# Down-regulation of EGFL8: A Novel Biomarker for Advanced Gastric Cancer

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**Abstract.** *Background: Recently, we have reported an important role of epidermal growth factor-like domain 8 (EGFL8) in the progression of colorectal cancer (CRC) and documented EGFL8 to be a novel prognostic biomarker for this malignancy. However, the function of EGFL8 in the other human gastroenterological malignancies such as gastric cancer remains largely unknown. Patients and Methods: EGFL8 expression in 53 cases of gastric cancer and the corresponding normal tissues were determined by quantitative real-time PCR and the EGFL8 down-regulation score for each patient was calculated. Subsequently, the correlations between EGFL8 down-regulation score and the clinicopathological features of gastric cancer were evaluated. Results: EGFL8 expression was significantly lower in the gastric cancer tissues than the corresponding normal tissues (p=0.0001) and the down-regulation of EGFL8 was evident in 73.6% (39/53) of the gastric carcinomas. More importantly, EGFL8 down-regulation was correlated significantly with peritoneal dissemination (p=0.037) and high TNM stage (p=0.025) of gastric cancer. Conclusion: The down-regulation of EGFL8 might be a novel biomarker for advanced gastric cancer.*

Gastric cancer is the fourth most common cancer worldwide and ranks first in incidence rate (age standardized) in Japan (1, 2). Though its prognosis has been improved in recent years, especially in Japan (2), gastric cancer remains the second most common cause of death from cancer in the world (1, 2). Accumulated evidence has indicated that gastric cancer results from various genetic and epigenetic alterations of oncogenes, tumor suppressor genes, cell cycle regulators, cell adhesion molecules and DNA repair genes (3). We have previously reported that the down-regulation of the *Mus81* (MMS and UV sensitive isolate number 81) gene, overexpression of the *PAI-1* (plasminogen activator inhibitor-1) gene and the methylation or demethylation of other genes such as *DCC* (deleted in colorectal cancer), *HACE1* (HECT domain and ankyrin repeat containing E3 ubiquitin-protein ligase 1) and *MGMT* (methylguanine DNA methyltransferase) were closely related to gastric cancer (4-8). However, further investigations to identify genetic alterations as new parameters for estimating the progression of gastric cancer are important in order to improve the success of treatment (9).

In a previous study, we demonstrated the overexpression of epidermal growth factor-like domain 7 (EGFL7), an essential gene in vascular development during embryogenesis (10), in hepatocellular carcinoma (HCC) tissues and revealed an important role of the EGFL7/FAK (focal adhesion kinase)/EGFR (epidermal growth factor receptor) signaling pathway in metastasis of HCC, which provided the first evidence for the expression pattern and role of EGFL7 in human malignancy (11). 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 $Ca^{2+}$ binding EGF-like domain and a N-terminal signal peptide (11, 12). 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 (12). Whereas Fitch *et al*. thought that EGFL7 and EGFL8 proteins might not overlap in their function during embryonic development (12), the similar structure shared by these two protein led us to hypothesize that EGFL8 may play an important role in human malignancies just like EGFL7.

Recently, our study has showed an important role of EGFL8 down-regulation in the progression of colorectal

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cancer (CRC) and documented EGFL8 to be a novel prognostic biomarker for CRC (13). However, the function of EGFL8 in the other human gastroenterological malignancies such as gastric cancer remains unknown. Therefore, the present study was carried out to determine the EGFL8 expression pattern in gastric cancer and to explore the correlations between EGFL8 expression and clinicopathological characteristics of this malignancy.

## Patients and Methods

*Patients and specimens*. Matched cancer and normal tissue specimens were obtained from 53 cases of patients with gastric cancer who underwent surgery at Showa University Fujikaoka Hospital from April 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˚C until analysis. Diagnoses of gastric cancer were all confirmed by histopathological examination. The clinicopathological profiles of all the patients are shown in Table I.

*RNA preparation and reverse transcription*. The total RNA was extracted from the gastric cancer tissues and the corresponding normal tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction as described previously (14). The quality of total RNA was measured by absorbance at 260 nm with a U-2001 spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). First-strand cDNA was generated from RNA as described previously (15).

*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 conducted in a final volume of 25 μl containing 1.0 μl of cDNA sample, 0.5 μl of each primer (forward and reverse, 100 nM), 12.5 μl 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˚C for 5 sec, 53˚C for 30 sec after an initial denaturation step [95˚C for 10 sec]). The PCR primers for EGFL8: forward, 5'-AGCCCTACCTGACCTTGTG-3'; reverse, 5'-GTGCGAGC-AGAGGGTGAT-3', were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). To correct for differences in both quality and quantity of the cDNA samples, the *β-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*. The relative expressions of EGFL8 in the tissue samples were normalized to the internal control *β-actin* and calculated by the $2^{-\Delta Ct}$ method. To show the degree of EGFL8 down-regulation in gastric cancer patients better, the down-regulation of EGFL8 was scored using the logarithmic scale as described previously (13):
EGFL8 down-regulation score=log *e* (the relative expression of EGFL8 in normal tissue/the relative expression of EGFL8 in cancer tissue).

An EGFL8 down-regulation score >0, indicated that EGFL8 expression did decrease in the gastric cancer patient, while a score ≤0, indicated that EGFL8 expression did not decrease in the gastric cancer patient.

*Statistical analysis*. The nonparametric Mann-Whitney *U*-test was applied to analyze the EGFL8 expression levels in the gastric cancer tissues and the corresponding normal tissues. The associations between EGFL8 down-regulation scores and the clinicopathological characteristics of gastric cancer were analyzed by Student's *t*-test. The continuous data were expressed as mean±SE. All the statistical analyses were two-sided and performed by SPSS 13.0 software package (SPSS, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

## Results

*Down-regulation of EGFL8 in gastric cancer tissues*. EGFL8 was detectable in all the gastric cancer tissue specimens and the corresponding normal tissue specimens. However, the relative expression of EGFL8 in the gastric cancer tissues was significantly lower than that in the corresponding normal tissues (0.092±0.026 *vs*. 0.214±0.050, $p=0.0001$) (Figure 1). And the EGFL8 down-regulation scores of the gastric cancer patients varied from –2.47 to 4.94 (1.08±0.24). In addition, the down-regulation of EGFL8 was evidenced in 73.6% (39/53) of the patients.

*Correlations between EGFL8 down-regulation and clinicopathological characteristics*. To explore the clinical association of EGFL8 down-regulation in gastric cancer, the clinicopathological data were correlated with the EGFL8 down- regulation scores. The high EGFL8 down-regulation scores were significantly correlated to peritoneal dissemination ($p=0.037$) and high TNM stage ($p=0.025$) of gastric cancer (Table I). However, no significant correlation was found between the EGFL8 down-regulation scores and the other clinicopathological features of gastric cancer such as gender, age, maximal tumor size, lymph node metastasis, invasion depth, distant metastasis and pathological type.

## Discussion

In addition to our earlier reports (12,13), the present study also showed a significant decrease of EGFL8 expression in human gastric tissues and the down-regulation of EGFL8 was found in most (73.6%) of patients. These data suggested that EGFL8 down-regulation is a common event during the carcinogenesis of gastroenterological malignancies and also indicated that EGFL8 may have a distinct expression pattern and mechanism of regulation from those of EGFL7 in human malignancies (11, 16-18). The EGFL8 gene is located on human chromosome 6 (6p21.32) and synthenic to the major histo-compatibility complex (MHC) regions (12). In light of the fact that the loss of heterozygosity (LOH) at 6p21.32 is

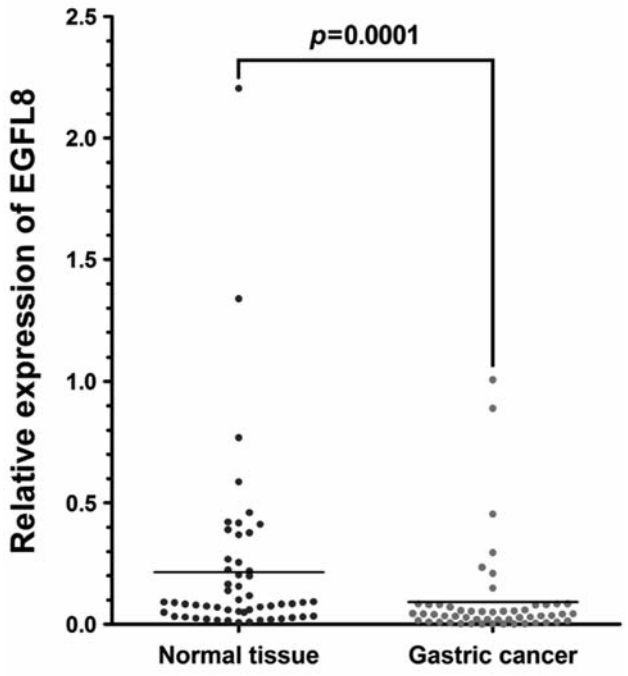


Figure 1. *EGFL8 expression levels in gastric cancer tissues and corresponding normal tissues determined by quantitative real-time PCR and normalized to internal control β-actin gene. Bars: means of relative expressions of EGFL8 in normal tissues and gastric cancer tissues, respectively.*

frequent in human malignancies and able to cause the down-regulated expression of human leukocyte antigen (HLA) class I in gastric cancer (19-21), we hypothesize that the down-regulation of EGFL8 may be also induced by LOH at its loci.

The down-regulation of EGFL8 was significantly correlated to high TNM stage (stage IV), suggesting EGFL8 might be down-regulated in line with the progression of gastric cancer. Moreover, the down-regulation of EGFL8 was significantly associated with peritoneal dissemination ($p$=0.037). Interestingly, there were also a trend for association between EGFL8 down-regulation and distant metastasis of gastric cancer ($p$=0.067). Both these clinicopathological features are well-accepted metastastic markers for advanced gastric cancer (22, 23), so the data suggested that EGFL8 may be a novel biomarker for advanced gastric cancer. Since the EGFL8 protein shares a similar structure with EGFL7 protein, which is also known as VE (vascular endothelial)-statin and inhibits the maturation of vessels by repressing the motility of smooth muscle cells (SMC) (24, 25), we propose that EGFL8 protein could also restrain the migration of SMCs and the down-regulation of EGFL8 might improve the maturation and validity of tumor vessels and subsequently prompt the metastasis and progression of gastric cancer. Further studies, of course, will be necessary to test this possibility.

Table I. *Correlations between clinicopathological features and down-regulation of EGFL8 in gastric cancer.*

| Clinicopathological features | Variables | n | Score of EGFL8 down-regulation (Mean±SE) | *P*-Value† |
|---|---|---|---|---|
| Gender | Male | 41 | 1.21±0.26 | 0.699 |
| | Female | 12 | 1.00±0.42 | |
| Age (years) | <65 | 15 | 1.12±0.35 | 0.917 |
| | ≥65 | 38 | 1.18±0.28 | |
| Maximal tumor size (mm) | <50 | 19 | 0.89±0.37 | 0.356 |
| | ≥50 | 34 | 1.32±0.28 | |
| Depth of tumor invasion‡ | ≤Mt | 6 | 0.99±0.81 | 0.780 |
| | >Mt | 47 | 1.19±0.23 | |
| Tumor differentiation‡ | Well-Mod | 18 | 1.00±0.32 | 0.593 |
| | Poor | 35 | 1.25±0.30 | |
| Lymph node metastasis | Presence | 22 | 1.23±0.38 | 0.793 |
| | Absence | 31 | 1.12±0.28 | |
| Peritoneal dissemination | Presence | 9 | 2.83±0.67 | 0.037* |
| | Absence | 44 | 0.83±0.28 | |
| Distant metastasis | Presence | 9 | 2.10±0.53 | 0.067 |
| | Absence | 44 | 0.98±0.36 | |
| TNM stage§ | I, II, III | 37 | 0.87±0.39 | 0.025* |
| | IV | 16 | 1.86±0.57 | |

†*P*-values were obtained from Student's *t*-test and all tests were two-sided. ‡Depth of tumor invasion and tumor differentiation according to General Rules for Gastric Cancer Study (26). Mt: Muscular tunic; Well or Mod: well- or moderately differentiated adenocarcinoma; Poor: poorly differentiated, mucinous or signet ring cell adenocarcinoma. §TNM classification is according to the International Union against Cancer (27). **P*<0.05.

In conclusion, EGFL8 expression is significantly down-regulated in human gastric cancer tissues and this decrease is related closely to high TNM stage and metastatic potential, which suggests that the down-regulation of EGFL8 might serve as a novel biomarker for advanced gastric cancer. However, further studies are still needed to elucidate the exact mechanisms underlying the down-regulation of EGFL8.

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