Nuclear Y-Box-binding Protein-1 Expression Predicts Poor Clinical Outcome in Stage III Colorectal Cancer

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Abstract. *Background/aim: Y-Box-binding protein-1 (YB-1), a DNA/RNA-binding protein, is an important oncogenic transcription and translation factor. We aimed to evaluate the relationships between nuclear YB-1 expression, epidermal growth factor receptor (EGFR) status, and poor clinical outcomes in patients with colorectal cancer (CRC). Materials and Methods: Nuclear YB-1 expression was immunohistochemically analyzed in CRC tissues obtained from 124 patients who underwent curative resection between 2005 and 2008. Correlations between nuclear YB-1 expression, various clinicopathological characteristics, EGFR status, and prognostic factors were evaluated. Results: High-grade nuclear YB-1 expression was detected in 62.9% of cases and was found to be an independent predictor of poorer overall survival (p<0.001) and relapse-free survival (p<0.001). A trend was also observed towards a positive correlation between nuclear YB-1 expression and EGFR status (p=0.051). Conclusion: Nuclear YB-1 expression is a useful prognostic biomarker that correlates with EGFR status in patients with CRC.*

Colorectal cancer (CRC) presents a serious threat to human health, with high incidence and mortality rates reported in many countries. In 2012, CRC represented the third most common form of cancer in men (746,000 cases, 10.0% of the total cancer cases) and the second most common form of cancer in women (614,000 cases, 9.2% of the total cancer cases), worldwide (1). Treatment for CRC has improved recently; however, almost 50.0% of patients who undergo radical surgery die of metastasis or recurrence. It is therefore, imperative, to identify factors predictive of CRC metastasis or recurrence (*e.g.* clinicopathological characteristics, preoperative tumour markers, and tumour-node-metastasis features). The understanding of the molecular regulatory mechanisms associated with CRC invasion and metastasis has been of great importance in guiding clinical diagnosis and treatment (2).

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein receptor tyrosine kinase that has been found overexpressed in approximately 80.0% of patients with CRC (3). Specific ligands, including epidermal growth factors, attach to form EGFR or a dimer with other molecules of the human EGFR family. EGFR is activated and signal transduction is triggered to downstream components of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and rat sarcoma (RAS)/recombinant activated factor (RAF)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signalling pathways occurs (4). EGFR signalling has been shown to play a critical role in cytodifferentiation, proliferation, and maintenance of normal tissues. On the other hand, EGFR signalling has also been shown to be involved in an increased risk of developing cancer, as well as invasion, metastasis, survival, and tumour angiogenesis in colon cancer tissue (5).

Y-Box-binding protein-1 (YB-1) is a member of a family of DNA/RNA-binding proteins that has a highly conserved cold-shock domain. It is an important oncogenic transcription and translation factor (6). YB-1 binds to the Y-box DNA sequence (an inverted CCAAT box) in the eukaryotic promoter (7). YB-1 plays a critical role in various cellular processes, including the regulation of transcription and translation, DNA repair, proliferation, and drug resistance.

Herein, we aimed to evaluate the relationships between nuclear YB-1 expression, EGFR status, clinicopathological characteristics, and survival outcomes in patients with stage III CRC.

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Key Words: Y- Box-binding protein-1, epidermal growth factor receptor, colorectal cancer, prognosis.

Materials and Methods

Patients and tumour samples. Formalin-fixed, paraffin-embedded tissue specimens were obtained from 124 patients with stage III CRC who underwent surgery at Kurume University Hospital (Fukuoka, Japan) between 2005 and 2008. A summary of the patients' clinicopathological characteristics is provided in Table I. Curative resection with regional lymphadenectomy was performed in all patients. None of the patients received preoperative chemotherapy or radiation therapy. Postoperative pathological staging was determined according to the Japanese Classification of Colorectal Carcinoma (8).

The study protocol was approved by the Institutional Review Board of Kurume University, Japan (no. 269). All participants provided informed, written consent. Research was conducted in accordance with the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001.

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm-thick sections, placed on coated glass slides, and labelled with antibodies against YB-1 and EGFR using the BenchMark XT (Ventana Medical Systems Inc., Tucson, AZ, USA) or ChemMate ENVISION (DakoCytomation, Glostrup, Denmark) methods. The BenchMark XT protocol was followed for YB-1, EGFR, and human EGFR2. This automated system utilizes the streptavidin-biotin complex method (iVIEW DAB Detection Kit; Ventana Medical Systems Inc., Tucson, AZ, USA). Antigen retrieval for YB-1 and EGFR2 was performed using heat treatment in Cell Conditioning 1 (Ventana Medical Systems Inc., Tucson, AZ, USA), while antigen retrieval for EGFR was performed using proteinase K treatment (Ventana Medical Systems Inc., Tucson, AZ, USA). Endogenous peroxidase activity was inhibited by incubating the slides in a 3.0% hydrogen peroxide solution for 5 minutes. Each slide was then incubated overnight with the designated antibody, at 4˚C. To detect staining, 3,3'-diaminobenzidine (Ventana Medical Systems Inc., Tuscon, AZ, USA) was used as the chromogen. Specimens were viewed using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan).

Immunohistochemical grading. Nuclear YB-1 expression was calculated from the mean values of three microscopic fields selected from those with the greatest accumulation of positive signals (hot spots), as described previously (9). A total number of hot spots of less than three in the three microscopic fields was categorized as low-grade, while a total of more than three was categorized as high-grade. EGFR expression was stratified into four groups as follows: no staining or weak membranous expression in <10% of cancer cells: score 0; negative; weak expression in >10% of cancer cells: score 1+, negative; weak to moderate expression along the entire membrane in >10% of cancer cells: score 2+, positive; and strong expression along the entire membrane in >10% of cancer cells: score 3+, positive. The results of the immunohistochemical studies were evaluated by two experienced reviewers (S.S. and T.M.) who were blinded to the clinical status of the patients (Figure 1).

Statistical analysis. Associations between nuclear YB-1 expression, clinicopathological characteristics of the patients, and molecular markers, including EGFR, were evaluated using Fisher's exact tests. Probability (*p*)-values below 0.05 were considered statistically significant, unless otherwise indicated. Survival curves were computed using the Kaplan–Meier method and statistical significance was assessed using a log-rank test. Overall survival (OS) and relapse-free survival (RFS) were defined as the time period extending from the operation to the date of death or disease recurrence, respectively. Univariate and multivariate analyses were performed using a Cox proportional hazards model. All statistical analyses were conducted using JMP, software version 11.0 (SAS Institute Inc., Cary, NC, USA).

Table I. *Clinicopathological characteristics of patients (N=124) with stage III colorectal cancer.*

Clinicopathological characteristic	Patients (N=124)
Age, years	
Mean±SD	67.2±12.1
Range	29-91
Gender, N (%)	
Male	77 (62.1)
Female	47 (37.9)
Tumour site, N (%)	
Colon	57 (46.0)
Rectum	67 (54.0)
Tumour depth, N (%)	
pT1-2	20 (16.1)
pT3-4	104 (83.9)
Tumour differentiation, N (%)	
Well/moderate	109 (87.9)
Other	15 (12.1)
Lymphatic invasion, N (%)	
Negative	33 (26.6)
Positive	91 (73.4)
Venous invasion, N (%)	
Negative	20 (16.1)
Positive	104 (83.9)
Lymph node metastasis, N (%)	
N1	93 (75.0)
N2-3	31 (25.0)
Adjuvant chemotherapy, N (%)	
Yes	53 (42.7)
No	71 (57.3)
Disease recurrence, N (%)	
Yes	34 (27.4)
No	90 (72.6)
Death, N (%)	
Yes	35 (28.2)
No	89 (71.8)

Results

Patients' clinicopathological characteristics and nuclear YB-1 expression. Relationships between the clinicopathological characteristics of Stage III CRC patients (N=124) and nuclear YB-1 expression are summarized in Table II. Nuclear YB-1 expression was not observed to correlate with the clinicopathological characteristics of patients with stage III CRC.

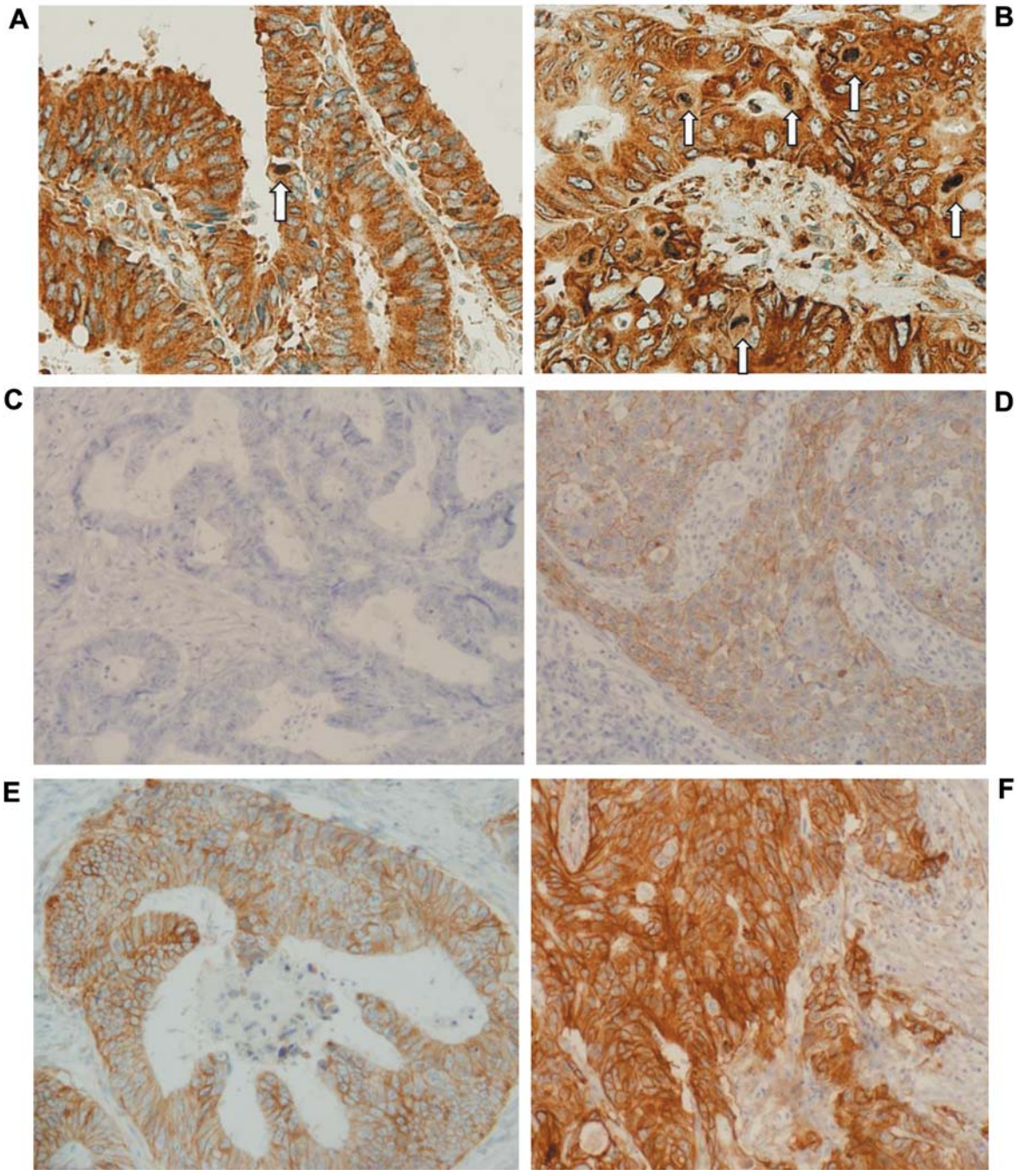

Figure 1. *Immunohistochemical staining of primary colorectal tumours, including representative cases of low-grade (A) and high-grade (B) nuclear Y-box binding protein-1 expression (arrows; ×200 magnification), as well as those with scores for epidermal growth factor receptor expression of 0 (negative) (C), 1+ (negative) (D), 2+ (positive) (E), and 3+ (positive) (F) (×40 magnification).*

Correlation between nuclear YB-1 and EGFR expression. Table III displays the result of a Fisher's exact test comparing nuclear YB-1 and EGFR expression. High-grade nuclear YB-1 expression was detected in 62.9% of cases (N=78), while EGFR expression was positive in 37.1% of cases (N=46). There was a trend towards a positive correlation between nuclear YB-1 and EGFR expression in patients with stage III CRC (p=0.051; Table III).

Table II. *Relationship between nuclear Y-box binding protein-1 (YB-1) expression and the clinicopathological characteristics of patients (N=124) with stage III colorectal cancer.*

Clinicopathological characteristic	Nuclear YB-1 expression		*p*-Value
	High (N=78)	Low (N=46)	
Age, N (%)			
≤68 Years	37 (64.9)	20 (35.1)	
>68 Years	41 (61.2)	26 (38.8)	0.669
Gender, N (%)			
Male	45 (58.4)	32 (41.6)	
Female	33 (70.2)	14 (29.8)	0.188
Tumour site, N (%)			
Colon	37 (64.9)	20 (35.1)	
Rectum	41 (61.2)	26 (38.8)	0.669
Tumour depth, N (%)			
pT1-2	10 (50.0)	10 (50.0)	
pT3-4	68 (65.4)	36 (34.6)	0.192
Tumour differentiation, N (%)			
Well/moderate	68 (62.4)	41 (37.6)	
Other	10 (66.7)	5 (33.3)	0.748
Lymphatic invasion, N (%)			
Negative	19 (57.6)	14 (42.4)	
Positive	59 (64.8)	32 (35.2)	0.460
Venous invasion, N (%)			
Negative	11 (55.0)	9 (45.0)	
Positive	67 (64.4)	37 (35.6)	0.424
Lymph node metastasis, N (%)			
N1	59 (63.4)	34 (36.6)	
N2-3	19 (61.3)	12 (38.7)	0.830
Adjuvant chemotherapy			
Yes	44 (62.0)	27 (38.0)	
No	34 (64.2)	19 (35.8)	0.803

Table III. *Correlation between nuclear Y-box binding protein-1 (YB-1) and epidermal growth factor receptor (EGFR) expression in patients (N=124) with stage III colorectal cancer.*

EGFR expression	Nuclear YB-1 expression		*p*-Value
	High (N=78)	Low (N=46)	
Positive	44 (56.4)	34 (43.6)	
Negative	34 (73.9)	12 (26.1)	0.051

Survival analysis. Patients with high-grade nuclear YB-1 expression (N=78) exhibited a significant reduction in their OS ($p<0.001$; Figure 2A) and RFS rates ($p<0.001$; Figure 2B) compared to patients with low-grade nuclear YB-1 expression (N=46). The 5-year OS and RFS rates of patients with high-grade nuclear YB-1 expression were 61.5% and 56.5%, respectively, while those for patients with low-grade nuclear YB-1 expression were 93.3% and 97.8%, respectively. The OS ($p<0.001$) and RFS rates ($p<0.001$) of the EGFR-positive group were significantly lower than those reported for the EGFR-negative group. In addition, we analyzed the effects of nuclear YB-1 and EGFR expression on the OS and RFS rates of patients with stage III CRC. Positive nuclear YB-1 and EGFR expression were associated with a significantly poorer prognosis. The OS ($p<0.001$; Figure 3A) and RFS rates ($p<0.001$; Figure 3B) of the EGFR-positive, high-grade nuclear YB-1 expression group (N=44) were significantly lower than those reported for the EGFR-negative, low-grade YB-1 expression group (N=12).

In the univariate analysis, increasing tumour depth ($p<0.05$), poor histological type ($p<0.01$), lack of adjuvant chemotherapy ($p<0.05$), and high-grade nuclear YB-1 expression ($p<0.001$) were found to be factors predictive of poorer OS (Table IV). Moreover, age above 68 years ($p<0.05$), poorer histological type ($p<0.001$), lymph node metastasis ($p<0.01$), and high-grade nuclear YB-1 expression ($p<0.001$) were found to be factors predictive of poorer RFS (Table V).

Applying a multivariate Cox proportional hazards model, increasing tumour depth [hazard ratio (HR)=6.36, 95% confidence interval (CI)=1.36-11.3; $p<0.05$], histological type (HR=3.56, 95% CI=1.48-7.68; $p<0.01$), lack of adjuvant chemotherapy (HR=2.10, 95% CI=1.07-4.19; $p<0.05$), and high-grade nuclear YB-1 expression (HR=4.98, 95% CI=2.08-14.7; $p<0.001$) were confirmed as independent prognostic factors for OS (Table IV). Age above 68 years (HR=2.25, 95% CI=1.12-4.74; $p<0.05$), poor histological type (HR=6.34, 95% CI=2.40-16.9; $p<0.001$), and high-grade nuclear YB-1 expression (HR=34.9, 95% CI=7.31-627; $p<0.001$) were confirmed as independent prognostic factors for RFS (Table V).

Discussion

Our present findings demonstrate that high-grade nuclear YB-1 expression is associated with poor prognosis and recurrence in patients with stage III CRC. Moreover, we also observed a trend towards a positive correlation between nuclear YB-1 and EGFR expression in patients with stage III CRC. These findings suggest that nuclear YB-1 expression correlates with EGFR expression, and high-grade expression of both nuclear YB-1 and EGFR are associated with a poor prognosis in patients with stage III CRC.

Previous reports have established YB-1 as an important regulator of the cell cycle, cell proliferation, DNA repair, oncogenic transcription, translation, and drug resistance (10, 11). In CRC, YB-1 expression correlates with prognosis and could serve as a poor prognostic biomarker, although the mechanism(s) by which nuclear YB-1 localization contributes to CRC is not clearly understood (12, 13).

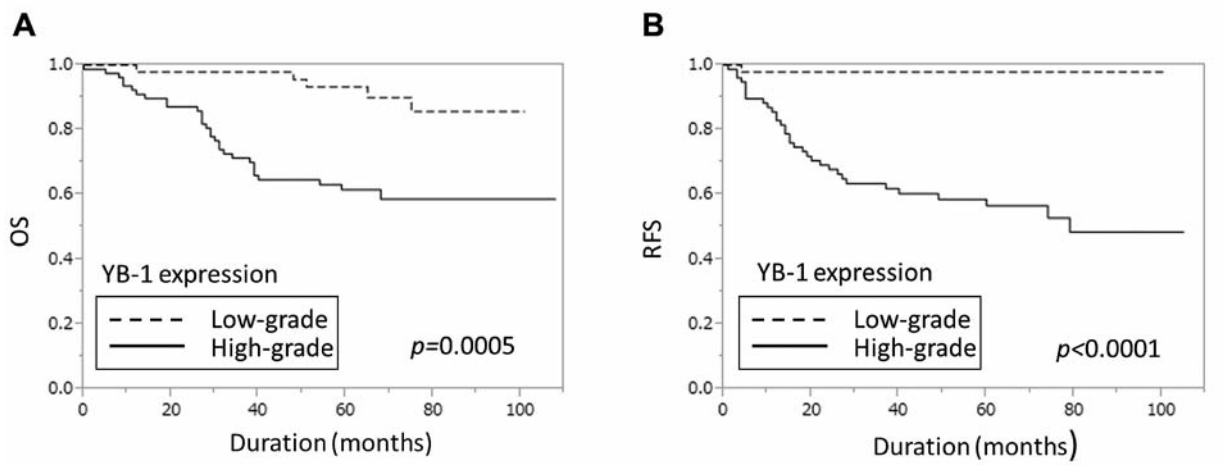

Figure 2. *Kaplan–Meier curves of overall survival (OS) (A) and relapse-free survival (RFS) (B) rates according to nuclear Y-box binding protein-1 (YB-1) expression in patients with stage III colorectal cancer. High-grade nuclear YB-1 expression, N=78; low-grade nuclear YB-1 expression, N=46.*

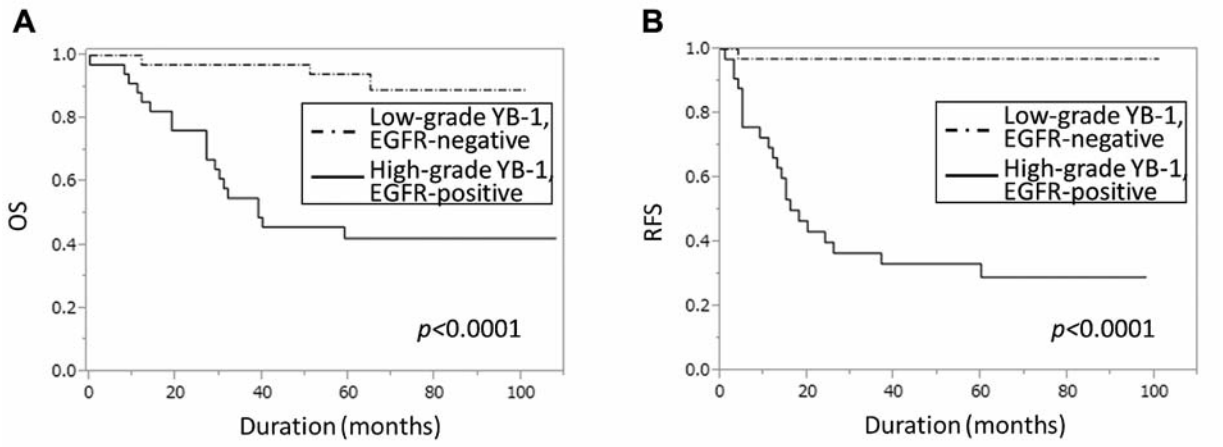

Figure 3. *Kaplan–Meier curves of overall survival (OS) (A) and relapse-free survival (RFS) (B) rates according to epidermal growth factor receptor (EGFR) status and nuclear Y-box binding protein-1 (YB-1) expression in patients with stage III colorectal cancer. EGFR-negative cases with low-grade nuclear YB-1 expression, N=34; EGFR-positive cases with high-grade nuclear YB-1 expression, N=34.*

Specifically, previous studies have reported on a correlation between nuclear YB-1 and EGFR expression in breast, lung, and cervical cancer (14-17).

Under normal cellular conditions, YB-1 localizes to the cytoplasm of benign cells (18). However, in response to DNA_damaging factors (*e.g.* xenobiotics and ultraviolet irradiation), YB-1 is often translocated to the nucleus (19). Moreover, Eliseeva *et al*. have described how nuclear translocation of YB-1 can be promoted by some growth factors and cytokines (20). Nuclear YB-1 expression is induced by the phosphorylation of serine residue 102 which is located in the cold-shock domain. Phosphorylation is promoted through the PI3K/AKT and MAPK/ERK signalling pathways and has been shown to be a poor prognostic factor in melanomas, as well as in breast and ovarian carcinomas (21-23).

In breast cancer, YB-1 phosphorylation was reported to be induced *via* EGFR, downstream of the PI3K/AKT and MAPK/ERK signalling pathways, in response to exposure to ionizing radiation (24). Hyogotani *et al*. also demonstrated a positive correlation between nuclear YB-1 and EGFR expression (15). Specifically, short interfering RNA-induced down-regulation of YB-1 expression was associated with a concomitant reduction in the up-regulation of *EGFR* expression in patients with non-small cell lung cancer. Therefore, nuclear translocation of YB-1 induces the up-

Table IV. *Univariate and multivariate analysis of overall survival using a Cox proportional hazards model in patients (N=124) with stage III colorectal cancer.*

Clinicopathological characteristic	Univariate analysis		Multivariate analysis	
	Patients (N=124)	*p*-Value	HR (95% CI)	*p*-Value
Age, years				
≤68	57		–	
>68	67	0.598	–	–
Tumour site				
Colon	57		–	
Rectum	67	0.835	–	–
Tumour depth				
pT1-2	20		1	
pT3-4	104	0.019*	6.36 (1.36-11.3)	0.013*
Tumour differentiation				
Well/moderate	109		1	
Other	15	0.006*	3.56 (1.48-7.68)	0.006*
Lymphatic invasion				
Negative	33		–	
Positive	91	0.166	–	–
Venous invasion				
Negative	20		–	
Positive	104	0.166	–	–
Lymph node metastasis				
N1	93		–	
N2-3	31	0.408	–	–
Adjuvant chemotherapy				
Yes	53		1	
No	71	0.027*	2.10 (1.07-4.19)	0.030*
Nuclear YB-1 expression				
Low	46		1	
High	78	<0.001*	4.98 (2.08-14.7)	<0.001*

CI, Confidence interval; HR, hazard ratio; YB-1, Y-box binding protein-1. *Indicates values that are statistically significant ($p<0.05$).

Table V. *Univariate and multivariate analysis of relapse-free survival using a Cox proportional hazards model in patients (N=124) with stage III colorectal cancer.*

Clinicopathological characteristic	Univariate analysis		Multivariate analysis	
	Patients (N=124)	*p*-Value	HR (95% CI)	*p*-Value
Age, years				
≤68	57		2.25 (1.12-4.74)	
>68	67	0.028*	1	0.022*
Tumour site				
Colon	57		–	
Rectum	67	0.395	–	–
Tumour depth				
pT1-2	20		–	
pT3-4	104	0.150	–	–
Tumour differentiation				
Well/moderate	109		1	
Other	15	<0.001*	6.34 (2.40-16.9)	<0.001*
Lymphatic invasion				
Negative	33		–	
Positive	91	0.338	–	–
Venous invasion				
Negative	20		–	
Positive	104	0.980	–	–
Lymph node metastasis				
N1	93		1	
N2-3	31	0.005*	1.59 (0.64-3.66)	0.304
Adjuvant chemotherapy				
Yes	53		–	
No	71	0.311	–	–
Nuclear YB-1 expression				
Low	46		1	
High	78	<0.001*	34.9 (7.31-62.7)	<0.001*

CI, Confidence interval; HR, hazard ratio; YB-1, Y-box binding protein-1.*Indicates values that are statistically significant ($p<0.05$).

regulation of *EGFR* expression (15). Moreover, it suggests that YB-1 binds directly to the *EGFR* promoter and that *EGFR* is a YB-1-responsive gene that relies on serine residue 102 for optimal DNA binding (25). Our present findings demonstrate that high-grade nuclear YB-1 expression tends to be positively correlated with EGFR expression in patients with stage III CRC. Moreover, patients with stage III CRC expressing high nuclear YB-1 and EGFR levels are had a poor prognosis. These findings suggest that nuclear YB-1 may be involved in EGFR signalling pathways that results in an unfavourable prognostic phenotype in CRC.

EGFR has been shown to be overexpressed in CRC and has also been associated with a poor prognosis (26). Therefore, molecularly targeted therapies (*e.g.*, EGFR inhibitors cetuximab and panitumumab) were introduced to treat unresectable and progressive or recurrent CRC. For EGFR-targeted therapy, expression of EGFR was initially considered important. However, it has subsequently been reported that mutations in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog(KRAS), encoding the downstream signal transmission protein, may be more significant than the expression of EGFR itself. In addition, it has become apparent that the KRAS mutation status of the tumour may be a negative predictive factor of EGFR-targeted therapy in CRC (27, 28).

In breast cancer tissues, *KRAS* mutations strongly induce phosphorylation of YB-1 (24). Furthermore, Kashihara *et al*. demonstrated that the absence or presence of nuclear YB-1 expression correlates with differences in the progression-free survival of patients with non-small cell lung cancer (N=26) who were treated with gefitinib (29). In addition, these authors also suggested that nuclear YB-1 expression may influence the effectiveness of EGFR-targeted therapies (29). In our study, regarding EGFR and its relationship with nuclear YB-1 expression, we

demonstrated that there was a tendency for the two to correlate with one another. In CRC, advances in treatment using molecularly targeted therapies have been made. However, further studies will be required to determine the mechanism(s) by which *EGFR* and *KRAS* mutations influence nuclear YB-1 translocation.

In conclusion, nuclear YB-1 expression is related to EGFR status and correlates with a poor prognosis in patients with stage III CRC. Nuclear YB-1 expression should be regarded as a potentially novel and useful biomarker for CRC. Moreover, it could be particularly beneficial for determining treatment options in predicting the effect of EGFR-targeted therapies, by detecting *KRAS* mutations in CRC. It will be necessary to further explore the relationship between *KRAS* mutations and nuclear YB-1 expression, as well as the effects of resistance to chemotherapeutic agents and molecularly targeted drugs.

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