

## Down-regulation of *EGFL8*: A Novel Prognostic Biomarker for Patients with Colorectal Cancer

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**Abstract.** *Background:* In a previous study, we reported a critical role of epidermal growth factor-like domain 7 (*EGFL7*) in the metastasis of hepatocellular carcinoma (HCC) and documented it to be a prognostic biomarker as well as a potential therapeutic target for HCC. However, the role of *EGFL8*, the only known paralog of *EGFL7*, in human malignancies is currently unclear. *Patients and Methods:* *EGFL8* expression in 101 cases of colorectal cancer (CRC) patients was determined by quantitative reverse transcription-polymerase chain reaction and the clinicopathological features of the CRC patients were correlated with the *EGFL8* down-regulation scores. In addition, the survival curve and Cox regression model were also employed to assess the prognostic value of *EGFL8* down-regulation. *Results:* *EGFL8* was significantly decreased in CRC tissues ( $p < 0.0001$ ) and the down-regulation of *EGFL8* was evidenced in 74.3% (75/101) of the CRC patients. *EGFL8* down-regulation correlated significantly to distant metastasis ( $p = 0.038$ ) and high TNM stage ( $p = 0.012$ ) of CRC. The CRC patients with high *EGFL8* down-regulation showed either poorer disease-free survival ( $p = 0.0167$ ) or poorer overall survival ( $p = 0.0310$ ) than those with low *EGFL8* down-regulation. Multivariable analysis identified *EGFL8* down-regulation as an independent prognostic factor for CRC patients (hazard ratio, 12.974;  $p = 0.037$ ). *Conclusion:* The reduced expression of *EGFL8* is closely related to metastatic potential and poor prognosis of CRC, suggesting the down-regulation of *EGFL8* as a novel prognostic biomarker for CRC patients.

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Colorectal carcinoma (CRC) ranks the third most common cancer in the world and the incidence of CRC is increasing rather rapidly in the Asia-Pacific region, especially in Japan (1-3). Abundant evidence has shown that a variety of genetic and epigenetic alterations in both oncogenes and tumor suppressors are involved in the pathogenesis of CRC. Activation of oncogenes such as rat sarcoma (*RAS*) gene and the inactivation of tumor suppressors such as adenomatous polyposis coli (*APC*) and *p53* genes have been well documented in CRC (4-6). In addition, we also have identified some genetic as well as epigenetic changes related to this disease (7-12). However, further investigations are still necessary to clarify the tumorigenic pathway of CRC (13).

In a previous study, we demonstrated the over-expression of epidermal growth factor-like domain 7 (*EGFL7*), an essential gene in vascular development during embryogenesis (14), in hepatocellular carcinoma (HCC) tissues and revealed an important role of the *EGFL7*/focal adhesion kinase (FAK)/epidermal growth factor receptor (EGFR) signaling pathway in metastasis of HCC, which provided the first evidence for the expression pattern and role of *EGFL7* in human malignancy (15). *Egfl8* is the only known paralog of *Egfl7* and the proteins they encode share the same overall domain structure, including an EGF-like domain, a  $\text{Ca}^{2+}$  binding EGF-like domain and an *N*-terminal signal peptide (14, 16). Moreover, the expression profiles of *Egfl8* and *Egfl7* are similar in adult mouse organs, with the highest levels of expression in kidney, brain, thymus and lung, but absolutely different in embryonic tissues (16). However, the expression pattern of *EGFL8* in human malignancies is currently unknown. Whereas Fitch *et al.* thought that *Egfl7* and *Egfl8* proteins might not overlap in their function during embryonic development (16), the similar structure shared by these two proteins led us to hypothesize that *EGFL8* may play an important role in human malignancies just like *EGFL7*. To test this hypothesis, the present study was carried out to determine the *EGFL8* expression pattern in human CRC tissues and to

explore the correlations between *EGFL8* expression and clinicopathological characteristics, as well as prognosis of this malignancy.

## Patients and Methods

**Patients and specimens.** Matched cancer and normal tissue specimens were obtained from 101 CRC patients who underwent surgery at Showa University Fujikaoka Hospital from February 2007 to August 2009. Informed written consent was obtained from all the patients and the study was approved by the Institutional Review Board of Showa University. All the specimens were collected and frozen in liquid nitrogen immediately after surgery and then stored at  $-80^{\circ}\text{C}$  until analysis. The diagnoses of CRC were all confirmed by histopathological examination. The clinicopathological data such as patient age, patient gender, tumor histology, lymph node status and metastasis situation were also collected and the characteristics of the colorectal tumors are summarized in Table I.

**RNA preparation and reverse transcription.** The total RNA was extracted from the CRC tissues and the corresponding normal tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction as described previously (17). The quality of the total RNA was measured by absorbance at 260nm with a U-2001 spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). First-strand cDNA was generated from RNA as described previously (18).

**Quantitative polymerase chain reaction (QPCR).** QPCR was performed by the Thermal Cycler Dice Real-time System TP800 (TaKaRa Bio Inc., Otsu, Shiga, Japan) using a SYBR Premix Ex Taq II kit (TaKaRa Bio Inc.). Thermocycling was carried out in a final volume of 25  $\mu\text{l}$  containing 1.0  $\mu\text{l}$  of cDNA sample, 0.5  $\mu\text{l}$  of each primer (forward and reverse, 100 nM), 12.5  $\mu\text{l}$  of the SYBR Premix Ex Taq II (including Taq DNA polymerase, reacting buffer, and deoxynucleotide triphosphate mixture). The PCR amplification consisted of 40 cycles ( $95^{\circ}\text{C}$  for 5 s,  $53^{\circ}\text{C}$  for 30 s after an initial denaturation step [ $95^{\circ}\text{C}$  for 10 s]). The PCR primers for *EGFL8* were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA): forward, 5'-AGCCCTA CCTGACCTTGTTG-3'; reverse, 5'-GTGCGAGCAGA GGGT-GAT-3'. To correct for differences in both quality and quantity of cDNA samples,  $\beta$ -actin gene was measured in the same samples as an internal control. All the QPCR analyses were performed in duplicate.

**Score of *EGFL8* down-regulation and grouping of CRC patients.** The relative expressions of *Egfl8* in the tissue samples were normalized to the internal control  $\beta$ -actin and calculated by the  $2^{-\Delta\text{Ct}}$  method. To show the degree of *Egfl8* down-regulation in CRC patients better, the down-regulation of *EGFL8* was scored using the logarithmic scale as described previously (19):

$\text{Egfl8 down-regulation score} = \log_e \left( \frac{\text{the relative expression of Egfl8 in normal tissue}}{\text{the relative expression of Egfl8 in cancer tissue}} \right)$ .

By the mean of all the CRC patients' *Egfl8* down-regulation scores, the CRC patients could be divided into two groups: high *Egfl8* down-regulation group (*Egfl8* down-regulation score  $\geq$  the mean) and low *Egfl8* down-regulation group (*Egfl8* down-regulation score  $<$  the mean).

**Follow-up and candidate prognostic factors of CRC.** Follow-up data were obtained from all the patients with CRC. The follow-up period was defined as the interval between the date of operation and the patient's death or the end of follow-up (February 23, 2010). The median follow-up time was 384 days. Deaths from other causes were treated as censored cases. Recurrence was diagnosed by clinical examination, colonoscopy, B-ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI). To determine factors influencing survival after surgery, 10 conventional variables along with the *EGFL8* down-regulation score were tested in all the CRC patients: age (year, continuous data), gender (male *versus* female), maximal tumor size (mm, continuous data), depth of tumor invasion ( $\leq$ muscular tunic invasion *versus*  $>$ muscular tunic invasion), pathological type (well/moderately differentiated CRC *versus* poorly-differentiated CRC), lymph node metastasis (presence *versus* absence), hepatic metastasis (presence *versus* absence), peritoneal dissemination (presence *versus* absence), distant metastasis (presence *versus* absence), TNM stage (I, II, III stage *versus* IV stage), and the *EGFL8* down-regulation score (continuous data).

**Statistical analysis.** The Mann-Whitney *U*-test was applied to analyze the *EGFL8* expression levels in the CRC tissues and the corresponding normal tissues. Student's *t*-test was used to analyze the correlations between the *EGFL8* down-regulation scores and the clinicopathological characteristics of CRC. Survival curves were constructed by using the Kaplan-Meier method and the differences in disease-free survival and overall survival were evaluated by log-rank test. A Cox proportional hazards regression model was constructed to identify the factors that were independently associated with overall survival of CRC. In this model, a step-wise selection was used for variable selection with entry and removal limits of  $p \leq 0.05$  and  $p > 0.10$ , respectively. The continuous data were expressed as mean  $\pm$  SE. All the statistical analyses were two-sided and performed by SPSS 13.0 software package (SPSS, Chicago, IL, USA). A *p*-value  $< 0.05$  was considered statistically significant.

## Results

***EGFL8* expression in CRC tissues.** *EGFL8* was detectable in all the tissue specimens, but its relative expression level in the CRC tissues was significantly decreased compared with the corresponding normal tissues ( $0.141 \pm 0.082$  *versus*  $0.523 \pm 0.233$ ,  $p < 0.0001$ ) (Figure 1). In addition, down-regulation of *EGFL8* was found in 74.3% (75/101) of the CRC patients. The *EGFL8* down-regulation score in the CRC tissues was  $1.63 \pm 0.27$ . According to the mean of *EGFL8* down-regulation scores, 51 cases of CRC patients fell into the low *EGFL8* down-regulation group and the other 50 cases fell into the high *EGFL8* down-regulation group.

**Correlations between *EGFL8* down-regulation and clinicopathological characteristics.** The high *EGFL8* down-regulation scores were significantly correlated to distant metastasis ( $p = 0.038$ ) and high TNM stage ( $p = 0.012$ ) of CRC (Table II). However, no significant correlation was found between *EGFL8* down-regulation scores and the other clinicopathological features of CRC.

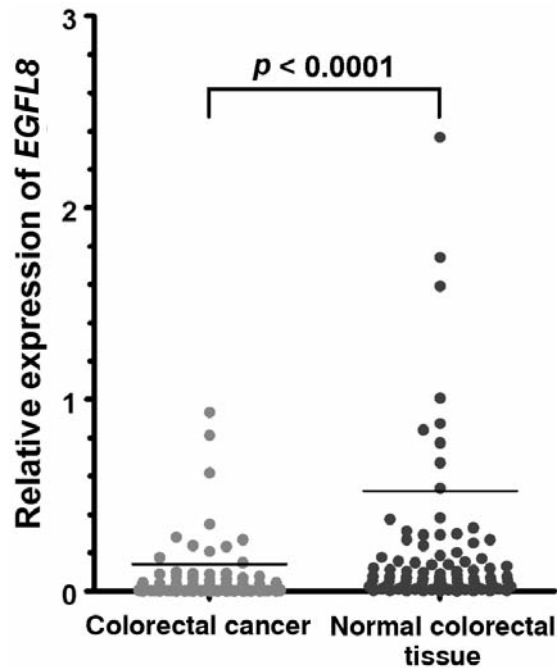


Figure 1. *EGFL8* expression in human colorectal cancer (CRC). *EGFL8* expression in 101 cases of CRC and corresponding normal colorectal tissues are shown. The bars in the figure indicate the means of the relative expressions of *EGFL8*.

**Correlation of down-regulation of *EGFL8* and poor prognosis.** The CRC patients in the high *EGFL8* down-regulation group had both poorer disease-free survival and poorer overall survival than those in the low *EGFL8* down-regulation group (2-year disease-free survival, 49% versus 96%,  $p=0.0167$ ; 2-year overall survival, 80% versus 97%,  $p=0.0310$ ) (Figure 2). By univariable Cox regression analysis, high TNM stage (hazard ratio [HR], 0.127;  $p=0.005$ ), peritoneal dissemination (HR, 0.150;  $p=0.010$ ), and high *EGFL8* down-regulation score (HR, 7.19;  $p=0.045$ ) were correlated to overall survival of the CRC patients. Four variables including patient gender, maximal tumor size, tumor differentiation and distant metastasis did not enter the multivariable Cox regression model. In this multivariable model, interestingly, only high TNM stage (HR, 0.012;  $p=0.034$ ) and high *EGFL8* down-regulation score (HR, 12.924;  $p=0.037$ ) were found to be independent prognostic factors for survival of CRC (Table III).

## Discussion

The present study showed for the first time that *EGFL8* expression significantly decreased in human CRC compared to the normal tissues and the down-regulation of *EGFL8* found in was most (74.3%) of the patients. These data suggested that *EGFL8* may have a distinct expression pattern and mechanism

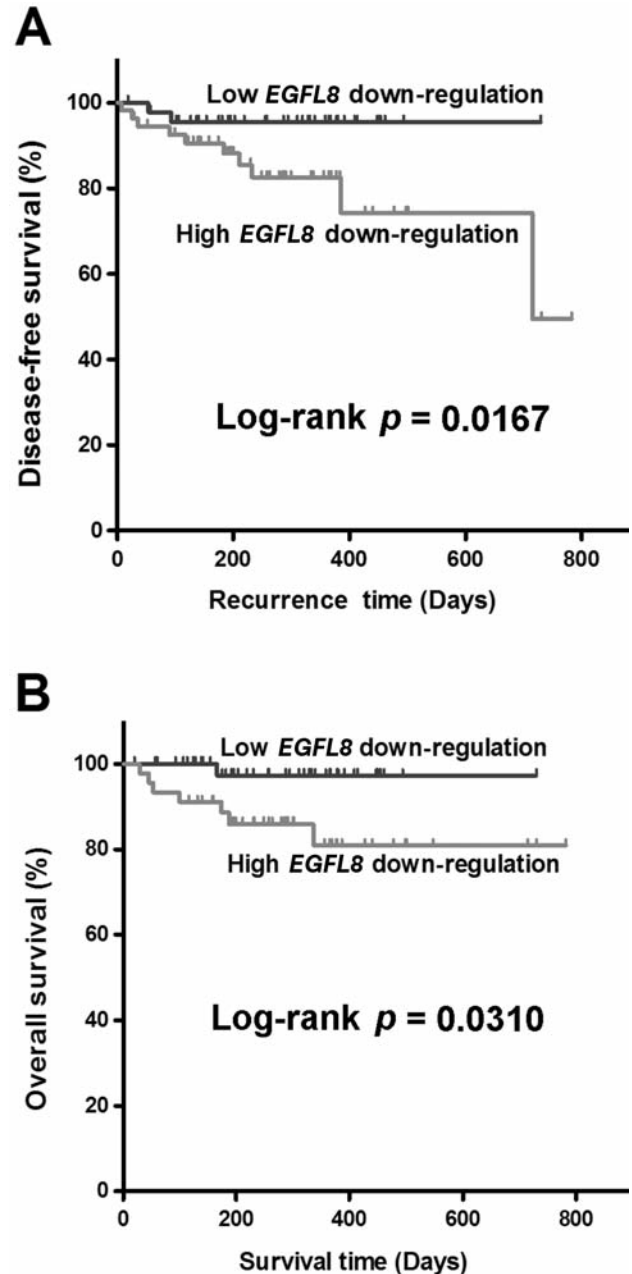


Figure 2. Estimated survival according to the expression of *EGFL8* in colorectal cancer (CRC) (Kaplan-Meier method).

of regulation from those of *EGFL7* in human malignancies (15, 20-22). The *EGFL8* gene is located on human chromosome 6 (6p21.32) and syntenic to the major histocompatibility complex regions (16). In light of the fact that loss of heterozygosity (LOH) at 6p21.32 is common in human malignancies and able to cause the down-regulated expression of human leukocyte antigen class I in CRC (23-25), the down-regulation of *EGFL8* may also be induced by LOH at its loci.

Table I. Characteristics of the colorectal tumors.

Characteristic	Number of cases
Tumor location	
Proximal colon <sup>†</sup>	41
Distal colon <sup>‡</sup>	28
Rectum	32
TNM stage <sup>§</sup>	
I	17
II	30
III	28
IV	26
T Category <sup>§</sup>	
T1	14
T2	10
T3	50
T4	27
N Category <sup>§</sup>	
N0	59
N1	30
N2	12
Histological type <sup>¶</sup>	
Adenocarcinoma	94
Mucinous adenocarcinoma	6
Signet ring cell	1

<sup>†</sup>Proximal colon includes cecum, ascending colon, and transverse colon; <sup>‡</sup>Distal colon includes descending colon and sigmoid colon; <sup>§</sup>TNM classification is according to the International Union against Cancer (UICC, 2002). <sup>¶</sup>Histological type is according to the Japanese Classification of Colorectal Carcinoma (7th ed., 2006)

When correlating the *EGFL8* down-regulation scores with the clinicopathological features of CRC, the down-regulation of *EGFL8* was significantly associated with distant metastasis of CRC. In addition, a trend for association with *EGFL8* down-regulation and hepatic metastasis of CRC was also found ( $p=0.071$ ). These criteria are well-accepted features of metastasis of CRC (26, 27), so these data suggested that *EGFL8* may be involved in metastasis of CRC. Since the *EGFL8* protein shares a similar protein structure with *EGFL7* protein, which is also called vascular endothelial statin and inhibits the maturation of vessels through repressing the motility of smooth muscle cells (SMC) (28, 29), we propose that *EGFL8* protein may also restrain the migration of SMCs and the down-regulation of *EGFL8* in CRC might improve the maturation and validity of tumor vessels and subsequently prompt the metastasis. Further studies, of course, will be necessary to test this possibility.

Down-regulation of *EGFL8* was also significantly correlated to high TNM stage (stage IV) of CRC, suggesting the down-regulation of *EGFL8* as a late event during the progression of gastroenterological malignancies. Because TNM stage is the most important prognostic factor for CRC (30), the Kaplan-Meier method was employed to assess the prediction power of *EGFL8* expression in the prognosis of CRC patients. The CRC

Table II. Correlations between clinicopathological features and the down-regulation of *EGFL8* in colorectal cancer.

Clinicopathological feature	Variables	n	Score of <i>EGFL8</i> down-regulation (Mean±SE)	p-Value
Gender	Male	57	1.32±0.34	0.193
	Female	44	2.04±0.45	
Age (years)	<65	36	1.28±0.37	0.343
	≥65	65	1.83±0.37	
Maximal tumor size (mm)	<50	53	1.22±0.40	0.112
	≥50	48	2.09±0.37	
Depth of tumor invasion <sup>†</sup>	≤Mt	24	2.10±0.67	0.277
	>Mt	77	1.48±0.29	
Tumor differentiation <sup>†</sup>	Well-Mod	82	1.67±0.31	0.800
	Poor	19	1.49±0.60	
Lymph node metastasis	Presence	40	1.83±0.33	0.365
	Absence	61	1.32±0.48	
Hepatic metastasis	Presence	16	2.81±0.49	0.071
	Absence	85	1.41±0.25	
Peritoneal dissemination	Presence	10	2.18±0.76	0.510
	Absence	91	1.57±0.29	
Distant metastasis	Presence	9	3.21±0.58	0.038*
	Absence	92	1.48±0.29	
TNM stage <sup>‡</sup>	I, II, III	75	1.22±0.27	0.012*
	IV	26	2.83±0.41	

<sup>†</sup>Depth of tumor invasion and tumor differentiation are according to the Japanese Classification of Colorectal Carcinoma (7th ed., 2006). Mt: muscular tunic; Well-Mod: well/moderately differentiated adenocarcinoma; Poor: poorly differentiated, mucinous or signet ring cell adenocarcinoma. <sup>‡</sup>TNM classification is according to the International Union against Cancer (UICC, 6th ed., 2002). \* $p<0.05$ .

patients with high *EGFL8* down-regulation scores had both poorer disease-free survival and poorer overall survival than those with low *EGFL8* down-regulation scores, further supporting the involvement of *EGFL8* in metastasis of CRC and implying the prognostic value of *EGFL8*. The multivariable Cox regression analysis identified the down-regulation of *EGFL8* as an independent risk factor for overall survival in CRC, suggesting *EGFL8* down-regulation as a novel prognostic marker for CRC patients.

Collectively, the current study has provided the first evidence indicating the significant decrease of *EGFL8* in CRC and its close association with metastasis and poor prognosis of CRC. The study suggests that the down-regulation of *EGFL8* may serve as a novel prognostic biomarker for CRC. However, further studies are required to elucidate the exact mechanisms underlying the down-regulation of *EGFL8*.

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Table III. Cox analysis for the prognostic factors of colorectal cancer.

Variable	Univariable analysis		Multivariable analysis	
	RR (95% CI)	p-Value	RR (95% CI)	p-Value
Gender (male <i>versus</i> female)	6.156 (0.756-50.133)	0.089	--	--
Age (years, continuous data)	0.223 (0.027-1.812)	0.160	0.092 (0.001-0.372)	0.072
Maximal tumor size (mm, continuous data)	0.301 (0.061-1.492)	0.142	--	--
Depth of tumor invasion <sup>†</sup> (≤Mt <i>versus</i> >Mt)	0.443 (0.054-3.601)	0.446	0.721 (0.029-18.082)	0.842
Tumor differentiation <sup>†</sup> (Well-Mod <i>versus</i> Poor)	0.273 (0.061-1.224)	0.090	--	--
Lymph node metastasis (presence <i>versus</i> absence)	1.025 (0.245-4.289)	0.973	5.134 (0.352-74.865)	0.232
Hepatic metastasis (presence <i>versus</i> absence)	0.267 (0.064-1.117)	0.071	1.618 (0.106-24.726)	0.730
Peritoneal dissemination (presence <i>versus</i> absence)	0.150 (0.036-0.631)	0.010*	0.095 (0.005-1.940)	0.126
Distant metastasis (presence <i>versus</i> absence)	0.216 (0.043-1.085)	0.063	--	--
TNM stage <sup>‡</sup> (Stage I, II, III <i>versus</i> IV)	0.127 (0.030-0.533)	0.005**	0.012 (0.000-0.719)	0.034*
EGFL8 down-regulation score (continuous data)	7.19 (0.889-18.453)	0.045*	12.924 (1.313-102.800)	0.037*

<sup>†</sup>Depth of tumor invasion and tumor differentiation are according to the Japanese Classification of Colorectal Carcinoma (7th ed., 2006). Mt: muscular tunic; Well-Mod: well/moderately differentiated adenocarcinoma; Poor: poorly differentiated, mucinous or signet ring cell adenocarcinoma. <sup>‡</sup>TNM classification is according to the International Union against Cancer (UICC, 6th ed., 2002). RR, relative risk; 95% CI, 95% confidence interval; \* $p < 0.05$ ; \*\* $p < 0.01$ .

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