

## ELF3 Overexpression as Prognostic Biomarker for Recurrence of Stage II Colorectal Cancer

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**Abstract.** *Background/Aim: Adjuvant chemotherapy for high-risk Stage II colorectal cancer (CRC) is weakly recommended; however, no consensus exists on “high-risk” definition. Prognostic biomarker identification is important for selecting patients with poor prognosis who may benefit from adjuvant chemotherapy. Materials and Methods: Using Microarray data analyses, ELF3 was identified as a candidate gene highly expressed in Stage II CRC with distant recurrences. ELF3 mRNA expression in 168 Stage II CRC patients was subjected to quantitative RT-PCR analysis and ELF3 protein expression in 185 patients was quantified by immunohistochemical analysis. The relationship between mRNA and protein expression levels and patient characteristics were also investigated. Results: The overall recurrence rate and relapse-free survival were significantly poorer in the ELF3 high-expression than the low-expression group at the mRNA and protein levels. High ELF3 mRNA and protein expression levels were independent poor prognostic factors. Conclusion: High ELF3 expression was associated with recurrence of Stage II.*

The incidence of colorectal cancer (CRC), which is the second leading cause of cancer-related death in Japan, continues to increase worldwide (1-3). The tumor-node-

metastasis (TNM) staging system of the International Union Against Cancer is the gold standard to determine an appropriate therapeutic strategy, including adjuvant chemotherapy (4-6). The risk of recurrence is an important factor to consider when choosing a postoperative treatment strategy and is therefore being intensively studied worldwide. Postoperative adjuvant chemotherapy for Stage III CRC patients is considered the standard treatment to improve survival. However, adjuvant chemotherapy is only weakly recommended for high-risk Stage II CRC, as there is currently no consensus on the definition of high-risk Stage II CRC (7-15). The prognosis of high-risk Stage II CRC is poor, similar to that of Stage III disease. Hence, the efficacy of postoperative adjuvant chemotherapy or more intensive surveillance is currently being investigated to improve the prognosis of these patients, and research is ongoing to identify factors that define high-risk disease. According to the American Society of Clinical Oncology guidelines, risk factors for recurrence of Stage II CRC include the dissection of less than 12 lymph nodes, T4, perforation, poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous carcinoma (16). According to the European Society for Medical Oncology guidelines, risk factors for the recurrence of Stage II CRC include T4, poorly differentiated gland cancer or undifferentiated carcinoma, vascular invasion, lymphatic invasion, perineural invasion, initial symptoms of intestinal obstruction or intestinal perforation, and dissection of less than 12 lymph nodes (17). Meanwhile, exploration of the risk classification for Stage II CRC patients is ongoing internationally. The aim of the study was to identify useful biomarkers for the selection of subgroups of Stage II CRC with poor prognosis using comprehensive data of gene copy number combined with gene expression analysis.

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**Key Words:** Stage II colorectal cancer, biomarker, prognostic marker, ELF3, microarray analysis.

**Materials and Methods**

*Identification of candidate genes by microarray gene expression analysis.* Microarray data from a previous study were used in the current study (18). The gene expression and the copy number data were deposited in the Gene Expression Omnibus repository [GSE64258, GSE64256 (gene expression), and GSE64257 (copy number analysis)]. Prognosis information for Stage II CRC in the previously analyzed microarray data was updated and re-analyzed. The copy number and gene expression data were compared between the two groups to identify candidate genes as potential biomarkers of the recurrence of Stage II CRC. Genes with a gain in copy number involved in disease recurrence were identified using logistic regression analysis ( $p < 0.05$ ), while genes that were significantly upregulated in disease recurrence were identified using the Welch *t*-test ( $p < 0.05$ ). Genes that were common to both groups were selected as candidates for further analysis. Finally, E74-like E26 transformation-specific transcription factor 3 (*ELF3*) was selected as the target gene for further analysis in the present study.

*Patients.* From 2009 to 2014, a total of 185 patients underwent curative surgery for Stage II CRC at the Tokyo Medical and Dental University (Tokyo, Japan). The median follow-up time for these patients was 59 (range=1-113) months. Tissue samples from 168 patients, including 27 with disease recurrence, were used for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. In addition, formalin-fixed paraffin-embedded samples were available for all 185 patients, including 32 with disease recurrence, and used for immunohistochemical (IHC) analysis. Clinical data were obtained from the medical records of each patient, and histopathological evaluations were assessed by reference to the TNM criteria of the Union for International Cancer Control, 8<sup>th</sup> edition. The study protocol was approved by the Institutional Review Board of Tokyo Medical and Dental University and conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients prior to inclusion in this study.

*Quantitative reverse transcription polymerase chain reaction.* Total RNA (10 ng) was isolated from bulk samples of cancer and adjacent non-neoplastic tissues and reverse-transcribed into complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). A TaqMan gene expression assay (Applied Biosystems: *ELF3*; Hs00963877\_g1, *ACTB*; Hs99999903\_ml) was used to investigate the expression of *ELF3*, with  $\beta$ -actin as an internal control. The qRT-PCR reaction was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). All calculated concentrations of the target genes were normalized to the amount of the endogenous reference using the comparative Ct ( $\Delta\Delta Ct$ ) method for relative quantification with Relative Quantification Study Software (StepOne Realtime PCR System, Applied Biosystems). Each assay was performed in duplicate. Gene expression was analyzed with the use of the ratio of cancer tissue expression to non-neoplastic tissue expression (T/N ratio).

*Immunohistochemical (IHC) analysis.* IHC analysis of *ELF3* was conducted using formalin-fixed paraffin-embedded tissue blocks from each patient. The slides were deparaffinized in xylene and rehydrated with a series of graded concentrations of ethanol. Antigen retrieval by microwave was conducted for 15 min in Antigen Retrieval Buffer (100 $\times$  ethylenediaminetetraacetic acid

Table I. Correlation between clinicopathological features and *ELF3* mRNA expression in Stage II CRC.

	ELF3 mRNA High expression (n=67)	ELF3 mRNA Low expression (n=101)	<i>p</i> -Value
Age			0.017*
<65	29	25	
$\geq 65$	38	76	
Gender			0.26
Male	37	65	
Female	30	36	
Histology			0.05
Well, Moderate	66	91	
Poor and others	1	10	
Depth			1
T3	60	90	
T4	7	11	
Location			Colon vs. Rectum
Colon (Right)	18	37	
Colon (Left)	36	45	1.0
Rectum	13	20	
Lymphatic invasion			1.0
-	50	76	
+	17	25	
Venous invasion			0.837
-	11	19	
+	56	82	
Pre-operative CEA (ng/dl)			0.873
<5	40	62	
$\geq 5$	27	39	
Perforation/Occlusion			0.52
-	67	67	
+	0	0	
Microsatellite Instability (MSI)			0.03*
High	1	11	
Low/MSS	66	90	
Harvested LN			0.20
$\geq 12$	53	88	
<12	14	13	

CEA, Carcinoembryonic antigen; MSS, microsatellite stable; LN, lymph node; \* $p < 0.05$ .

buffer, pH 8.0; Abcam, Cambridge, UK) at 98°C. Afterward, the slides were incubated in a solution of 3% hydrogen peroxide in 100% methanol for 15 min at room temperature in order to quench endogenous peroxidase activity and then incubated overnight with rabbit polyclonal antibody against *ELF3* (dilution, 1:100; Sigma-Aldrich Corporation, St. Louis, MO, USA) at 4°C. The slides were then incubated with peroxidase-labeled antibody (Histofine® Simple Stain™ MAX PO (MULTI); Nichirei Bioscience, Inc., Tokyo, Japan) for 30 min at room temperature. Peroxidase activity was detected with Histofine Simple Stain DAB Solution (Nichirei BioScience, Inc.) for 20 min at room temperature. Finally, the slides were counterstained with 1% Mayer's hematoxylin for 12 s. Staining intensity was graded by two independent observers who were blinded to the patient information.

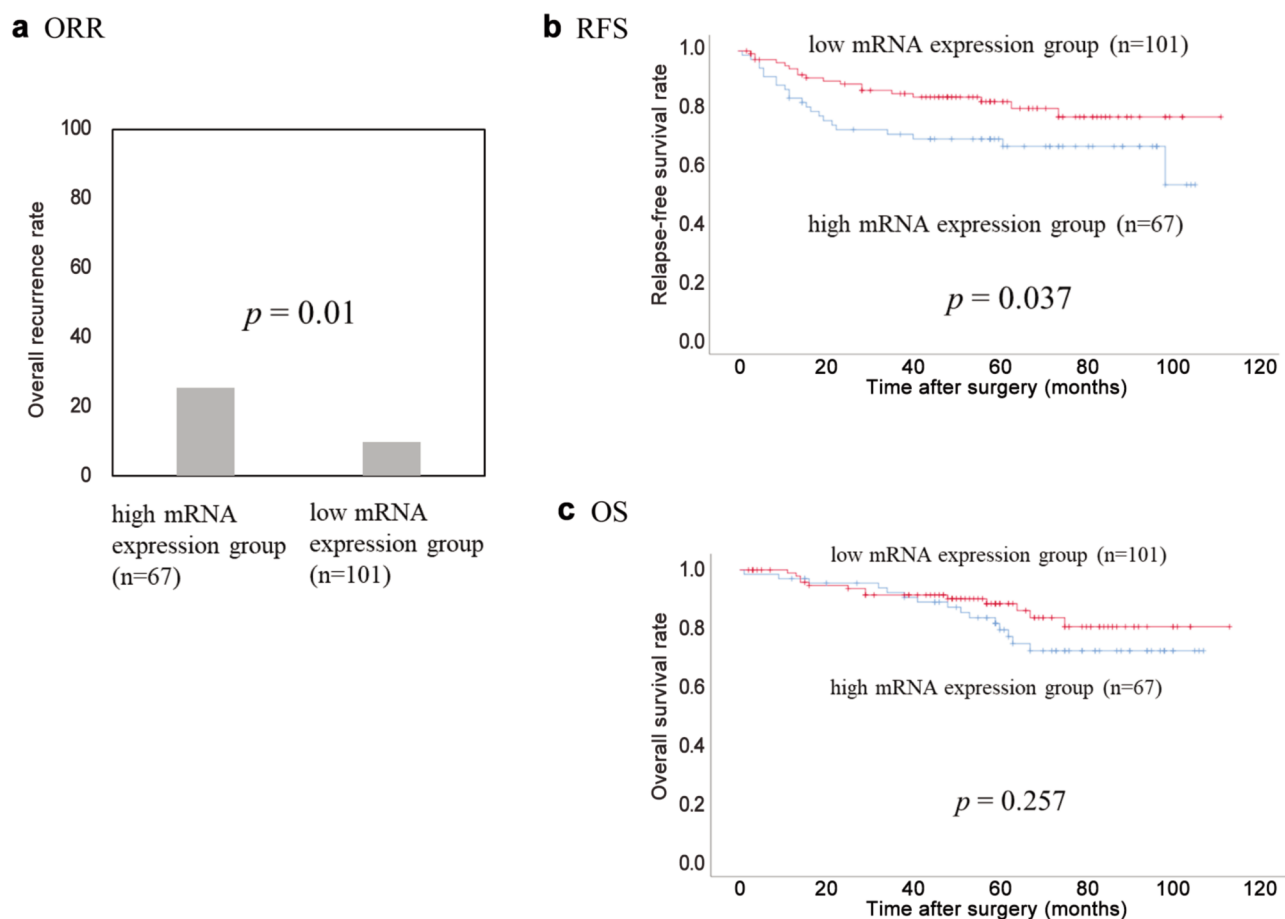


Figure 1. Bar graph showing overall recurrence rate (ORR) (a) and Kaplan-Meier curves showing relapse-free survival (RFS) (b) and overall survival (OS) (c) of 168 Stage II CRC patients by *ELF3* mRNA expression level.

**Statistical analysis.** All statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0. (IBM Corporation, Armonk, NY, USA). The Mann-Whitney *U*-test and the Chi-square test were used where appropriate to identify significant differences between groups. The overall recurrence rate (ORR) was defined as the rate of any recurrence after surgical resection. Relapse-free survival (RFS) was defined as the time from the date of surgical resection to any recurrence or death other than from the primary cancer. Overall survival (OS) was defined as the time from the date of surgery to death from any cause. Survival curves were plotted using the Kaplan-Meier method, and differences between the curves were estimated using the log-rank test. Potential prognostic factors were screened using univariate and multivariate analyses with a Cox proportional hazards model. A Cox model was used to estimate the hazard ratio (HR) and the corresponding 95% confidence interval (CI). A probability (*p*) value of <0.05 was considered statistically significant.

## Results

**Gene expression and copy number analyses for identification of candidate genes.** Comprehensive analysis of gene expression and copy number showed that 396 genes were up-

regulated in the recurrence group compared to the non-recurrence group, and 72 genes had gains in copy number. Seven genes were common in both groups. Among these genes, *ELF3* was selected as a candidate gene because it is reported to be up-regulated or down-regulated in other solid tumors, although the clinical significance of this gene in CRC remains unclear. Therefore, *ELF3* was the focus of the subsequent analyses.

**Validation of *ELF3* mRNA expression.** qRT-PCR analysis of *ELF3* mRNA expression showed that the T/N ratio of the recurrence group was marginally higher than that of the non-recurrence group ( $p=0.093$ ).

**Relationship between *ELF3* mRNA expression and prognosis of CRC.** For statistical evaluation purposes, the 168 samples used for qRT-PCR analysis were allocated to one of the following two groups based on the cutoff value determined from a receiver operating characteristic (ROC) curve in order to predict recurrence after surgery: a high-expression group

Table II. Univariate and multivariate analyses of clinicopathological features affecting the ORR, RFS, and OS based on *ELF3* mRNA levels.

Variables	n	ORR				RFS				OS			
				MA				MA				MA	
		ORR	UA <i>p</i> -Value	OR (95% CI)	<i>p</i> -Value	5y RFS	UA <i>p</i> -Value	Relative risk (95% CI)	<i>p</i> -Value	5y OS	UA <i>p</i> -Value	Relative risk (95% CI)	<i>p</i> -Value
Age													
<65	54	24.0%	0.07	0.617	0.317	77.1%	0.756			91.4%	0.024*	2.679	0.055
≥65	114	12.3%		(0.240-1.589)		76.8%				80.0%		(0.980-7.319)	
Gender													
Male	102	17.6%	0.52			73.6%	0.390			85.1%	0.675		
Female	66	13.6%				82.1%				85.9%			
Histology							0.555						
Well,	157	16.6%	1.0			76.8%				85.7%	0.956		
Moderate													
Poor and others	11	9.1%				81.8%				81.8%			
Depth													
T3	150	15.3%	0.50			76.3%	0.390			86.5%	0.649		
T4	18	22.2%				72.2%				76.0%			
Location													
Colon	135	13.3%	0.064	3.137	0.032*	79.3%	0.346			87.2%	0.269		
Rectum	33	27.3%		(1.100-8.947)		68.1%				74.3%			
Lymphatic invasion													
-	126	10.3%	0.001	5.527	<0.001	83.1%	0.001	2.804	<0.001	87.0%	0.196		
+	42	33.3%	**	(2.136-14.298)	**	59.6%	**	(1.487-5.286)	**	80.5%			
Venous invasion													
-	30	6.7%	0.171			81.5%	0.601			84.6%	0.756		
+	138	18.1%				76.0%				85.6%			
Pre-operative CEA (ng/dl)													
<5	102	15.7%	1.0			82.4%	0.021*	1.970	0.033*	87.8%	0.037*	2.256	0.033*
≥5	66	16.7%				68.5%		(1.057-3.671)		78.5%		(1.067-4.771)	
Perforation/ Occlusion													
-	166	16.2%	1.0			76.80%	0.464			84.1%	0.56		
+	2	0%				100%				100%			
MSI													
High	12	0%				100%				100%			
Low/MSS	156	16.2%				75.2%				83.0%			
Harvested LN													
≥12	141	15.6%	0.77			81.3%	0.038*	1.569	0.214	62.3%	0.012*	1.970	0.106
<12	27	18.5%				56.4%		(0.771-3.194)		90.0%		(0.865-4.487)	
<i>ELF3</i> mRNA expression (T/N value)													
Low	101	9.9%	0.01*	3.150	0.017*	82.3%	0.037*	1.965	0.036*	88.4%	0.257		
High	67	25.4%		(1.230-8.068)		79.4%		(1.045-3.697)		69.3%			

ORR, Overall recurrence rate; RFS, relapse-free survival; OS, overall survival; UA, univariate analyses; MA, multivariate analyses; OR, odds ratio; \*\**p*<0.01; CI, confidence interval.

(T/N value>1.2, n=67) or a low-expression group (T/N value<1.2, n=101) (Table I). The ORR was significantly higher in the high *ELF3* mRNA expression group than in the low-expression group (Figure 1a). Univariate analysis

indicated that lymphatic invasion (*p*=0.001) and *ELF3* mRNA expression (*p*=0.01) were significantly associated with the ORR. Multivariate analysis indicated that *ELF3* overexpression is an independent and significant poor



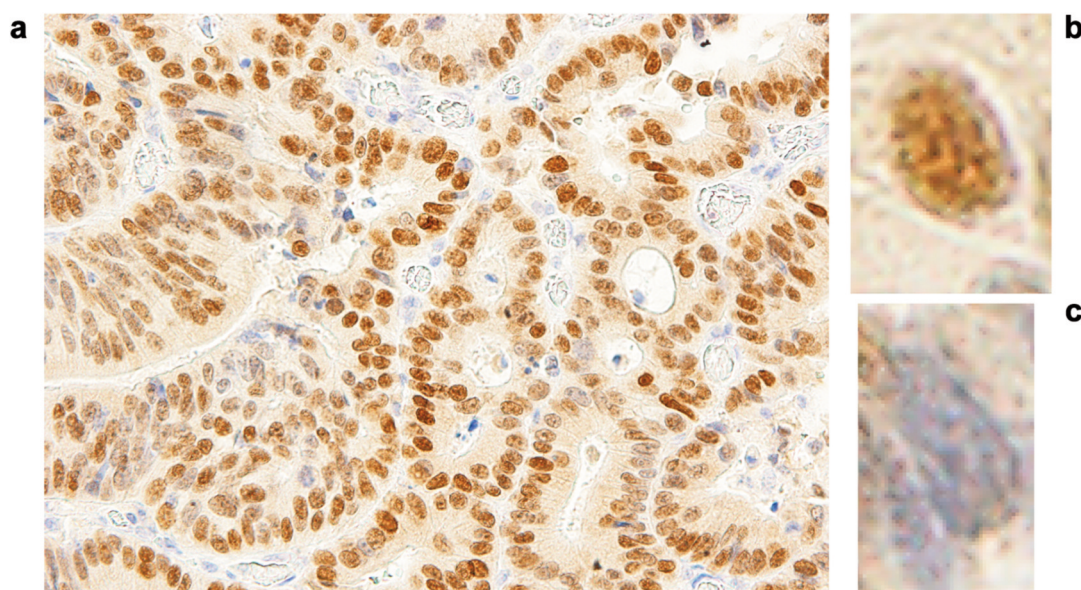


Figure 2. Representative immunostaining for *ELF3* in ulcer randwall of colorectal cancer: (a) ulcer randwall ( $\times 400$ ), (b) positive staining, (c) negative staining.

prognostic factor of the ORR ( $p=0.017$ ; RR=3.150; 95% CI=1.230-8.068) (Table II). RFS was significantly lower in the high mRNA *ELF3* expression group than in the low mRNA expression group (Figure 1b). Univariate analysis indicated that lymphatic invasion ( $p<0.001$ ), preoperative carcinoembryonic antigen (CEA) level ( $p=0.021$ ), number of harvested lymph nodes ( $p=0.038$ ), and *ELF3* mRNA expression ( $p=0.037$ ) were significantly associated with RFS. Multivariate analysis indicated that *ELF3* mRNA overexpression is an independent and significant poor prognostic factor of RFS ( $p=0.036$ ; RR=1.965; 95% CI=1.045-3.697). OS was significantly lower in the high *ELF3* mRNA expression group than in the low mRNA expression group (Figure 1c). Univariate analysis indicated that age ( $p=0.024$ ), preoperative CEA level ( $p=0.037$ ), and number of harvested lymph nodes ( $p=0.012$ ) were significantly associated with OS. *ELF3* mRNA expression was not significantly associated with OS. All patients with high microsatellite instability (MSI) had no recurrence, therefore MSI was excluded from further analysis.

**Validation of *ELF3* protein expression.** IHC analysis indicated that *ELF3* was located in the nuclei of CRC cells and staining for *ELF3* at the invasive tumor front tended to be weak, but tended to be strong at the ulcer randwall. Therefore, staining intensity was graded at three sites with strong expression in each tumor ulcer randwall: blue staining of the nucleus was considered negative, while the lack of blue staining was considered positive (Figure 2). The average scores of three positively stained sites (0-100) was adopted as the protein

score. IHC analysis showed that *ELF3* protein expression was significantly higher in the recurrence group than in the non-recurrence group ( $p<0.001$ ) (Figure 3a).

**Relationship between expression of *ELF3* protein and prognosis.** The 185 samples subjected to IHC analysis were allocated to one of the two following groups based on the cutoff value determined from a ROC curve to predict recurrence after surgery: a high-expression group (IHC score  $>40$ ,  $n=43$ ) or a low-expression group (IHC score  $<40$ ,  $n=142$ ) (Table III). ORR was significantly higher in the high *ELF3* protein expression group than in the low protein expression group (Figure 3a). Univariate analysis indicated that lymphatic invasion ( $p<0.001$ ) and *ELF3* protein expression ( $p<0.001$ ) were significantly associated with the ORR. Multivariate analysis indicated that *ELF3* protein overexpression is an independent and significant poor prognostic factor of the ORR ( $p<0.001$ ; RR=30.701; 95% CI=9.769-96.488) (Table IV). RFS was significantly lower in the high *ELF3* protein expression group than in the low protein expression group (Figure 3b). Univariate analysis indicated that lymphatic invasion ( $p<0.001$ ), preoperative CEA level ( $p=0.004$ ), number of harvested lymph nodes ( $p=0.006$ ), and *ELF3* protein expression ( $p<0.001$ ) were significantly associated with RFS. Multivariate analysis indicated that *ELF3* protein overexpression is an independent and significant prognostic factor of RFS ( $p<0.001$ ; RR=6.648; 95% CI=3.519-12.559). OS was significantly lower in the high *ELF3* protein expression group than in the low protein expression group (Figure 3c). Univariate analysis indicated that age ( $p=0.005$ ),

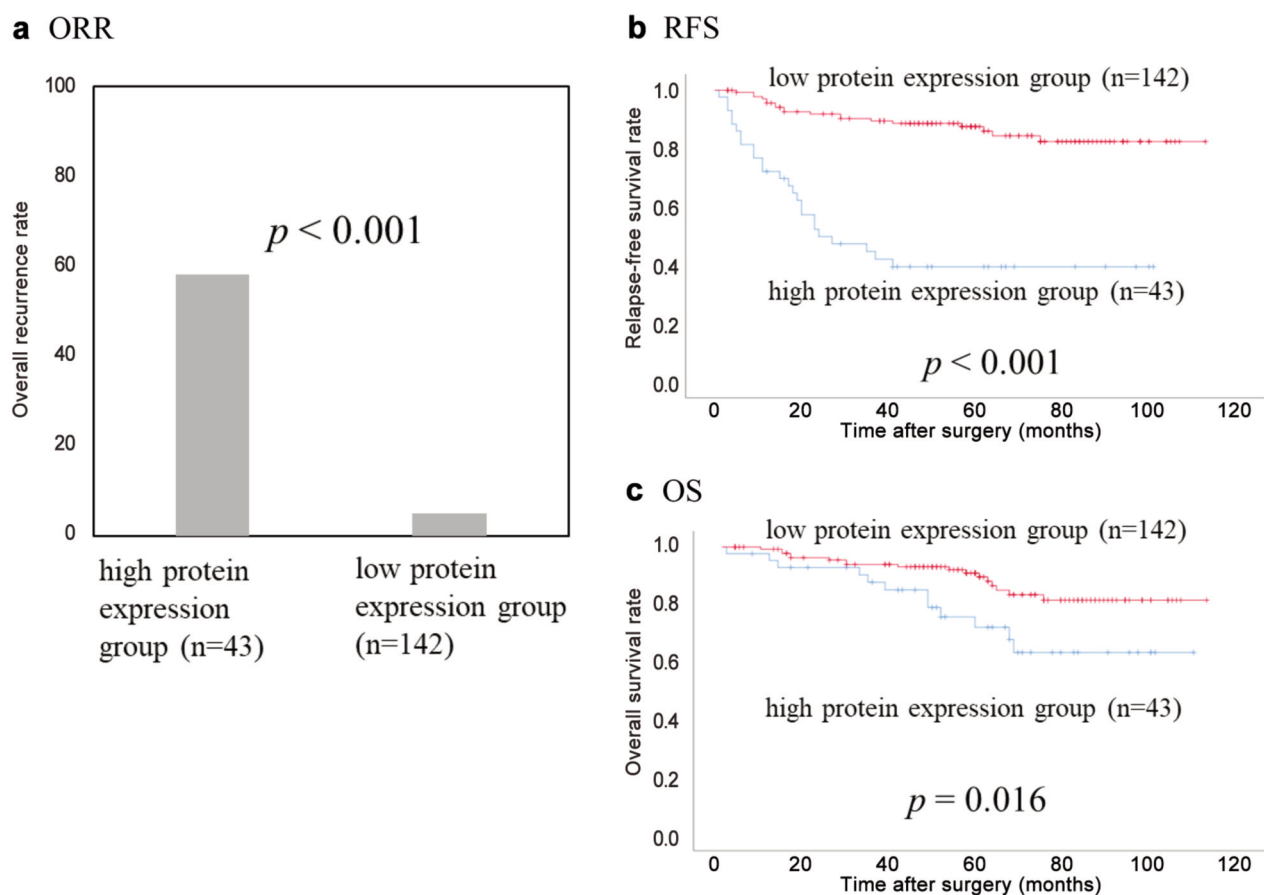


Figure 3. Bar graph showing overall recurrence rate (ORR) (a) and Kaplan-Meier curves showing relapse-free survival (RFS) (b) and overall survival (OS) (c) of 185 Stage II CRC patients by *ELF3* protein expression level.

preoperative CEA level ( $p=0.025$ ), number of harvested lymph nodes ( $p=0.002$ ), and *ELF3* protein expression ( $p=0.016$ ) were significantly associated with OS. Multivariate analysis indicated that *ELF3* protein overexpression is an independent and significant poor prognostic factor of OS ( $p=0.003$ ; RR=3.085; 95% CI=1.450-6.563).

## Discussion

This study is the first to demonstrate the clinical significance of *ELF3* expression to predict recurrence of Stage II CRC. Gene expression and copy number analyses identified an association between *ELF3* expression and disease recurrence. High *ELF3* expression was an independent and important poor prognostic factor of ORR and RFS after curative surgery for Stage II CRC. Similar results were obtained at both the mRNA and protein levels.

The *ELF3* gene, a E26 transformation-specific member of the ETS family of transcription factors, is located on

chromosome 1q32.1 and encodes a protein consisting of 371 amino acid residues. Analysis of the mRNA expression profile of the *ELF3* gene across normal human tissues showed strong expression in the gastrointestinal tract (19). Diseases associated with *ELF3* include cancers of the bladder, ovary, biliary tract, stomach, cervix, breast, prostate, lung, liver, and colon (20-27, 31). These studies reported that mutations to the *ELF3* genes have a tumor suppressive role in some cancers. Conversely, other groups reported high *ELF3* expression in cancer cells.

*ELF3* has been suggested to be involved in CRC malignancy via  $\beta$ -catenin accumulation and regulation of TGF $\beta$ R II. Aberrant regulation of the Wnt/ $\beta$ -catenin pathway plays a key role in colorectal carcinogenesis, as many cases of CRC are characterized by  $\beta$ -catenin accumulation. It has been reported that overexpression of *ELF3* induces  $\beta$ -catenin accumulation in both the nucleus and cytoplasm without input from the Wnt/ $\beta$ -catenin pathway (28, 29).  $\beta$ -catenin accumulates in the cytoplasm

Table III. Correlation between clinicopathological features and *ELF3* protein expression in stage II CRC.

	ELF3 protein High expression (n=43)	ELF3 protein Low expression (n=142)	p-Value
Age			
<65	23	62	0.30
≥65	20	80	
Gender			
Male	26	87	1.0
Female	17	55	
Histology			
Well, Moderate	41	129	0.53
Poor and others	2	13	
Depth			
T3	34	129	0.53
T4	9	13	
Location			
Colon (Right)	17	45	Colon vs. Rectum 1.0
Colon (Left)	18	70	
Rectum	8	27	
Lymphatic invasion			
-	24	114	0.002**
+	19	28	
Venous invasion			
-	5	27	0.36
+	38	115	
Pre-operative CEA (ng/dl)			
<5	23	90	1.0
≥5	16	52	
Perforation/Occlusion			
-	42	139	1.0
+	1	3	
MSI			
High	2	10	0.74
Low/MSS	39	128	
unknown	2	4	
Harvested LN			
≥12	35	116	1.0
<12	8	26	

and nucleus of colon cancer cells; therefore, overexpression of cancer-related genes, such as cyclinD1 and c-myc, induces abnormal cell proliferation (30). Abnormal cell proliferation promotes cancer growth. Therefore, *ELF3* overexpression is considered to be a poor prognostic factor in Stage II CRC. Wang *L et al.* reported that suppressing *ELF3* in CRC cells attenuates  $\beta$ -catenin signaling and decreases cell proliferation, migration and survival, and targeting *ELF3* in xenograft tumors suppressed tumor progression *in vivo* (28). It is considered that the high expression of *ELF3* increases the intracellular accumulation of  $\beta$ -catenin, although high expression of  $\beta$ -catenin could not be confirmed in *ELF3* protein high-expression patients by

IHC staining in the present study. The detailed mechanism of  $\beta$ -catenin accumulation due to overexpression of *ELF3* is expected to be elucidated in the future. Nakarai *et al.* reported that *ELF3* may be practical to detect lymph node metastasis of CRC, and *ELF3* controls the intestinal epithelial differentiation by regulation of the expression of transforming growth factor  $\beta$  receptor type II (TGF $\beta$ R II), which behaved as a tumor suppressor. *ELF3* activated the TGF $\beta$ R II promoter and regulates TGF $\beta$ R II, which is connected to extracellular matrix remodeling and tumorigenesis (31). Although our study did not examine the association between TGF $\beta$ R II and *ELF3*, it is worth investigating the association in clinical tissues of CRC in the future. Overexpression of *ELF3* may have been related to poor prognosis of Stage II CRC *via*  $\beta$ -catenin and TGF $\beta$ R II.

Recent studies have been conducted to identify useful biomarkers other than clinicopathological factors. Although MSI status, the Oncotype DX Colon Cancer Assay, and the ColoPrint genomic classifier have been applied for the evaluation of genes as predictors of recurrence, none of these technologies have been clinically applied in Japan (32-38). A study on the validity of the recurrence score generated by the Oncotype DX Colon Cancer Assay in patients following curative resection of Stages II and III colon cancer (SUNRISE study) is currently underway in Japan. A prospective observational study on risk stratification by ColoPrint (PARSC study) of 1,200 patients with Stage II/III CRC, including 575 with Stage II disease, is ongoing in Europe, the USA, and Asia. The results of the present study showed that high *ELF3* expression might be a high-risk factor for recurrence of Stage II CRC patients and a potential candidate marker for preoperative adjuvant chemotherapy to decrease the rate of recurrence.

The primary limitation of this study was the small number of samples of Stage II CRC with recurrence because the recurrence rate of Stage II CRC recurrence is not particularly high. A further verification study with a larger number of Stage II CRC patients with recurrence is needed to confirm our results.

In conclusion, the results of the present study showed that *ELF3* expression was significantly increased in the Stage II CRC recurrence group, suggesting the potential of the *ELF3* protein as a prognostic factor of recurrence. The focus of this study was highly expressed genes and the identified gene was further investigated as a biomarker for use in clinical practice, such as preoperative endoscopic biopsy samples. In the future, it is expected that *ELF3* will be confirmed as a candidate biomarker, while attempts are being made to classify and stratify recurrence risk using other technologies, such as the Oncotype DX genomic test.

### Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Table IV. Univariate and multivariate analyses of clinicopathological features affecting the ORR, RFS, and OS based on ELF3 protein levels.

Variables	n	ORR				RFS				OS			
		UA		MA		UA		MA		UA		MA	
		ORR	UA p-Value	OR (95% CI)	p-Value	5y RFS	UA p-Value	Relative risk (95% CI)	p-Value	5y OS	UA p-Value	Relative risk (95% CI)	p-Value
Age													
<65	85	21.2%	0.24			76.7%	0.714			91.8%	0.005	1.777	0.008
≥65	100	14.0%				75.3%				79.4%	**	(1.160-2.720)	**
Gender													
Male	113	18.6%	0.69			73.4%	0.243			84.7%	0.83		
Female	72	15.3%				80.4%				87.0%			
Histology													
Well,	170	18.2%	0.47			75.2%	0.317			85.4%	0.721		
Moderate													
Poor and others	15	6.7%				86.7%				86.7%			
Depth													
T3	162	15.4%	0.08	1.352	0.678	78.5%	0.054	1.336	0.48	86.1%	0.569		
T4	23	30.4%		(0.325-5.628)		60.9%		(0.598-2.958)		73.9%			
Location													
Colon	150	14.7%	0.08	6.829	0.005	78.3%	0.193			88.3%	0.244		
Rectum	35	28.6%		(1.802-25.884)	**	67.1%				69.7%			
Lymphatic invasion													
-	138	10.1%	0.001	5.075	0.004	84.5%	<0.001	2.804	0.002	86.5%	0.148		
+	47	38.3%	**	(1.674-15.384)	**	52.7%	**	(1.464-5.371)	**	82.4%			
Venous invasion													
-	32	6.3%	0.08	3.546	0.164	82.8%	0.287			85.6%	0.745		
+	153	19.6%		(0.597-21.076)		74.7%				85.5%			
Pre-operative CEA (ng/dl)													
<5	117	16.2%	0.69			81.6%	0.004	2.913	0.001	89.1%	0.025*	2.631	0.01*
≥5	68	19.1%				66.5%	**	(1.568-5.412)	**	78.9%		(1.255-5.516)	
Perforation/Occlusion													
-	181	17.1%	0.53			76.2%	0.992			85.2%	0.385		
+	4	25%				75%				100%			
MSI													
High	12	0%				100%				100%			
Low/MSS	156	19.2%				73.6%				86.8%			
unknown	6	0%											
Harvested LN													
≥12	151	15.9%	0.31			81.2%	0.006	1.996	0.04*	64.4%	0.002	2.416	0.026*
<12	34	23.5%				54.5%	**	(1.031-3.865)		90.2%	**	(1.112-5.249)	
ELF3 protein expression													
Low	142	4.9%	<0.001	30.701	<0.001	87.5%	<0.001	6.648	<0.001	89.6%	0.016*	3.085	0.003
High	43	58.1%	**	(9.769-96.488)	**	39.4%	**	(3.519-12.559)	**	72.1%		(1.450-6.563)	**

ORR, Overall recurrence rate; RFS, relapse-free survival; OS, overall survival; UA, univariate analyses; MA, multivariate analyses; OR, odds ratio; \*\*p<0.01; CI, confidence interval.

### Author's Contributions

AT, TI, SO and HU were involved in the conception and design of the study, and development of the methodology. AT performed the

experiments and collected clinicopathological data. AT, SW, FM and TT analyzed the results. AT, TI and SO edited the manuscript. AK, SY, TM assisted with all assays. TI, MT, HU, YK supervised the study. All Authors have read and approved the final manuscript.



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