Clinical implications of Fas/Fas ligand expression in patients with esophageal squamous cell carcinoma following neoadjuvant chemoradiotherapy

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Abstract. Recent epidemiological studies demonstrated that the incidence of esophageal squamous cell carcinoma (ESCC) is on the increase. Although neoadjuvant chemoradiotherapy (CRT) followed by surgery may improve long-term survival and reduce local recurrence in patients with esophageal cancer, the overall cure rate of esophageal cancer is low. Fas/Fas ligand (FasL) signaling initiates the cell death pathway. The roles of FasL in tumor growth, progression and resistance to treatment have been demonstrated in several malignancies. The aim of this preliminary study was to evaluate Fas/FasL expression in ESCC with neoadjuvant CRT. A total of 20 patients who received neoadjuvant CRT (30-40 Gy; 5-fluorouracil plus cisplatin followed by surgery) were enrolled. We evaluated the expression of Fas, FasL and Ki67 (a proliferative marker) using immunohistochemistry and analyzed the correlations between their expression and clinical outcomes. Additionally, we investigated the association of Fas/FasL expression with peritumoral immune CD8-positive and Foxp3-positive cells. High FasL expression was significantly correlated with disease recurrence (P=0.0134). Patients with high FasL expression exhibited poorer recurrence-free and overall survival (P=0.0102 and 0.0385, respectively). Patients with low Fas and high FasL exhibited significantly poorer recurrence-free survival (P=0.0035). Although statistical significance was not reached, Fas expression appeared to be inversely correlated with Foxp3-positive cells and FasL expression appeared to be inversely correlated with CD8-positive cells. In conclusion, FasL expression was associated with tumor relapse and poor prognosis in patients with ESCC following CRT. Pharmacological control of Fas/FasL signaling may improve therapeutic efficacy and outcome in ESCC patients receiving preoperative CRT.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is a highly malignant tumor and its prognosis is generally poor. Recent epidemiological studies demonstrated that the incidence of ESCC is on the increase (1). Neoadjuvant chemoradiotherapy (CRT) followed by surgery may improve long-term survival and reduce local recurrence in patients with esophageal cancer. However, the overall cure rate of esophageal cancer remains <20% (2-4).

Fas and Fas ligand (FasL) are transmembrane proteins that belong to the tumor necrosis factor family and their signaling pathway is a key regulator of apoptotic cell death, i.e., Fas binding of FasL induces an apoptotic cascade. Furthermore, the Fas/FasL pathway regulates the tumor microenvironment, including the host immune system and extracellular matrix (5-12). Several authors have reported that Fas/FasL expression is correlated with tumor progression and poor prognosis in esophageal cancer (6,13,14). By contrast, Takikita *et al* (15) reported that the Fas/FasL apoptotic pathway, including Fas-associated death domain protein and caspases 8 and 10 were not prognostic factors in ESCC.

In the present study, we evaluated the expression of Fas, FasL and Ki67 (a proliferation marker) in ESCC following neoadjuvant CRT and analyzed the correlation of their expression with clinical outcome. Additionally, the association of Fas/FasL expression with peritumoral immune cells was investigated.

Materials and methods

Patients and specimens. A total of 20 patients who had received neoadjuvant CRT followed by surgery were enrolled in this study. All the formalin-fixed, paraffin-embedded (FFPE) specimens of the patients were available for evaluation. The study protocol was approved by the Ethics Review Board of Mie University Hospital and all the included patients provided written informed consent for their tissues to be used in this study.

5-Fluorouracil (5-FU) and cisplatin (CDDP)-based CRT. All the patients received systemic 5-FU and CDDP chemotherapy with concurrent radiotherapy. The regimen included 4 cycles of 5-FU (600 mg/m^2 administered intravenously over 24 h), plus tegafur and uracil (400 mg/kg of body weight administered orally for 5 days) and CDDP (4 mg/day administered intravenously for 5 days) with concurrent 40 Gy radiation followed by surgery. Preoperative radiotherapy was delivered to the primary tumor as well as the peritumoral area at a dose of 40 Gy in 20 fractions within 4 weeks. The time interval between neoadjuvant CRT and surgery was 2-3 weeks.

Clinical response and histopathological analysis of tumor regression following CRT. The clinical response following preoperative CRT was evaluated by barium esophagography, endoscopy and computed tomography. The results were graded as complete response (CR), partial response (PR), no change (NC) or progressive disease. The pathological response to CRT was evaluated using the Mandard tumor regression grade (TRG) (16). The tumors were classified according to the Mandard system into 5 grades as follows: i) TRG1, CR with absence of residual cancer and fibrosis extending through the wall; ii) TRG2, presence of residual tumor cells scattered through the fibrotic area; iii) TRG3, increased numbers of residual cancer cells, with predominant fibrosis; iv) TRG4, residual cancer outgrowing the fibrotic area; v) TRG5, absence of regressive changes. We categorized patients in categories TRG1 and 2 as responders and those in categories TRG3-5 as non-responders.

Immunohistochemistry (IHC). The FFPE specimens were cut into 2- to 3-μm sections. Following deparaffinization and dehydration, the sections were placed in a 10 mmol/l sodium citrate buffer (pH 6.0) and autoclaved at 121˚C for 10 min for antigen retrieval. The sections were incubated in 3% hydrogen peroxide for 10 min, blocked and incubated with a primary antibody overnight at 4˚C. Monoclonal mouse anti-human Fas antibody (B-10, catalog no. sc-8009; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100), polyclonal rabbit anti-human FasL antibody (C-20, catalog no. sc-957; Santa Cruz Biotechnology; dilution 1:100) and monoclonal mouse anti-human Ki67 antibody (MIB-1, code M7240; Dako Cytomation, Glostrup, Denmark; dilution 1:100) were the primary antibodies used in a labeled streptavidin-biotin system (EnVision™ + Dual Link System-HRP; Dako Cytomation). Antibody binding was visualized using 3,3'-diaminobenzidine (Dako Cytomation). All the sections were counterstained with hematoxylin prior to being dehydrated and mounted. At least 2 sections per specimen were stained to confirm reproducibility. Negative controls were prepared simultaneously with pre-immune immunoglobulin.

IHC evaluation. The sections were observed under a light microscope (BX50, Olympus, Tokyo, Japan). We calculated IHC scores by multiplying the percentage of positive epithelial cells (0-100%) by the staining intensity, as previously described (17). The staining intensities were scored as follows: 0, negative; 1, weak; and 2, strong for Fas and Ki67; and 0, negative; 1, weak; 2, moderate; and 3, strong for FasL. The IHC scores ranged between 0 and 200 for Fas and Ki67; and between 0 and 300 for FasL. Each sample was scored by two investigators (K.T. and Y.O.) who were blinded to the clinicopathological information regarding the origin of the samples.

Enumeration of peritumoral CD8- and Foxp3-positive cells. We previously recorded the numbers of peritumoral

Table I. Patient characteristics (n=20).

Characteristics	Values (%)
Age, years	
Median	69
Range	52-77
Gender	
Male	18 (90)
Female	2 (10)
Location	
Upper	1 (5)
Middle	7 (35)
Lower	12 (60)
ypT	
T1/2	10 (50)
T3/4	10 (50)
ypN	
Absent	10 (50)
Present	10 (50)
Postoperative stage	
I	1 (5)
II	9 (45)
III	7 (35)
IV	3 (15)
Lymphatic invasion	
Absent	4 (20)
Present	16 (80)
Vascular invasion	
Absent	12 (60)
Present	8 (40)
Histological differentiation	
High/moderate	17 (85)
Poor	3 (15)
R0 resection	
Yes	16 (80)
No	4 (20)
Mandard TRG	
5	0 (0)
4	4 (20)
3	7 (35)
2	9 (45)
1	0 (0)
Recurrence (R0, n=16)	
Absent	10 (62.5)
Present	6 (37.5)

TRG, tumor regression grade.

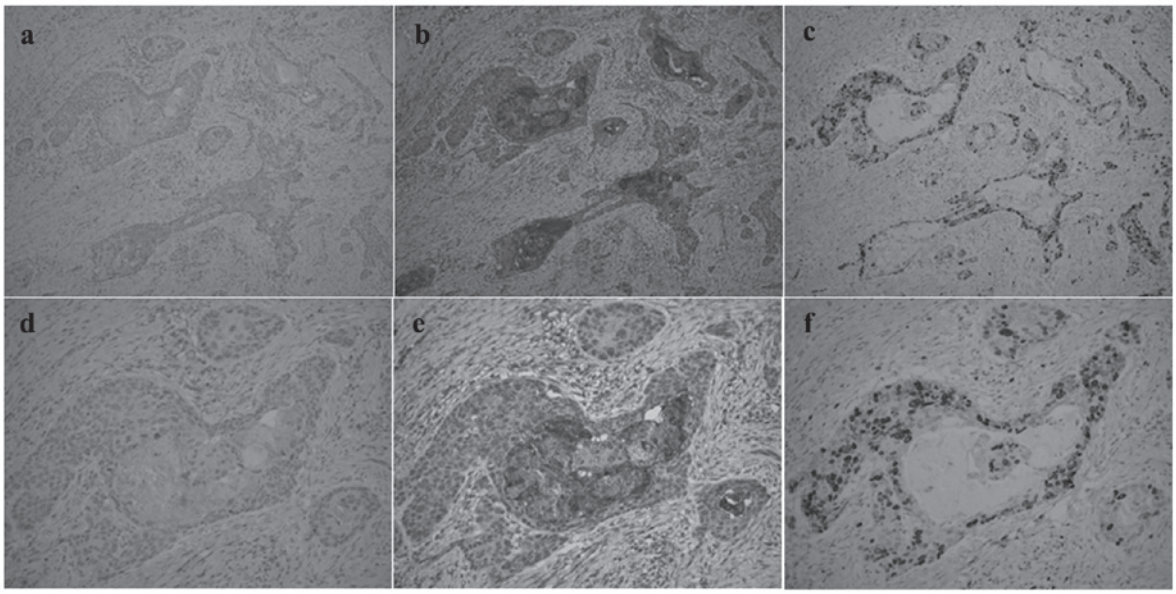

Figure 1. Immunohistochemical findings for Fas, Fas ligand and Ki67. (a and d) Fas, (b and e) Fas ligand and (c and f) Ki67. Original magnification, x100 (a-c) and x200 (d-f).

CD8- and Foxp3-positive cells using light microscopy and the median count was calculated for each sample. The median values for the number of CD8- and Foxp3-positive cells were 70 (range, 18-250) at a magnification x200 and 13 (range, 1-57) at a magnification x100 (18). We analyzed the correlation of Fas/FasL expression with peritumoral CD8- and Foxp3-positive cells.

Statistical analysis. Statistical analysis was performed using StatView v5.0 software (SAS Institute Inc., Cary, NC, USA). Significant differences were analyzed using the Chi-square test. The Pearson's correlation test was used to determine statistical correlations. Recurrence-free and overall survival probabilities were calculated from the date of surgery to the date of recurrence or death, using the Kaplan-Meier product limit method; intergroup differences were determined using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The patient characteristics are summarized in Table I. The median age of the patients was 68 years (range, 52-77 years) and the male:female ratio was 9:1. The median follow-up period was 16 months (range, 4-91 months). Of the 20 tumors, 12 were located in the lower, 7 in the middle and 1 in the upper part of the esophagus. The mean size of the tumors was 40 mm (range, 20-82 mm). The post-CRT pathological T stages were as follows: pT1 (n=1), pT2 (n=9), pT3 (n=9) and pT4 (n=1). A total of 10 patients (50%) presented with lymph node metastases. The median number of total dissected lymph nodes was 14 (range, 0-39) and the median value of lymph node density was 5.45% (range, 0-55.6%). A total of 17 tumors (85%) exhibited well or moderately differentiated squamous cell carcinoma histology. R0 resection was performed in 80% of the cases. The clinical response was classified as follows: NC, 9 patients; PR, 10 patients; and CR, 1 patient. The Mandard TRG grades were as follows: TRG1, no patients; TRG2, 9 patients; TRG3, 7 patients; TRG4, 4 patients; and TRG5, no patients (non-responders, n=11; responders, n=9).

IHC findings for Fas, FasL and Ki67 in ESCC following neoadjuvant CRT. Fig. 1 shows the IHC findings for Fas, FasL and Ki67 in ESCC following neoadjuvant CRT. Fas and FasL were expressed in the cytoplasm and nuclei of cancer cells. Additionally, FasL expression was diffusely detected in stromal cells. Ki67 expression was detected in cancer cell nuclei. There was no correlation between the IHC findings for Fas/FasL and Ki67. The median values of the IHC scores for Fas, FasL and Ki67 were 80 (range, 1-200), 190 (range, 30-300) and 10 (range, 1-180), respectively. We classified those cases with values above the median IHC score as the high-expression group and the remainder as the low-expression group.

Correlations of Fas, FasL and Ki67 expression with clinicopathological variables. High FasL expression was significantly correlated with disease recurrence in patients treated with curative intent (P=0.0134). However, there were no correlations between Fas/FasL expression and other clinicopathological characteristics (Table II). High Ki67 expression was significantly correlated with lymph node metastasis and lymphatic invasion (P=0.007 and 0.025, respectively) (data not shown).

Survival analysis based on Fas and FasL expression. The recurrence-free survival and overall survival according to Fas and FasL expression using the Kaplan-Meier product limit method are shown in Fig. 2. High expression of FasL was found to be significantly associated with poor recurrence-free and overall survival (P=0.0102 and 0.0385, respectively). Patients with low Fas and high FasL expression exhibited a poorer recurrence-free survival (P=0.0035).

Table II. Correlations of Fas, Fas ligand (FasL) and Ki67 expression with clinicopathological variables.

Variables	Fas-low	Fas-high	P-value[a]	FasL-low	FasL-high	P-value[a]
Median age, years						
≤69	6	4	0.371	4	6	0.371
>69	4	6		6	4	
Gender						
Male	8	10	0.136	9	9	0.999
Female	2	0		1	1	
Location						
Upper	1	0	0.270	1	0	0.565
Middle	2	5		3	4	
Lower	7	5		6	6	
Size, mm						
<40	6	8	0.329	7	7	0.999
≥40	4	2		3	3	
ypT						
T0/1/2	7	3	0.074	4	6	0.371
T3/4	3	7		6	4	
Lymph node metastasis						
Absent	3	7	0.074	6	4	0.371
Present	7	3		4	6	
Postoperative stage						
0/I/II	5	5	0.999	6	4	0.371
III/IV	5	5		4	6	
Lymphatic invasion						
Absent	1	3	0.264	3	1	0.264
Present	9	7		7	9	
Vascular invasion						
Absent	6	6	0.999	6	6	0.999
Present	4	4		4	4	
Histological differentiation						
High/moderate	9	8	0.531	9	8	0.531
Poor	1	2		1	2	
R0 resection						
Yes	9	7	0.264	9	7	0.264
No	1	3		1	3	
Pathological response						
Non-responders	6	5	0.653	5	6	0.653
Responders	4	5		5	4	
Recurrence (R0, n=16)			0.0907			**0.0134**
Absent	4	6		8	2	
Present	5	1		1	5	

[a]Chi-square test. Bold print denotes statistical significance.

Correlation of Fas/FasL expression with peritumoral CD8- and Foxp3-positive cells. Although the differences did not achieve statistical significance, Fas expression was inversely correlated with peritumoral Foxp3-positive cells, whereas FasL expression appeared to be inversely correlated with peritumoral CD8-positive cells and directly correlated with Foxp3-positive cells (Fig. 3).

Discussion

In esophageal cancer, upregulation of FasL and downregulation of Fas have been reported (19). In the present study, the median value of the FasL IHC score was significantly higher compared to that of the Fas IHC score in ESCC following preoperative CRT (P=0.006). Gratas *et al* (19) demonstrated

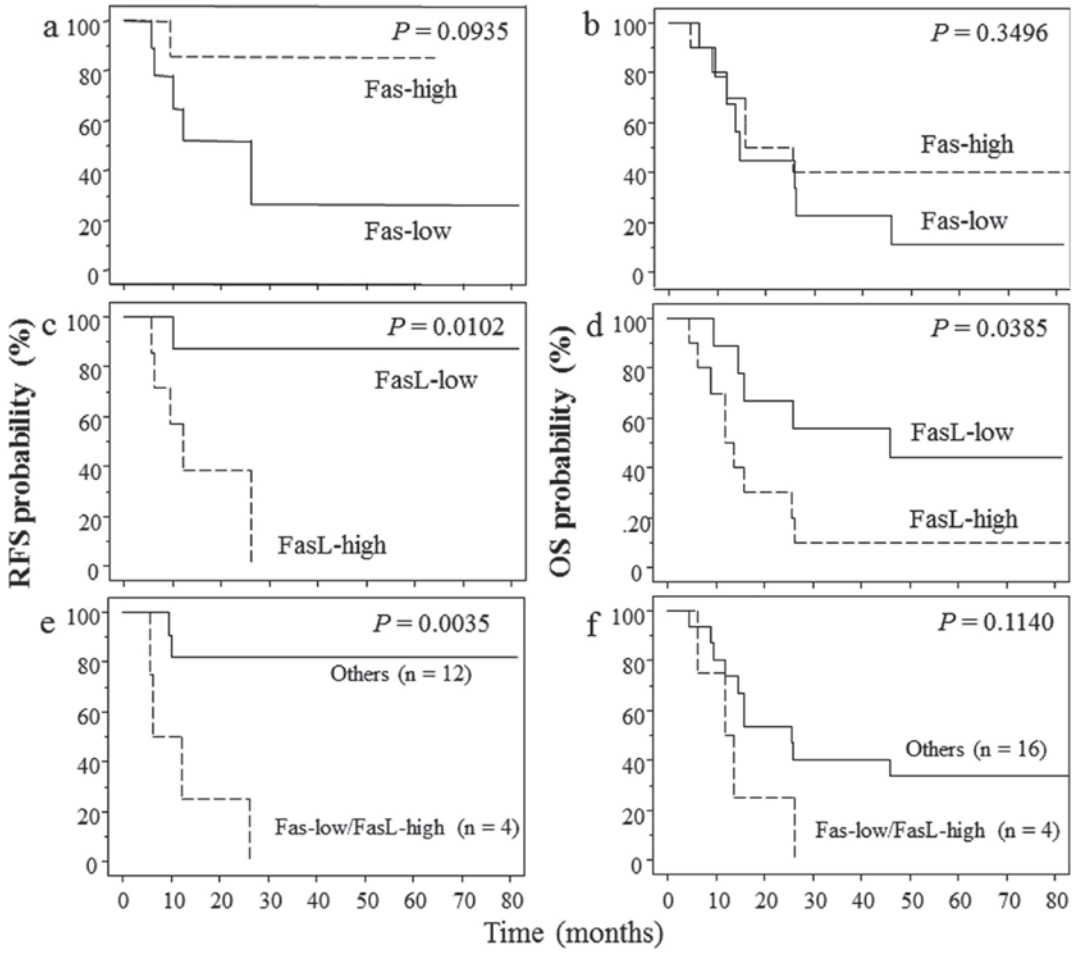

Figure 2. Kaplan-Meier survival curves based on the expression of (a and b) Fas, (c and d) FasL and (e and f) combined Fas/FasL. (e) Patients with low Fas and high FasL expression exhibited poorer RFS. OS, overall survival; RFS, recurrence-free survival; FasL, Fas ligand.

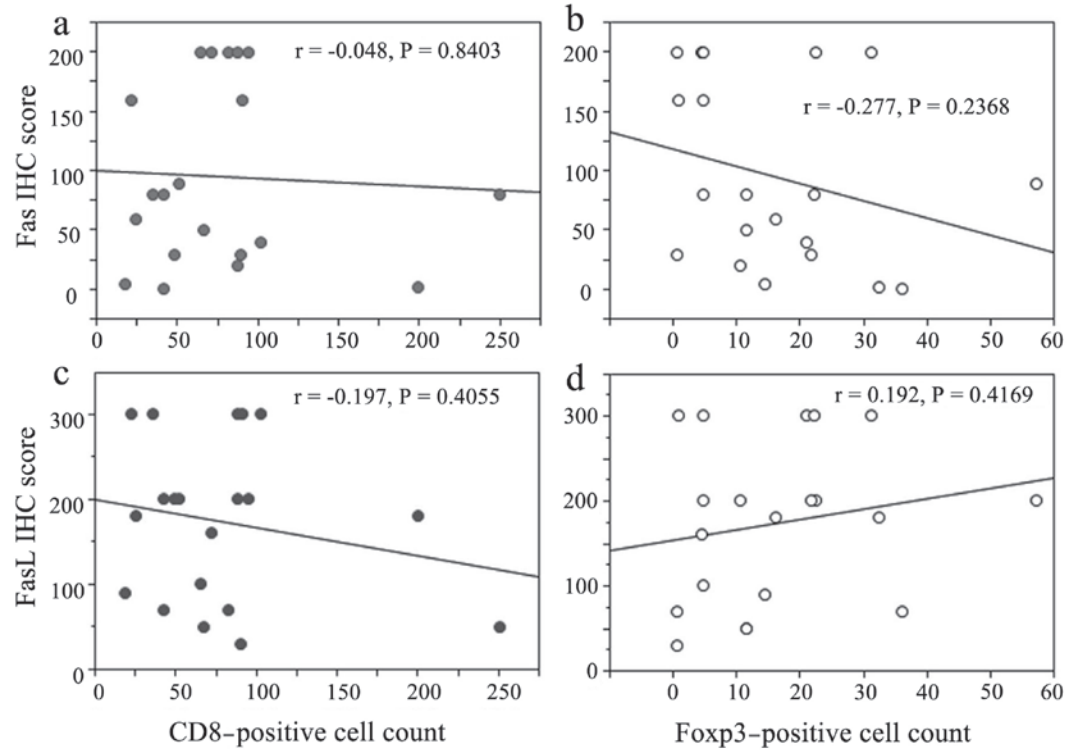

Figure 3. Correlation of Fas/Fas ligand (FasL) immunohistochemistry (IHC) score with count of peritumoral CD8- and Foxp3-positive cells. Correlation of Fas expression with (a) CD8-positive and (b) Foxp3-positive cells. Correlation of FasL expression with (c) CD8-positive and (d) Foxp3-positive cells.

that FasL expression was found in over half of the examined cancer cells, whereas Fas expression was rarely observed in tumor cells in ESCC. We observed that Fas expression was present in all the cases (median percentage of Fas-positive cancer cells, 80%; range, 1-100%). To explain this difference, we surmised that Fas expression may have been upregulated by radiation based on a previous study reporting that Fas expression in ESCC was increased by irradiation *in vitro* in a dose-dependent manner (20). In the present study, we did not compare the expression of Fas pre- and post-CRT.

We observed that a high FasL expression was correlated with tumor relapse. Moreover, patients with high FasL expression exhibited poorer recurrence-free and overall survival among ESCC patients treated with preoperative CRT. Shibakita *et al* (6) reported that FasL expression did not affect survival, whereas Fas expression was an independent favorable prognostic factor for patient survival in ESCC. In our data, patients with low Fas expression tended to have poor recurrence-free survival compared to those with high Fas expression.

One possible mechanism explaining the involvement of Fas/FasL expression in the prognosis of ESCC is evasion of the host immune response. Tumor-derived FasL counteracts the host immune system by eliminating Fas-sensitive cytotoxic T cells, such as CD8-positive cells (6,19). In the present study, FasL expression was diffusely observed in stromal cells in ESCC following CRT. Although significant differences were not observed, Fas appeared to be negatively correlated with peritumoral Foxp3-positive cells (regulatory T cells) and FasL appeared to be positively correlated with peritumoral CD8-positive cells. Rigberg *et al* (20) hypothesized that Fas/FasL proteins may provide certain tumors with an immune privilege and increase their resistance to radiotherapy. Moreover, several authors have reported that Fas/FasL signaling plays a role in chemoresistance to doxorubicin and oxaliplatin and immune responses by interaction with matrix metalloproteinase-7 (10-12). Although there were no correlations between Fas/FasL expression and TRG in this study, pharmacological control of Fas/FasL signaling may result in improved therapeutic efficacy and outcome in ESCC patients who receive preoperative CRT.

Rigberg *et al* (20) demonstrated that neither anti-Fas monoclonal antibody nor transduction of FasL directly inhibited tumor cell growth in an *in vitro* study. Thus, the main role of Fas/FasL signaling in tumor progression is likely the regulation of the tumor microenvironment. Sun *et al* (21) reported that polymorphisms of Fas/FasL genes appeared to be associated with an increased risk of developing ESCC. Taken together, the Fas/FasL pathway may be considered to be a candidate therapeutic target in ESCC, as the Fas/FasL signaling pathway plays an important role in tumorigenesis and tumor progression (22,23).

In conclusion, high FasL expression was associated with poor prognosis and the evaluation of FasL expression may provide clinically useful prognostic information for ESCC patients receiving neoadjuvant CRT. Moreover, the control of Fas/FasL signaling may lead to improved therapeutic efficacy and outcome in ESCC patients following neoadjuvant CRT. However, the data in this study should be interpreted with caution. The major limitations were the limited patient sample and the retrospective nature of the study. A larger study population is required to validate our conclusions.

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