Prognostic Significance of Fas and Fas Ligand Expressions in Human Esophageal Cancer

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ABSTRACT
Esophageal carcinomas have recently been shown to express Fas ligand (FasL) and down-regulate Fas to escape from host immune surveillance. However, the prognostic importance of Fas/FasL and their correlation with clinicopathological characteristics are yet to be delineated in this highly malignant carcinoma. Specimens from 106 esophageal squamous cell carcinoma patients were used for immunohistochemical evaluation of Fas, FasL, and CD8 expressions. Fifty-two (49%) and 34 (32%) patients were positive for FasL and Fas, respectively. There were no associations between FasL expression and clinicopathological characteristics except lymph vessel invasion. Strong FasL expression correlated with significant ($P = 0.0011$) decrease in tumor nest CD8+ cells. However, neither FasL nor CD8+ had any impact on patient survival. Strong Fas expression was correlated with depth of invasion (40.3% in pT1,T2 versus 20.5% in pT3,T4; $P = 0.0308$), histological differentiation (45.7% in well versus 25.4% in nonwell; $P = 0.0347$), and lymph node metastasis (22.6% in positive versus 45.5% in negative; $P = 0.0129$). Fas expression was one of the independent favorable prognosticators for patients’ survival (risk ratio, 3.26; $P = 0.0103$) in esophageal SCC. Fas expression was an independent prognosticator for recurrence-free survival, whereas FasL expression did not influence the survival in esophageal squamous cell carcinoma. Down-regulation of tumor Fas may be the hallmark of immune privilege for the tumor, thus causing the patients’ poorer outcome. Tumor FasL may counterattack the host immune cells to such an extent that the prognosis is not affected.

INTRODUCTION
Immune privilege is a unique trick adopted by specialized organs including testis, brain, and corneal tissues to avoid inflammatory reaction and is executed by constitutive expression of FasL ($^1$). Fas (CD95) and FasL have been known as transmembrane proteins and as members of the tumor necrosis factor receptor family. Binding of FasL to Fas induces trimerization of the Fas receptor, which recruits caspase-8 via an adaptor, FADD/MORT1. The oligomerization of caspase-8 may result in self-activation of proteolytic activity and trigger the ICE protease cascade. The activated ICE members can cleave various substrates, such as poly(ADP) ribose polymerase, lamin, rho-GDI, and actin, and cause morphological changes to the cells and nuclei ($^1$). FasL induces suicidal death (apoptosis) of accumulated cytotoxic T cells by binding to Fas expressed on T cells. Cancer cells that arise after several mutations from their ancestor normal cells come under host immune attack for elimination from the body. However, most of the tumors escape from the host immune attack by imitating themselves as immune-privileged sites by either overexpressing FasL or down-regulating Fas. FasL expression has been recently reported in several carcinomas including melanoma ($^2$), colon cancer ($^3$), hepatocellular carcinoma ($^4$, $^5$), lung cancer ($^6$), brain tumors ($^7$), ovarian cancer ($^8$), and liver metastases of colon cancer ($^9$).

Similarly, in esophageal cancer, up-regulation of FasL expression and down-regulation of Fas expressions were noted ($^10$). The majority of esophageal SCCs had up-regulation of FasL, which helped the tumor cells to counterattack the immune system by killing Fas-sensitive cytotoxic T cells. Also in the majority of tumors, the function of Fas was subverted by the down-regulation of expression, suggesting that this may be a self-defense mechanism for esophageal carcinoma cells to evade Fas-mediated killing from the host cytotoxic and/or killer cells ($^10$).

Tumor infiltration by lymphocytes have been considered to be a manifestation of host immune reaction to cancer cells ($^11$). Immunophenotypic evaluations have shown that CD8+ T cells form the predominant subset of TIL. The effect by CD8+ T cells in TIL could be theoretically related to the effector function of activated killer T cells and could induce apoptosis of cancer cells with Fas and FasL system ($^12$).

Fas and FasL expressions have been noted in a small number of patients with esophageal carcinoma ($^10$, $^13$), but their relative prognostic importance and correlation with clinicopathological characteristics and CD8 expressions are yet to be delineated in this highly malignant carcinoma with dismal prognosis. Therefore, we examined Fas, FasL, and CD8 expressions immunohistochemically in 106 surgical specimens of esophageal SCC, and the results were correlated with clinicopathological features and patients’ survival.

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2 The abbreviations used are: FasL, Fas ligand; ICE, interleukin 1β converting enzyme; TIL, tumor-infiltrating lymphocyte; SCC, squamous cell carcinoma.
MATERIALS AND METHODS

Surgically resected specimens were collected from 106 consecutive patients with esophageal SCC who had been operated on with a curative intent between December 1980 and December 1995 at the Second Department of Surgery, Shimane Medical University. None of them had preoperative radiotherapy or chemotherapy. Clinicopathological characteristics of these patients were investigated based on the tumor-node-metastasis classification of the malignant tumors (14).

The macroscopic tumor classification (Borrmann classification) was done according to the Japanese criteria set for the esophageal patients (15), which conforms with the well-recognized Borrmann classification defined for gastric carcinoma patients (16).

Immunohistochemical Detection of FasL, Fas, and CD8 Expressions. Paraffin-embedded sections of esophageal tumors were deparaffinized in xylen and rehydrated in graded ethanol. The sections were boiled in 10 mm sodium citrate (pH 6.0) for 10 min and incubated in H2O2 (0.3-0.5% for FasL and Fas and 3.0% for CD8) for 30 min. The slides were then washed with PBS three times for 5 min. After treatment with blocking serum for 30 min, the sections were incubated with the primary antibodies (1:200 dilution overnight for FasL, 1:400 dilution 1 h for Fas, and 1:30 dilution overnight for CD8). Rabbit polyclonal antibody against FasL (Santa Cruz Biochemistry Inc., Santa Cruz, CA), rabbit monoclonal antibody against Fas (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and mouse monoclonal antibody against CD8 (Novocastra Laboratories, Ltd., Newcastle, United Kingdom) were used in this study. The immunohistochemical staining was done using the streptavidin-biotin kit (Nichirei, Tokyo, Japan) by the avidin-biotin-peroxidase method, according to the manufacturer’s instructions. Aminoethylcalbasol was used as the chromogen, and the slides were counterstained with hematoxylin. Formalin-fixed, paraffin-embedded sections of human testis (17) were used as positive controls for FasL; sections of human liver served as positive controls for Fas; and tonsil sections were used in CD8 staining. Sections without primary antibodies as well as those with nonimmunized rabbit serum for FasL and Fas, and mouse serum for CD8, served as negative controls. All of the histological slides were examined by two observers (M. S. and Y. K. D.), who were unaware of the clinical data or the disease outcome. When the interpretation differed between the two observers, reevaluation was done for a final decision on a conference microscope.

Evaluation of Fas and FasL Expressions. Intensity and extent of Fas and FasL expressions were evaluated by a comprehensive score formula originally designed by Busch et al. (18). The intensity of staining was scored as follows: (a) 1, weak expression; (b) 2, moderate expression; and (c) 3, strong expression. The extent of staining in the samples was scored as: (a) 1, less than one-third of the tumor cells had positive staining; (b) 2, one-third to two-thirds of the tumor cells positive staining; and (c) 3, more than two-thirds of the tumor cells had positive staining. The results obtained with the two scales were multiplied against each other, yielding a single scale with steps of 1, 2, 3, 4, 6, and 9, in which 1, 2, 3, and 4 were considered to be weak staining, and 6 and 9 were considered to be strong staining.

Classification of CD8-stained T Cells by Location. Cytotoxic T-cells stained by anti-CD8 were classified into two groups according to their locations (19); those distributed along the margin of invasive cancer (margin CD8) and those infiltrated within cancer cell nests (nest CD8). Three high power fields (×200) with most abundant distribution of CD8-stained T cells were selected for counts, and the result was expressed as the mean number of CD8+ cells per high-power field.

Statistical Analyses. The standard χ2 test with or without Yates’ correction was used for comparative analyses. The survival rates were estimated by the Kaplan-Meier method (20), and the statistical analysis was carried out by the log-rank test to test for equality of the survival curves. In calculating 5-year recurrence-free survival rates, those who died of causes unrelated to esophageal cancer were considered to be recurrence-free at end point, and those who were alive more than 5 years without any happening were considered to be recurrence-free at the end of 5th year. In multivariate analysis, independent prognostic factors were determined by the Cox proportional hazards model (Ref. 21; StatView J4.5: Abacus Concepts, Inc., Berkeley, CA). The level of significance was set at P < 0.05.

RESULTS

Patient and Tumor Characteristics. Pathological tumor stages (pT) were pT1 in 30, pT2 in 32, pT3 in 26, and pT4 in 18 patients. Ninety-seven patients were male and 9 were female. The median age was 63.1 years (range, 44–83 years).

CD8 Expression. FasL protein was detected in all of the 106 primary esophageal SCCs analyzed. Fifty-two patients (49.1%) showed strong expression, whereas 54 (50.9%) demonstrated weak expression. FasL was predominantly expressed in the basal and suprabasal layers of mucosal and submucosal tumors (Fig. 1a) and homogeneously expressed in more advanced tumors (Fig. 1b). There was no relationship between the depth of tumor invasion and FasL expression, which indicated no up-regulation of FasL expressions with tumor progression in this study. There was no statistically significant association between FasL expression and clinicopathological characteristics except for the lymph vessel invasion; strong expression of FasL was correlated with positive lymph vessel invasion (P = 0.0070; Table 1).

Fas Expression. Fas expression was predominant in the outer layers of the superficial tumors and remained positive near the center of tumor nests of the more advanced tumors (Fig. 1, c and d). Strong Fas expression was detected in 34 (32.1%) of 106 tumors. Tumor Fas expression was down-regulated with the advancement of the disease process. Strong Fas expression was noted in 40.3% of pT1 and pT2 tumors and in 20.5% of pT3 and pT4 tumors (P = 0.0308). Also, well-differentiated tumors were predominant (P = 0.0347), and lymph node metastasis was less frequent in the strong Fas group (P = 0.0129; Table 1). Age, size of tumor, Borrmann classification, and vessels invasion were not different between the two groups.

CD8 Expression and Its Correlation with Fas and FasL. The counts of CD8+ cells were 0–195 and 1–99 in tumor margin and nest, respectively. The cutoff point between strong...
and weak CD8+ expressions was determined as 30 for nest CD8+ and 70 for margin CD8+ cells, respectively. Strong margin CD8+ was expressed in 29 (27.4%) of 106 tumors, and strong nest CD8+ was counted in 32 (30.2%) tumors. As shown in Table 2, strong FasL expression correlated with weak nest CD1 expression \( (P = 0.0011) \), but there was no significant correlation between the margin CD8+ and FasL expression \( (P = 0.9214) \). A significant number of tumors having an excess number of CD8+ cells in the tumor margin \( (P = 0.0446) \) and nest \( (P = 0.0045) \) had simultaneous down-regulation of Fas expression in tumor cells.

**Clinicopathological Factors Influencing Recurrence-free Survival Rate.** Thirty-one patients are alive and free from cancer at the time of this analysis. Twenty-eight patients died of causes unrelated to esophageal cancer and the remaining 47 patients died of recurrence of the disease. Of 47 recurrences, 15 were in the locoregional area, 15 in the distant area, and 17 were in both local and distant areas.

The factors influencing recurrence-free survival rate by univariate analyses are Borrmann classification \( (0, I \) versus \( II, III, IV; P < 0.0001) \), size of tumor \( (\leq 5 \ versus > 5 \ cm; P < 0.0001) \), amount of blood transfusion \( (22; \leq 2 \ versus \geq 3 \ units; P = 0.0295) \), lymph vessel invasion (positive versus negative; \( P = 0.0003) \), blood vessel invasion (positive versus negative; \( P = 0.0004) \), tumor stage \( (0, I \ versus III, IV; P < 0.0001) \), and degree of Fas expression (strong versus weak; \( P = 0.0001) \). FasL and CD8 expressions did not influence the survival rate.

Recurrence-free survival curves in terms of Fas expression...
are shown in Fig. 2. The recurrence-free rate was significantly (P = 0.0001) lower in patients with Fas down-regulation. There was no significant correlation between the Fas expression and recurrence pattern (local alone versus local with distant versus distant metastases; P = 0.6475). After stratification of patients according to the pT stage, Fas expression remained a significant predictor of recurrence-free survival in individual pT stages (P = 0.0168 for pT2 and P = 0.0023 for pT3 and pT4 tumors).

The independent risk factors for disease recurrence as determined by the multivariate analysis were tumor stage (risk ratio = 6.98), Fas expression (risk ratio = 3.26), and amount of blood transfusion (risk ratio = 2.02; Table 3).

Moreover, an assessment of multivariate analysis of independent prognostic indicators was done with the overall death as the end point. In this analysis, although the level of significance and the risk ratios for survival were different, the conclusions did not differ (tumor stage: risk ratio = 5.58, P = 0.0002; Fas expression: risk ratio = 2.12, P = 0.0206; and amount of blood transfusion: risk ratio = 2.14, P = 0.0056) with those achieved by the recurrence-free survival as the end point.

**DISCUSSION**

SCC of the esophagus is one of the most malignant tumors with dismal prognosis. Despite dramatic advances in diagnostic method and meticulous execution of radical surgery with or without adjuvant treatments, one-half of the patients die within 3 years, and the overall 5-year survival rate is only 20.0–23.8% (15, 23, 24). The most significant indicator of survival in patients with esophageal SCC is the presence of regional lymph node metastasis. Several genetic alterations including those of p53 and bcl-2 are thought to be responsible for the worst prognosis. These genes regulate the cell cycle, and the derangement of functions of these genes produces inappropriate turnover of rapidly dividing mucosal cells. Apoptosis, the programmed cell death, plays an instrumental role in cellular turnover, and the death execution is mediated through an apoptotic cascade in which the Fas/FasL system plays the key role in activation of downstream signals to the death machinery.

Recently, two pioneer studies have evaluated Fas and FasL expressions in esophageal SCC (10, 13), and one of the studies by Gratas et al. (10) has conceivably shown that most of the esophageal tumors [15 (79%) of 19] had weak Fas expression, which suggests that this may be a common mechanism for tumors to escape from the Fas-mediated host immune surveillance. Our results conform well with their study having negative

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**Table 1** FasL and Fas expressions and clinicopathological features in esophageal carcinomas (n = 106)

<table>
<thead>
<tr>
<th>Variable</th>
<th>FasL</th>
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<tr>
<td></td>
<td>Strong (n = 52)</td>
<td>Weak (n = 54)</td>
<td>Strong (n = 34)</td>
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<td>Age (yr)</td>
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<td>≤60</td>
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<tr>
<td>&gt;60</td>
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<td>39</td>
<td>22</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>49</td>
<td>48</td>
<td>31</td>
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<td>Female</td>
<td>3</td>
<td>6</td>
<td>3</td>
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<td>Size of tumor</td>
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<tr>
<td>≤5.0</td>
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<td>25</td>
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<tr>
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<tr>
<td>II, III, IV</td>
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<td>20</td>
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<tr>
<td>Well</td>
<td>19</td>
<td>16</td>
<td>16</td>
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<tr>
<td>Non-well</td>
<td>33</td>
<td>38</td>
<td>18</td>
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<tr>
<td>Depth of invasion</td>
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<tr>
<td>pT1</td>
<td>21</td>
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<td>13</td>
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<td>pT2</td>
<td>10</td>
<td>22</td>
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<td>11</td>
<td>7</td>
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<tr>
<td>pT4</td>
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<td>12</td>
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<td>Lymph node metastasis</td>
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<tr>
<td>Positive</td>
<td>27</td>
<td>35</td>
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<tr>
<td>Negative</td>
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<td>19</td>
<td>20</td>
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<tr>
<td>Lymph vessel invasion</td>
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<tr>
<td>Positive</td>
<td>35</td>
<td>48*</td>
<td>25</td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Blood vessel invasion</td>
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<tr>
<td>Positive</td>
<td>33</td>
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<td>22</td>
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<tr>
<td>Negative</td>
<td>19</td>
<td>14</td>
<td>12</td>
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* P = 0.0347.
* P = 0.0308 (pT1, T2 versus pT3, T4).
* P = 0.0129.
* P = 0.0070.

**Table 2** Correlations between CD8 positivity and FasL and Fas expressions

<table>
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<tr>
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<th>FasL</th>
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<tbody>
<tr>
<td></td>
<td>Strong</td>
<td>Weak</td>
<td>Strong</td>
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<tr>
<td>Nest CD8</td>
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<td></td>
</tr>
<tr>
<td>Strong</td>
<td>8</td>
<td>24*</td>
<td>4</td>
</tr>
<tr>
<td>Weak</td>
<td>44</td>
<td>30</td>
<td>30</td>
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<tr>
<td>Margin CD8</td>
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</tr>
<tr>
<td>Strong</td>
<td>14</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Weak</td>
<td>38</td>
<td>39</td>
<td>29</td>
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* P = 0.0011.
* P = 0.0045.
* P = 0.0446.
or weak Fas expression in 72 (68%) of 106 tumors, and we have furthered their results by showing that, in esophageal SCC, Fas may be an independent predictor for recurrence-free survival. In the multivariate analysis, Fas expression became one of the independent prognosticators for long-term survival (risk ratio = 3.26), whereas FasL expression did not influence the patients’ survival. This is the first study showing a significant correlation between Fas expression and disease recurrence, and, therefore, screening esophageal SCC patients for Fas expression may be of worth to predict prognosis and to design appropriate therapies in the future. Regardless of the pT tumor stages, Fas down-regulation became a worse prognostic indicator with higher incidences of regional lymph node metastasis. A significant number of well-differentiated tumors and large numbers of tumors in early stages had strong Fas expression indicating that by down-regulating Fas expression, tumors manage to escape the host immune attack for further growth and progression to more advanced stages. Ohbu et al. (25) reported that the rate of apoptosis was higher in well-differentiated esophageal tumors than in poorly differentiated tumors. Therefore, it would be possible that the survival advantage seen in Fas-positive cases is due to the occurrence of frequent apoptosis in well-differentiated esophageal SCC. Also, it may be unique in weakly positive esophageal SCC that Fas-expressing tumor cells are usually concealed at the center of the tumor nests to avoid contact with the host immune cells at the advancing margin as is shown in Fig. 1d. Moreover, to clarify the association of down-regulation of tumor Fas and patients’ poorer outcome, evaluating apoptosis may be important.

The battle between the host and tumor cells is a two-way mechanism, in which the tumor cells subvert the host attack by down-regulation of Fas expression and fight back by up-regulating FasL expression. Bennett et al. (13) have shown that esophageal tumor-derived-FasL acted upon surrounding TIL to induce apoptosis as a mechanism of host immune evasion. In our study, a significant inverse correlation was seen between the tumor FasL expression and nest CD8+ cells except, however, there was no correlation with the margin CD8+ cells. Gratas et al. (10) demonstrated that 18 (94.7%) of 19 invasive esophageal SCCs showed up-regulation of FasL. In this series, however, strong FasL was noted in only 52 cases (49.1%), and FasL did neither correlate with the histological differentiation nor influence the patients’ survival rate. The ineffectiveness of FasL as a prognostic indicator may be due to the absence of any impact on marginal CD8+ cells in esophageal SCC. In this study, most of the CD8+ cells could be located in the interstitial tissue at the tumor margin rather than in the nest, indicating that these cells escaped the attack from tumor FasL and may have had an aberrant signal transduction in lymphocytes making them insensitive to the tumor-derived FasL. Similarly, Ademmer et al. (26) found aggregation of cytotoxic CD8+CD103+ T cells in the fibrous tissue distant from the tumor cells in pancreatic cancer. It has been shown that the murine thymoma line EL-4 expresses FasL without any cytotoxicity against Fas+ cells (27), and gld mice express a mutant form of nonfunctional FasL (28), which might explain the ineffectiveness of FasL as a prognostic indicator in some tumors. Moreover, the absence of Fas in FasL-positive tumors precluded autocrine FasL attack and also prevented an attack from the CTLs (8).

We also examined the correlation between Fas and cytotoxic T-cell (CD8) expression and found that a significant number of CD8+ cells, both in the margin and in the tumor nest, were present in Fas-negative or weak cases indicating that the CD8+ cells remain idle in the absence of tumor Fas and did not participate in active immune surveillance. Eventually, the number of CD8+ cells had no impact on patients’ recurrence-free survival in this study. Freeman et al. (29) have shown that TIL in human prostate and bladder cancers are able to secrete vascular endothelial growth factor in situ at bioactive concentrations. Therefore, it may be possible that malignant tumors simultaneously down-regulate apoptosis and enhance neovascularization by down-regulating Fas expression and inviting TIL for angiogenic growth factor, respectively.

In conclusion, our results indicate that Fas expression may be a useful independent predictor of prognosis in esophageal SCC, and significant down-regulation of tumor Fas identifies it as a therapeutic target in this carcinoma with worse prognosis. Tumor-derived FasL had significant impact on the number of CD8+ cells in the tumor nest, but neither FasL nor the number of CD8+ cells could be a predictor of survival in the absence of tumor Fas.

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