INTRODUCTION

Esophageal carcinoma is one of the most common malignancies in the world. Since the growth of tumor is relatively fast, patients with esophageal carcinoma generally have a worse prognosis than those with any other gastrointestinal tumors. It has been thought that several factors such as stage, histological grade, DNA ploidy, epidermal growth factor receptor, p53, and lymph node metastasis influence the survival. Angiogenesis, which is essential for tumor growth and metastasis, depends on the production of angiogenic factors by tumor cells and normal cells. Increased vascularity enhances the growth of the primary neoplasm and provides a greater chance for a hematogenous metastasis. It has been shown to have a prognostic value in several solid tumors such as breast, lung, prostate, cervical, and colon cancer (1-5). Previous studies also have demonstrated that the vascular density of a tumor directly correlates with metastasis and poor outcome in patients with solid tumors (9-14).

Vascular endothelial growth factor (VEGF) is an angiogenic factor that stimulates the growth of endothelial cells (15-17). It consists of four isoforms that have 121, 165, 189, and 206 amino acid residues and all four types of VEGF are secreted in abundance by many kinds of carcinoma cells (18). Recent studies showed a positive correlation among the VEGF expression, tumor microvessel density (MVD), and tumor aggressiveness (19-21). Alterations of the p53 tumor suppressor gene have known to be the most common genetic changes in solid tumors including esophageal carcinomas. Recent reports showed that mutations of p53 might be associated with angiogenesis by regulating the VEGF expression (22, 23). To clarify the prognostic significance and relationship between VEGF expression and clinicopathological features and its correlation with microvessel density and p53 mutations, we retrospectively analyzed 81 primary esophageal carcinomas.
Cases of adenocarcinoma from Barrett's esophagus were excluded. Six cases of basaloid squamous cell carcinoma and one case of carcinosarcoma were included. Tumor staging was based on the pTNM pathological classification system. They included three patients with stage I, 28 with stage IIA, four with stage IIB, 36 with stage III, and ten with stage IV. Histological grades, tumor stages, lymph node metastasis, and the depth of invasion are shown in Table 1. Information concerning the date of initial diagnosis, other clinical characteristics, and death were obtained by a retrospective study. Subjects were followed up until any of the followings: the date of death, the last date they were known to be alive, or the end of the follow-up. Observations were censored either at the date of last follow-up or at the last date of the follow-up period if death had not occurred. The median duration of follow-up for surviving patients was 16.25 (3.9-128.0) months.

### Immunohistochemical staining

The avidin-biotin complex (ABC) method was used for the immunostaining. Formalin-fixed paraffin-embedded tissue blocks were sectioned at 4-μm thickness and immunostaining was performed according to following methods. Sections were deparaffinized in xylene for 10 min three times and rehydrated in serial graded alcohol in the following order: 100% for 5 min, 90% for 5 min, 70% for 5 min, and 50% for 5 min. Antigen retrieval was performed for VEGF and p53. For antigen retrieval, we microcoooked the prepared 10 mM citrate buffer (pH 6.0) in a microwave for 3 min from the boiling point. The slides were put on the preheated citrate buffer and microwaved for 2 min three times. The container was put in a margin and cooled for 20 min at room temperature. The slides were washed in phosphate-buffered saline (PBS) once for 5 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in a 45 mL methanol solution for 15 min. The slides were washed in 1 × PBS for 5 min three times. All the slides were preincubated with two drops of normal blocking solution (goat serum) at 37℃ for 20 min (100 μL/slide). Cautions should be exercised not to let the slides dry. The antibodies used were a mouse monoclonal IgG antibody (Santa Cruz Biotech, U.S.A.) at a 1:50 dilution for VEGF, a mouse monoclonal IgG antibody (Santa Cruz) at a 1:100 dilution for p53, and a mouse monoclonal antibody (Immunotech, Cedex, France) at a 1:100 dilution for CD 34. Each antibody was incubated overnight at 4℃ and washed in 1 × PBS twice. Each of the biotinyalted secondary antibodies was added for 30 min at 37℃ followed by the avidin-biotinylated peroxidase complex (Immunotech) for additional 30 min at room temperature. After washing with PBS, the samples were stained by 3,3′-diaminobenzidine (Immunotech). Counterstaining was performed with hematoxylin for 30 sec.

### Evaluation of immunostaining for p53, VEGF, and microvessel density

All slides were coded and evaluated without knowledge of patients’ identity or clinical status by two experienced pathologists. Each experiment was independently performed twice. More than 10% of nuclear staining was defined as positive for p53. VEGF can be expressed in various human tissues including esophageal squamous cells and stromal cells. To determine the expression status of VEGF in cancer cells, we examined adjacent normal squamous epithelium. In our experiment, VEGF expression was largely confined in basal part of the epithelium (Fig. 1) and was not exceed 30% of the squamous cells. Accordingly, we considered positive for VEGF if more than 30% of tumor cells stained in their cytoplasm with stronger intensity than nonspecific background staining.

The degree of angiogenesis was determined by the MVD in defined areas of tissue sections according to the method
of Weidner et al. (24). All slides were coded and evaluated by an experienced pathologist without knowledge of patient’s identity or clinical status. Each microvessel counting was performed twice. Each slide was first scanned at \( \times 100 \) magnification to determine three “hot spots” defined as areas with the maximum number of microvessels. The slides were then examined at \( \times 200 \) magnification. Microvessels were counted within the area defined in each of the three hot spots. Areas of staining with no discrete breaks were counted as a single vessel. Microvessel density was estimated by adding the number of vessels in each of the three hot spots and then expressed as the mean number of vessels.

**Statistical analysis**

Statistical significance was evaluated using the Mann-Whit-
ney U test for independent groups. Survival curves were calculated using the Kaplan-Meier method and compared with other prognostic variables using the log-rank test. Correlation between variables was assessed by the Pearson’s coefficient (r). Univariate analysis and multivariate stepwise Cox’s regression analyses were performed to identify prognostic factors for survival. All statistical analyses were two-sided at a significance level of \(p=0.05\), and performed using SPSS 10R statistical software.

### RESULTS

The expression of VEGF was identified mainly in the cytoplasm of the cancer cells, and 41 (51.3%) of the 80 cases were evaluated as VEGF-positive. The representative data are shown in Fig. 1. When the patients were divided into two groups, that is, VEGF-positive and negative groups, there were no significant differences in clinicopathological findings between the two groups according to the results from \(\chi^2\) analysis (Table 2). Since all of the pT4 cases underwent palliative resection, cases of esophagectomy with thoracotomy for radical lymphadenectomy in pT1b to pT3 cases were selected and compared. The average number of metastatic lymph nodes at surgery in this group of patients was 1.17 in the patients with VEGF-negative tumors and 2.18 in those with VEGF-positive tumors and there was no statistical significance (\(p=0.46\)). The recurrence rate was 48.7% (19 of 39) in the VEGF-negative group and 43.9% (18 of 41) in the VEGF-positive group.

Eighty-one cases were evaluated for MVD. The range of MVD in esophageal cancer was 13.3-179.0 and the median number was 59.0. The representative data are shown in Fig. 1. The mean MVD in VEGF-negative group was 68.68 ± 31.91 (mean ± SE) and that in VEGF-positive group was 70.88 ± 43.11 (mean ± SE). MVD in the patients with VEGF-negative tumors was higher than in those with VEGF-positive tumors and there was no statistical significance (\(p=0.72\)) (Fig. 2).

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The p53 expression was evaluated in 79 of 81 patients. Forty-one of 79 (51.9%) were p53 positive. There were no significant differences in various clinicopathological findings, such as age, tumor size, histological grade, depth of invasion, lymph node metastasis, stage, and venous or lymphatic invasion between p53-positive and negative groups. The recur-

### Table 2: VEGF (n=80) and p53 (n=79) expression in esophageal cancer patients

|              | VEGF (-) | VEGF (+) | p value | p53 (-) | p53 (+) | p value |
|--------------|----------|----------|---------|---------|---------|---------|
| Age (years)  |          |          |         |         |         |         |
| <60          | 19       | 21       | 0.823   | 19      | 20      | 0.914   |
| >60          | 20       | 20       | 19      | 21      |         |         |
| Sex          |          |          |         |         |         |         |
| Male         | 36       | 39       | 0.603   | 33      | 41      | 0.016   |
| Female       | 3        | 2        | 5       | 0       |         |         |
| p53          |          |          |         |         |         |         |
| Negative     | 16       | 22       | 0.358   |         |         |         |
| Positive     | 21       | 19       |         |         |         |         |
| Tumor size   |          |          |         |         |         |         |
| <5 cm        | 24       | 22       | 0.645   | 22      | 23      | 0.862   |
| >5 cm        | 15       | 17       | 15      | 17      |         |         |
| Grade        |          |          |         |         |         |         |
| WD*          | 8        | 5        | 0.564   | 8       | 5       | 0.354   |
| MD           | 20       | 24       | 19      | 24      |         |         |
| PD           | 6        | 5        | 7       | 4       |         |         |
| Depth of invasion |      |          |         |         |         |         |
| T1           | 3        | 1        | 0.458   | 2       | 1       | 0.453   |
| T2           | 9        | 8        | 10      | 7       |         |         |
| T3/T4        | 27       | 32       | 26      | 33      |         |         |
| Lymph node   |          |          |         |         |         |         |
| Negative     | 19       | 19       | 0.832   | 19      | 17      | 0.447   |
| Positive     | 20       | 22       | 19      | 24      |         |         |
| Metastasis   |          |          |         |         |         |         |
| Negative     | 34       | 36       | 0.933   | 35      | 34      | 0.220   |
| Positive     | 5        | 5        | 3       | 7       |         |         |
| Stage        |          |          |         |         |         |         |
| I/II/IIIB    | 17       | 18       | 0.978   | 19      | 14      | 0.153   |
| III/IV       | 22       | 23       | 19      | 27      |         |         |
| Venous/lymphatic invasion |       |          |         |         |         |         |
| Negative     | 5        | 10       | 0.204   | 7       | 9       | 0.836   |
| Positive     | 32       | 30       | 28      | 32      |         |         |
| Relapse      |          |          |         |         |         |         |
| No           | 20       | 23       | 0.666   | 24      | 20      | 0.199   |
| Yes          | 19       | 19       | 14      | 21      |         |         |
| Death        |          |          |         |         |         |         |
| No           | 24       | 22       | 0.476   | 19      | 26      | 0.229   |
| Yes          | 15       | 19       | 19      | 15      |         |         |

*WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated.
rence rate and death rate were 36.8% (14 of 38) and 50.0% (19 of 38) in the p53-negative group and 51.2% (21 of 41) and 36.5% (15 of 41) in the p53-positive group, respectively ($p=0.199; 0.229$) (Table 2). When we analyzed the correlation between the VEGF and p53 expression, there was no association between two groups.

By univariate analysis, depth of invasion, lymph node metastasis, stage, tumor size, and distant metastasis were correlated with overall survival (Table 3). Among those variables, the presence of distant metastasis was the most significant factor for the survival. For the patients with distant metastasis, the median survival was only 4.4 months, compared with 43.5 months for patients without metastasis. However, we could not find any difference in overall survival between VEGF-positive and negative groups ($p=0.3129$) (Fig. 3A). Some findings were noted in the p53 expression ($p=0.4144$) (Fig. 3B).

Since MVD is a continuous variable, Cox’s regression hazard model was used for the analysis. By multivariate analysis, the presence of distant metastasis was the only factor that influences overall survival (Table 4).

**Table 3.** Univariate analysis of factors that influence the overall survival

| Factors                  | Patients (n) | Median survival (m) | p value |
|--------------------------|--------------|---------------------|---------|
| Depth of invasion (n=81) |              |                     |         |
| T1/T2                    | 21           | 0.044               |         |
| T3/T4                    | 60           | 27.4                |         |
| Lymph node (n=81)        |              |                     |         |
| Negative                 | 39           | 60.4                | 0.040   |
| Positive                 | 42           | 27.9                |         |
| Metastasis (n=81)        |              |                     |         |
| Negative                 | 71           | 43.5                | 0.009   |
| Positive                 | 10           | 4.4                 |         |
| Stage (n=81)             |              |                     |         |
| I/II/III                 | 35           | 60.4                | 0.034   |
| IV                       | 46           | 19.2                |         |
| Tumor size (n=79)        |              |                     |         |
| <5 cm                    | 46           | 53.9                | 0.053   |
| >5 cm                    | 33           | 17.8                |         |
| VEGF (n=80)              |              |                     |         |
| Negative                 | 39           | 53.9                | 0.313   |
| Positive                 | 41           | 39.6                |         |
| p53 (n=79)               |              |                     |         |
| Negative                 | 38           | 27.9                | 0.414   |
| Positive                 | 41           | 39.8                |         |

**Table 4.** Multivariate analysis of factors that influence the overall survival

| Factors                  | $b$ | SE ($b$)* | p value | Odd ratio (95% CI)* |
|--------------------------|-----|-----------|---------|---------------------|
| T (T3/4 vs T1/2)         | 0.71| 0.48      | 0.14    | 2.04 (0.79-5.24)    |
| N (N1 vs N0)             | 0.214| 0.40     | 0.60    | 1.24 (0.56-2.73)    |
| M (M1 vs M0)             | 1.29| 0.50      | 0.01    | 3.65 (1.36-9.78)    |
| Tumor size (>5 cm vs <5 cm) | 0.48| 0.37      | 0.20    | 1.61 (0.78-3.35)    |
| VEGF (positive vs negative) | 0.24| 0.37      | 0.53    | 1.27 (0.61-2.63)    |
| p53 (positive vs negative) | -0.55| 0.38    | 0.15    | 0.58 (0.27-1.23)    |

Cox’s proportional hazards regression model is as follows: $h_i(t)= h_0(t)\exp (\beta_1 T + \beta_2 lymph node + \beta_3 distant metastasis + \beta_4 tumor size + \beta_5 VEGF + \beta_6 p53 + \beta_7 microvessel density)$.

*SE: standard error; 95% CI: 95% confidence interval.

**DISCUSSION**

In this study, we analyzed the relationships between VEGF expression, MVD, p53, and clinicopathological features in esophageal carcinoma.
esophageal carcinomas. VEGF expression was noted in more than half of the patients and these data are consistent with the findings of the previous reports (24-28). However, the association between VEGF expression and clinicopathological findings is controversial. Some reports showed that lymphatic and/or venous invasion, tumor stage, or histological grade was correlated with VEGF expression (24-27). However, recently Shih et al. (28) reported that VEGF expression was not correlated with any of the clinical features, such as, tumor size, lymph node metastasis, histological grade, and lymphatic or venous invasion. We could not find any correlation between VEGF expression and the clinicopathological findings, either. In this study, the median number of lymph node metastasis in the VEGF-positive group was slightly higher than that of the VEGF-negative group. It has been reported by Shih et al. that the average number of metastatic lymph nodes at surgery was 5.6 in the patients with VEGF-positive tumors and 3.0 in those with VEGF-negative tumors, and was significantly higher in those with VEGF-positive tumors.

To investigate the association between VEGF expression and tumor angiogenesis, we examined MVD immunohistochemically using anti-CD34 antibody. In the present study, the MVD in esophageal carcinoma tissues had a wide range, and the MVD of VEGF-positive carcinomas tended to be higher than that of VEGF-negative carcinomas. These results suggest that VEGF may be one of the key angiogenic factors, and promotes tumor angiogenesis in esophageal carcinoma tissues, in the same way as previously described in other carcinomas. However, we could not find a significant correlation between VEGF expression and MVD, as compared with previous reports (25-27). Several studies also reported that there was no correlation between VEGF expression and MVD (24, 28).

Factors that regulate VEGF expression in tumor and non-tumor cells have been investigated. Hypoxia has been known to be one of the most important mediators inducing the increase of VEGF. Mutations of the ras and p53 genes have been shown to up-regulate the VEGF expression (22, 23). Some reports showed that mutations in this gene might be connected with angiogenesis by regulating VEGF expression in human cancers (29, 30). Although in this study the p53 expression was noted in 51.9% of esophageal carcinomas, there was no significant correlation between VEGF expression and accumulation of p53 protein in esophageal carcinomas. Recent study by Shimada et al. also reported that p53 and VEGF were highly expressed in esophageal carcinoma tissues by immunohistochemical analysis, however, by multivariate analysis both molecular and biological markers were not associated with poor survival. Rather, cyclin D1, E-cadherin, and epidermal growth factor receptor were revealed to have prognostic relevance for survival and recurrence (31). These results suggest that numerous cytokines and growth factors, such as epidermal growth factor, platelet-derived growth factor, transforming growth factor β, and insulin-like growth factors produced by tumor and normal cells may affect the VEGF expression.

The prognostic factors influencing disease-free and overall survival in esophageal carcinomas are based on both the histological type and the tumor stage. In the present study, by univariate analysis, tumor stage, depth of invasion, lymph node metastasis, distant metastasis, and tumor size were correlated with poor overall survival. However, the distant metastasis was the only prognostic factor affecting disease-free and overall survival in this cohort of esophageal carcinoma patients by multivariate analysis. This finding is inconsistent with other studies demonstrating that tumor stage and lymph node metastasis are the important prognostic factors. This can be explained by the facts that our study was a retrospective one and many patients underwent palliative esophageal surgery, so that the definitive lymph node dissection and accurate pathological staging were not possible in these patients. However, even if we had selected patients under curative surgery, we could not have found any specific prognostic findings. These conflicting results should be verified by prospective studies with a large number of patients. Previous studies have demonstrated that the angiogenesis is associated with the prognosis of patients with several malignancies. We followed the patients to determine whether a higher vessel count and VEGF expression could predict the risk for recurrence. We found that the prognosis of patients was not associated with the microvessel density or VEGF positivity. These findings suggest that the angiogenesis is not simply controlled by the presence of VEGF but may be mediated by other angiogenic factors. Since the angiogenic process is complex, additional studies concerning other angiogenic regulators are warranted.

In conclusion, this study has demonstrated that VEGF is highly expressed in human esophageal carcinomas. Since the VEGF expression is not correlated with the p53 expression, MVD or clinicopathological findings, further studies with other angiogenic molecules are needed to determine the role in esophageal carcinomas.

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