Abstract. Background: Circulating vascular endothelial growth factor-C (VEGF-C) levels were measured in patients with esophageal cancer to assess the value of VEGF-C as a biomarker for predicting tumor recurrence. Patients and Methods: Preoperative serum samples were acquired from 80 patients and healthy volunteers who served as normal controls. VEGF-C levels were assessed using enzyme-linked immunosorbent assay (ELISA). Results: The preoperative serum VEGF-C level in patients with esophageal cancer was significantly higher than in healthy volunteers. Furthermore, patients with recurrence had significantly higher preoperative serum VEGF-C levels than patients without recurrence, and a high preoperative serum VEGF-C level was found to be an independent risk factor for recurrence, in addition to lymph node metastasis. Conclusion: Preoperative VEGF-C levels may reflect malignancy and predict recurrence in patients with esophageal cancer. Therefore, the preoperative VEGF-C level may be a useful biomarker for choice of multimodality therapy.

Lymphatic vessels play an important role in the maintenance of tissue homeostasis (1) and transport of immune cells (2), and also serve as the primary conduit for malignant tumor cell metastasis to regional lymph nodes (3). Lymph node metastasis is a characteristic of malignant cancers and is observed more frequently in esophageal cancer than in other digestive tract cancers, making it one of the most important prognostic factors (4). Since the induction of tumor lymphangiogenesis by vascular endothelial growth factor (VEGF)-C or -D was first reported to promote cancer metastasis (3, 5), many investigations have shown that tumor expression of lymphangiogenesis factors is correlated with clinico-pathological features (especially metastatic tumor spread) and prognosis in various human cancers (6).

VEGF-C expression in tumor or stromal cells has been directly correlated with clinicopathological features, including lymph node metastasis in human esophageal squamous cell carcinoma (7). Kimura et al. reported that tumor expression of VEGF-C is correlated with lymphatic involvement and prognosis in esophageal squamous cell carcinoma (8), and Ishikawa et al. suggested that VEGF-C might play a positive role in the early stage of esophageal carcinogenesis based on the active production of VEGF-C in some dysplastic lesions, as well as in esophageal carcinomas (9). However, serum VEGF-C levels in patients with esophageal cancer have not been measured. Therefore, we examined the association of VEGF-C with clinicopathological features in patients with esophageal cancer and assessed the serum VEGF-C level as a predictor of recurrence.

Patients and Methods

Patients and samples. The study was performed in patients with esophageal cancer who underwent potentially curative surgery without preoperative therapy at the Department of General Surgical Science, Gunma University Graduate School of Medicine, between 1998 and 2005. Tumor stage was classified according to the 6th edition of the tumor-node-metastasis classification of the International Union Against Cancer (UICC) (1). Healthy volunteers were recruited as normal controls. All patients and healthy volunteers signed informed consent forms according to our institutional guidelines. Information on gender, age, stage of disease and histopathological factors was abstracted from medical records.
Preoperative serum samples were acquired from 80 patients (71 males and 9 females). The average age of the patients was 62.8, with a range of 41 to 80 years. The last date of follow-up was February 1, 2006, and the median follow-up time for all survivors was 39 months, with a range of 5 to 80 months. Serum samples from 20 healthy volunteers without cancer (average age, 65.4 years) were assayed as normal controls. Before surgery, venous blood was obtained from each patient. Blood samples were stored at 4 °C after collection and the serum was separated from the blood by centrifugation at 1000 xg for 15 min. The serum samples were kept frozen at -80 °C until the assay was performed.

**Serum VEGF-C quantification.** The VEGF-C concentration in the serum samples was determined by ELISA using a Quantikine® Human VEGF-C Immunoassay Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Before running the assay, the samples were thawed at 4 °C overnight and then 5-fold diluted with animal serum. One hundred microliters of buffered protein base and 50 µl of sample were added to the wells of a microtiter plate coated with anti-VEGF-C mouse monoclonal antibody, and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker. The plate was washed 4 times and 200 µl of conjugated antibody solution were added. After a further incubation, the plate was washed again, 200 µl of substrate solution were added, and a third incubation was carried out for 30 minutes at room temperature with protection from light. After the addition of 50 µl of stop solution, color development was determined immediately at 450 nm using a microtiter plate reader. The mean minimum detectable dose was 13.3 pg/ml. All samples were assayed in duplicate in a blinded fashion and the mean was used for data analysis.

**Statistical analysis.** Statistical analysis was performed using non-parametric techniques such as a Mann-Whitney U-test and a Kruskal-Wallis test. Survival curves were calculated using the Kaplan-Meier method and analysis was performed using a log-rank test, with p<0.05 considered to be significant. A logistic regression model was used to assess the relative risk ratio of recurrence, again with p<0.05 considered significant. All statistical analyses were performed with the SPSS software package (version 13.0., SPSS, Inc., Chicago, IL, USA).

**Results**

The preoperative serum VEGF-C level in patients with esophageal cancer was 5680±2891 pg/ml (median±interquartile range), which was significantly higher than that in the level in healthy volunteers (4819±1255 pg/ml, p=0.0245) (Figure 1). The postoperative serum VEGF-C level (4751±2876 pg/ml) was significantly lower than the preoperative level (p<0.0001, Figure 1). Associations between clinicopathological characteristics and serum VEGF-C levels in patients with esophageal cancer are summarized in Table I. The serum VEGF-C level was associated with regional lymph node metastasis (p=0.0068), lymphatic invasion (p=0.0081) and blood vessel invasion (p=0.0219). However, there was no significant association between serum VEGF-C and factors such as age, gender, location, histological grade, histological type, tumor depth, stage grouping, infiltrative growth pattern, intraepithelial spread, or intramural metastasis.

For assessing the prognostic value, patients with serum VEGF-C levels less than the median level of 5680 pg/ml were assigned to the low level group (n=40), whereas those with levels >5680 pg/ml were assigned to the high level group (n=40). There were no significant differences in age or gender between these groups. The overall survival rates of patients with high serum VEGF-C levels in the peripheral vein were significantly lower than those of patients with low serum VEGF-C levels (p=0.0183) (Figure 2A). The 5-year overall survival rate of patients with low VEGF-C was 70.7% and that of patients with high VEGF-C was 36.1%. The disease-free survival rates of patients with high VEGF-C levels were also significantly lower than those of patients with low VEGF-C levels (p=0.0236) (Figure 2B). The 5-year disease-free survival rates of patients with low and high VEGF-C were 73.6% and 41.1%, respectively.

In follow-up, recurrence was observed in 32 out of 80 patients and it was found that patients with recurrence had a significantly higher preoperative serum VEGF-C level than those without recurrence (7078±2611 vs. 5310±2125 pg/ml, p=0.0041) (Figure 3). The initial site of recurrence was classified as local recurrence in 9 patients, hematogenous recurrence in 14, and lymph node recurrence in 16; these classifications include 7 patients with composite recurrence. The serum VEGF-C levels (median±IR) in patients with local recurrence, hematogenous recurrence and lymph node recurrence were 4179±1952, 7314±1801, and 7507±1966.
The preoperative serum VEGF-C was significantly higher in patients with hematogenous recurrence and lymph node recurrence, compared to patients without recurrence ($p=0.0014$ and $p=0.0001$, respectively), whereas there was no significant difference in VEGF-C between patients with local recurrence and those without recurrence ($p=0.2043$) (Table II).

The sensitivity and specificity of serum VEGF-C for predicting recurrence in patients with esophageal cancer were calculated by construction of an ROC curve (Figure 4). A sensitivity of 71.9% and a specificity of 66.7% were obtained using a cut-off value of 5744 pg/ml. Logistic regression models were used to calculate the relative risk for recurrence. A high preoperative serum VEGF-C level was found to be an independent risk factor for recurrence ($p=0.0069$, relative risk 5.60), in addition to lymph node metastasis ($p=0.0061$, relative risk 8.50) (Table III).
In this study, we investigated serum VEGF-C levels in clinical samples from patients with esophageal cancer. The preoperative serum VEGF-C level in the patients was significantly higher than the level in healthy volunteers, and the postoperative serum VEGF-C level was significantly lower than the preoperative level. The preoperative serum VEGF-C level was associated with regional lymph node metastasis, lymphatic invasion and blood vessel invasion. Patients with high preoperative serum VEGF-C in the peripheral vein had poorer prognoses than patients with low VEGF-C, and a high preoperative serum VEGF-C level was an independent risk factor for recurrence.

Secretion of VEGF-C seems to be increased in primary cancer of the esophagus, since the preoperative serum VEGF-C level in patients with esophageal cancer was significantly higher than the level in healthy volunteers, and their postoperative serum VEGF-C levels showed a significant decrease. Given the association of the preoperative serum VEGF-C level with regional lymph node metastasis, lymphatic invasion and blood vessel invasion, we suggest that the preoperative serum VEGF-C level might reflect formation of a metastatic focus through angiogenesis and lymphangiogenesis in the interstitium surrounding the primary tumor. Such active angiogenesis and lymphangiogenesis could lead to systemic spread of cancer cells, and this may explain the significance of a high preoperative serum VEGF-C level as an independent risk factor for recurrence.

Table II. The association between recurrence pattern and serum VEGF-C level measured from a peripheral vein.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Serum VEGF-C level (median±IR) pg/ml</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No recurrence</td>
<td>48</td>
<td>5310±2125</td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td>32</td>
<td>7078±2611</td>
<td>0.0041</td>
</tr>
<tr>
<td>Local recurrence</td>
<td>9</td>
<td>4179±1952</td>
<td>0.2043</td>
</tr>
<tr>
<td>Hematogenous recurrence</td>
<td>14</td>
<td>7314±1801</td>
<td>0.0014</td>
</tr>
<tr>
<td>Lymph node recurrence</td>
<td>16</td>
<td>7507±1966</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

IR: Interquartile range, *Mann-Whitney U-test (vs. no recurrence).

Table III. Multivariate analysis using the logistic regression model.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Reference factor</th>
<th>P</th>
<th>Relative risk</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-</td>
<td>0.8828</td>
<td>1.01</td>
<td>0.94-1.07</td>
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<tr>
<td>Gender</td>
<td>Female vs. Male</td>
<td>0.1241</td>
<td>5.27</td>
<td>0.63-43.9</td>
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<tr>
<td>Tumor depth</td>
<td>T1 vs. T2-4</td>
<td>0.1176</td>
<td>3.18</td>
<td>0.75-13.5</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>N0 vs. N1</td>
<td>0.0061</td>
<td>8.5</td>
<td>1.84-39.3</td>
</tr>
<tr>
<td>Intramural metastasis</td>
<td>IM(−) vs. IM(+)</td>
<td>0.3275</td>
<td>2.31</td>
<td>0.43-12.3</td>
</tr>
<tr>
<td>Serum VEGF-C level</td>
<td>Low vs. High</td>
<td>0.0069</td>
<td>5.6</td>
<td>1.60-19.6</td>
</tr>
</tbody>
</table>

CI: confidence interval.

Discussion

In this study, we investigated serum VEGF-C levels in clinical samples from patients with esophageal cancer. The preoperative serum VEGF-C level in the patients was significantly higher than the level in healthy volunteers, and the postoperative serum VEGF-C level was significantly lower than the preoperative level. The preoperative serum VEGF-C level was associated with regional lymph node metastasis, lymphatic invasion and blood vessel invasion. Patients with high preoperative serum VEGF-C in the peripheral vein had poorer prognoses than patients with low VEGF-C, and a high preoperative serum VEGF-C level was an independent risk factor for recurrence.

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Following the finding that induction of tumor lymphangiogenesis by VEGF-C or VEGF-D promotes cancer metastasis (3, 5), many studies have shown that tumor expression of lymphangiogenesis factors correlates with clinicopathological features (especially metastatic tumor spread) and prognosis in human cancers (6). In esophageal squamous cell carcinoma, Kitadai et al. showed that tumor VEGF-C expression is correlated with clinicopathological features including depth of tumor invasion, tumor stage, venous invasion, lymphatic invasion and lymph node metastasis in human esophageal squamous cell carcinomas (7). Mobius et al. also reported that patients with squamous cell carcinoma and lymph node metastases had significantly higher VEGF-C expression, and that high VEGF-C expression tended to be correlated with poor survival in squamous cell cancer, but not in esophageal adenocarcinoma (10).

Serum VEGF-C levels have been investigated in non-small cell lung cancer and cervical cancer. Tamura and Ohta reported that serum VEGF-C levels in patients with non-small cell lung carcinoma correlated with pathological stage, lymph node metastasis and lymphatic vessel invasion, and suggested that preoperative prediction of lymph node metastasis was possible using a combination of VEGF-C and VEGF levels (11). Mitsuhashi et al. reported that the pre-therapeutic serum levels of VEGF-C correlated significantly with FIGO stage, tumor size, and disease recurrence or persistence after treatment in squamous cell carcinoma of the uterine cervix (12). Our data are consistent with these studies and we suggest that preoperative circulating VEGF-C levels might reflect formation of a metastatic focus through tumor-associated angiogenesis and lymphangiogenesis.

We give adjuvant chemotherapy in the subgroup with lymph node metastasis, since it has been confirmed that postoperative chemotherapy reduces the risk of recurrence (13). Moreover, extensive lymph node metastasis may be a factor in the indication for neoadjuvant therapy, suggesting that lymph node metastasis may be important in selecting combined modality therapy in esophageal cancer. In this study, a preoperative high serum VEGF-C level was an independent risk factor for recurrence, in addition to lymph node metastasis, and this suggests that the preoperative serum VEGF-C level is a useful biomarker for assessment of the need for multimodality therapy. We conclude that the preoperative VEGF-C level may reflect a malignancy such as lymph node metastasis, and may predict recurrence in patients with esophageal cancer.

References


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