

CSCs were shown to be involved in tumor progression, relapse and treatment resistance (13, 27-28). EMT has recently been considered to be associated with aggressive phenotype and metastatic potential of cancer cells (12, 29-30). We investigated immunohistochemistry of CD44, CD133, E-cadherin, TWIST1, and the association of their

Figure 2. Kaplan-Meier curves for overall (A), disease-free (B), locoregional recurrence-free (C), and distant metastasis-free (D) survival according to survivin expression.

expression with treatment response and survival outcomes was evaluated. Expression of these four single molecular markers was not associated with TRG, down-staging, and survival outcomes, despite evidence supporting that the CSC/EMT phenotype is related to poor treatment response and poor prognosis in patients with rectal cancer (19, 31). However, discrepancies can exist between studies, and these discrepancies may be associated with the characteristics of antibody used (monoclonal vs. polyclonal), methodology, scoring criteria, treatment method, sample size, and follow-up period.

Accumulating evidence supports the notion that survivin overexpression is associated with CSC/EMT phenotypes (19, 21, 32-33). Several mechanisms such as DNA damage repair capability, longer G₂ arrest, reactive oxygen species mechanism as well as apoptosis, are associated with the therapeutic resistance of CSCs (34). Therefore, studies have investigated the relationship between survivin, which plays an important role in suppressing caspase-mediated apoptosis, and the CSC phenotype. Kim *et al.* reported that interleukin-4 was significantly up-regulated in CD133-positive cells compared to CD133-negative cells, and

Figure 3. Kaplan-Meier survival curves for overall (A) and disease-free (B) survival according to combined survivin/CD44 expression.

Table IV. Univariate analysis for overall (OS), disease-free (DFS), and distant metastasis-free (DMFS) survival according to markers and combined markers expression

Variable	OS			DFS			DMFS		
	HR	95% CI	p-Value	HR	95% CI	p-Value	HR	95% CI	p-Value
Survivin	2.79	1.16-6.72	0.022	3.35	1.55-7.25	0.002	4.50	1.58-12.83	0.005
CD44	1.70	0.65-4.45	0.279	1.86	0.78-4.39	0.159	1.23	0.42-3.60	0.705
CD133	1.30	0.53-3.18	0.569	1.31	0.61-2.81	0.481	1.16	0.42-3.20	0.775
E-cadherin loss	0.59	0.20-1.77	0.346	0.84	0.32-2.22	0.721	0.76	0.21-2.70	0.673
Twist1	1.52	0.60-3.84	0.374	1.33	0.62-2.87	0.468	1.33	0.47-3.74	0.589
Survivin+/CD44+	4.25	1.66-10.89	0.003	3.89	1.69-8.94	0.001	3.89	1.36-11.10	0.011
Survivin+/CD133+	2.66	1.09-6.51	0.032	3.56	1.59-7.95	0.002	4.20	1.50-11.73	0.006
Survivin+/E-cadherin loss	2.37	0.98-5.72	0.055	3.20	1.47-6.93	0.003	5.18	1.82-14.78	0.002
Survivin+/TWIST1+	2.48	0.99-6.22	0.054	3.85	1.71-8.69	0.001	3.26	1.14-9.29	0.027
Survivin+/CD44+/CD133+	4.33	1.52-12.29	0.006	4.52	1.73-11.78	0.002	3.84	1.21-12.20	0.022
Survivin+/CD44+/TWIST1+	4.93	1.72-14.12	0.003	5.05	2.01-12.68	0.001	3.37	1.06-10.73	0.040
Survivin+/CD44+/E-cadherin loss	4.49	1.76-11.46	0.002	4.51	1.96-10.39	<0.001	4.59	1.59-13.20	0.005
Survivin+/CD133+/TWIST1+	2.41	0.93-6.29	0.071	3.85	1.62-9.12	0.002	3.31	1.12-9.82	0.031
Survivin+/CD133+/E-cadherin loss	2.25	0.90-5.64	0.084	3.50	1.54-7.95	0.003	5.07	1.81-14.2	0.002
Survivin+/Twist1+/E-cadherin loss	2.07	0.79-5.39	0.136	3.82	1.67-8.74	0.002	3.95	1.38-11.28	0.010
Survivin+/CD44+/CD133+/E-cadherin loss	5.44	1.91-15.51	0.002	6.60	2.59-16.83	<0.001	4.27	1.32-13.79	0.015
Survivin+/CD44+/CD133+/TWIST1+	4.03	1.31-12.39	0.015	4.06	1.47-11.16	0.007	3.01	0.84-10.73	0.090
Survivin+/CD44+/TWIST1+/E-cadherin loss	5.44	1.91-15.51	0.002	6.60	2.59-16.83	<0.001	4.27	1.32-13.79	0.015
Survivin+/CD133+/TWIST1+/E-cadherin loss	1.99	0.72-5.48	0.184	3.93	1.61-9.61	0.003	4.22	1.42-12.57	0.010
Survivin+/CD44+/CD133+/TWIST1+/E-cadherin loss	4.52	1.48-13.84	0.008	5.62	2.02-15.64	0.001	4.03	1.12-14.52	0.033

HR, Hazard ratio; CI, confidence interval. Bold values indicate significant difference.

several studies showed that survivin was significantly associated with CSC phenotype in colorectal cancer tissue (21, 32). Moreover, recent evidence showed the association between apoptosis and EMT. Zhu *et al.* showed that

TWIST1 had an anti-apoptotic function as well as effects on EMT (19), and Vesuna *et al.* reported that an increased expression of TWIST1 inhibited apoptosis by suppressing proapoptotic genes (33).

Table V. Multivariate analysis for overall survival according to positivity for survivin and CD44 expression.

Variable	HR	95% CI	p-Value
Survivin+/CD44+			
High vs. low	6.45	1.87 22.25	0.003
Age			
>50 vs. ≤50 Years	3.37	0.59 19.23	0.172
CEA			
>5 ng/ml vs. ≤5 ng/ml	2.91	0.88 9.70	0.081
Pathological T category			
3-4 vs. 0-2	0.34	0.05 2.16	0.255
Differentiation			
PD vs. WD/MD	1.65	0.38 7.16	0.502
Perineural invasion			
Present vs. absent	0.77	0.18 3.30	0.726
Circumferential resection margin			
Positive vs. negative	118.64	7.95 1769.21	0.001
TRG			
2-4 vs. 0-1	0.17	0.04 0.77	0.022
T Down-staging			
Yes vs. no	0.47	0.10 2.27	0.349

TRG, Tumor regression grade; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; PD, poorly differentiated; WD, well-differentiated; MD, moderately differentiated. Bold values indicate significant difference.

Co-expression of biomarkers may be better at reflecting the degree of acquisition of biological behaviors of cancer cells (5, 14-15). In order to identify useful biomarkers predicting treatment response and prognosis, combined biomarker analysis of the altered expressions of five markers (survivin, CD44, CD133, E-cadherin, and TWIST1) was performed herein. The majority of significantly predictive biomarker combinations included co-expression of both survivin and CD44. Given this observation, we performed further evaluation of the survivin/CD44-positive phenotype to clarify whether this combined biomarker could predict tumor response. Tumors harboring positivity for both survivin and CD44 had poor response to preoperative CRT, that is, TRG 0-1 ($p=0.019$). Five-year OS and DFS of patients with survivin/CD44-positive phenotype were 51.8% and 27.2%, respectively. However, for the remaining phenotypes corresponding values were 86.2% and 68.1%, respectively. Multivariate analysis for OS revealed the group with survivin/CD44-positive phenotype had a 6.45-fold increased risk of death ($p=0.003$). The simultaneous expression of these biomarkers may reflect more aggressive biological behavior of rectal cancer than the alteration of single biomarkers.

Certain limitations exist to this study. Firstly, there are inherent limitations of retrospective studies such as a small number of patients and potential bias from physicians regarding treatment selection. However, there was no

significant difference in the treatment regimens between the two groups. Secondly, in the present study, only nuclear expression of survivin was semi-quantitatively assessed. Qi *et al.* suggested that subcellular localization of survivin may have different protein functions and reported nuclear survivin expression was associated with a favorable outcome (35). It remains controversial whether the subcellular location of survivin is associated with prognosis and further evaluation is needed.

In conclusion, survivin overexpression in pretreatment biopsy specimens was an independent adverse prognostic factor for DFS and DMFS, and the survivin/CD44-positive phenotype was an independent predictor for a shorter OS in patients receiving preoperative CRT for rectal cancer. Further research with a large population is needed to validate these results.

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