Flt-4–Positive Endothelial Cell Density and Its Clinical Significance in Non–Small Cell Lung Cancer

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ABSTRACT

Purpose: Experimental studies have revealed that fms-like tyrosine kinase (Flt)-4 plays important roles in lymphangiogenesis in malignant tumors, but the clinical significance remains unclear. We assessed Flt-4 expression in tumor cells and in endothelial cells in correlation with clinical outcomes in non–small cell lung cancer (NSCLC).

Experimental Design: A total of 206 consecutive patients with resected pathological stage I–IIIA NSCLC were reviewed. Expression of Flt-4 was examined immunohistochemically, and Flt-4–positive microvessels were quantitatively evaluated (Flt-4–positive endothelial cell density).

Results: There was no significant correlation between Flt-4–positive endothelial cell density and any characteristic of patients including nodal metastases. A significant correlation between Flt-4–positive endothelial cell density and Flt-4 status in tumor cells was documented ($P < 0.001$), but there was no significant difference in the mean Flt-4–positive endothelial cell density according to vascular endothelial growth factor-C or -D status in tumor cells. The 5-year survival rate for higher Flt-4–positive endothelial cell density tumor (56.4%) was significantly lower than that of lower Flt-4–positive endothelial cell density tumor (69.0%, $P = 0.046$); the prognostic significance was enhanced in pIIIA-N2 patients (5-year survival rates, 18.8% for higher Flt-4–positive endothelial cell density tumor, respectively; $P = 0.012$). A multivariate analysis confirmed that higher Flt-4–positive endothelial cell density was a significant and independent prognostic factor ($P = 0.019$). CD34-positive vessel density or Flt-4 status in tumor cells was not a significant prognostic factor.

Conclusions: Flt-4–positive endothelial cell density, not Flt-4 status in tumor cells, was a significant prognostic factor in NSCLC.

INTRODUCTION

Primary lung cancer is the leading cause of cancer death in most industrialized countries, and non–small cell lung cancer (NSCLC) accounts for 70 to 80% of primary lung cancer. Tumor-node-metastasis (TNM) system is generally used in the evaluation of tumor progression, and nodal involvement as well as distant metastasis is the critical factor to determine the prognosis of NSCLC (1–3). In addition, several clinical studies have shown that lymphatic invasion is also a significant prognostic factor in NSCLC (4, 5). Thus, lymphatic spread is a critical factor to determine tumor progression and prognosis of NSCLC, and it should be a new target in the diagnosis and therapy of NSCLC.

Recent advances in molecular biology have revealed mechanism of lymphatic spread in malignant tumors (6). The vascular endothelial growth factor (VEGF) family and their receptor family are a group of growth factors and their receptors to regulate growth of endothelial cells (7). Among the vascular endothelial growth factor receptor (VEGFR) family members, Flt (fms-like tyrosine kinase)-4, also known as VEGFR-3, is a receptor for VEGF-C and VEGF-D (7, 8). Expression of Flt-4 is developmentally regulated: Flt-4 is expressed on all of the embryonic endothelia, but its expression is largely restricted to the lymphatic endothelium in adult tissues (9). Thus, Flt-4 is a marker of lymphatic endothelial cells (10–13), and experimental studies have revealed that activation of Flt-4 induces lymphangiogenesis (14). In fact, expression of Flt-4 is up-regulated in a variety of malignant tumors such as lymphangioma, vascular skin tumors, and Kaposi’s sarcoma, especially in the lymphatic endothelium in metastatic lymph nodes (11, 13, 15). Moreover, in experimental studies, inhibition of Flt-4 signaling can suppress tumor lymphangiogenesis and lymph node metastasis (16, 17). Thus, Flt-4 can be an important diagnostic and therapeutic target for treating a variety of malignant tumors.

In NSCLC, however, only a few clinical studies on Flt-4 expression have been reported (18–22), and clinical significance of Flt-4 expression on tumor cells and/or on endothelial cells remains controversial. Particularly, no study has documented a prognostic value of Flt-4 expression in endothelial cells. Thus, we assessed Flt-4–positive endothelial cell density as well as Flt-4 expression on tumor cells in correlation with clinical outcomes of resected NSCLC.
MATERIALS AND METHODS

Patients and Tissue Preparation. A total of 206 consecutive patients with pathological stage I-III A NSCLC who underwent complete tumor resection without any preoperative therapy at Kyoto University Hospital between January 1985 and December 1990 and whose histologic specimens are available for immunohistochemical staining were retrospectively reviewed (Table 1). Pathological stage was re-evaluated and determined with the present TNM classification as revised in 1997 (1). Histologic type and cell differentiation were determined with the current classification by WHO as revised in 1999 (23). For analyses according to the differentiation of cancer cells, well-differentiated squamous cell carcinoma and adenocarcinoma were classified as well-differentiated tumors and moderately differentiated squamous cell carcinoma and adenocarcinoma as moderately differentiated tumors; large cell carcinoma and poorly differentiated squamous cell carcinoma and adenocarcinoma were classified as poorly differentiated tumors, and the other histologic types were excluded in the analyses. For all of these patients, records of surgery, inpatient medical records, chest X-ray films, whole-body computed tomography films, and bone scanning films were reviewed. Follow-up of postoperative clinical course was conducted by outpatient medical records and by inquiries by telephone or letter.

All of the primary tumor specimens were immediately fixed in 10% (v/v) formalin, and then embedded in paraffin. Serial 4-μm/L sections were prepared from each sample and were served for H&E staining and immunohistochemical staining. Slides were reviewed independently by two investigators (Fengshi Chen and Kazumasa Takenaka) without knowledge of any clinical data. This study was approved by the Ethics Committee (Faculty of Medicine, Kyoto University).

Immunohistochemical Staining. Expression of Flt-4 was evaluated with immunohistochemical staining with a streptavidin-biotinylated horseradish peroxidase detection system (LSAB+ kit/HRP; DAKO, Kyoto, Japan). After retrieval of the antigen with heating in a microwave oven for 15 minutes, sections were incubated overnight at 4°C with an anti-Flt-4 goat polyclonal antibody (AF349, R&D Systems, Inc., Minneapolis, MN) diluted at 1:100. As a chromogen, diaminobenzidine-tetrahydrochloride (0.03%) containing 0.1% hydrogen peroxide was used, and sections were counterstained with hematoxylin. Flt-4 expression on tumor cells was classified based on the staining intensity as follows: score 0 if no staining was detected; score 1 if the staining intensity was weak; score 2 if the intensity was moderate; and score 3 if the intensity was high. Flt-4 status on tumor cells was finally classified to low expression (score, 0 or 1) or high expression (score, 2 or 3). For the negative control, all of the reagents except for the primary antibody were used. Flt-4–positive endothelial cell density was determined following a standard method for determination of microvessel density (24) as follows: the 5 most stained areas (i.e., the so-called hot-spots) within a section were selected for determination of Flt-4–positive endothelial cell density; and microvessels highlighted with the anti-Flt-4 antibody were counted under light microscopy with a 200-fold magnification. Because some vessels, especially lymphatic vessels, may be collapsed, all of the spots highlighted with the Flt-4 antibody that were not identified as tumor cells were counted. The average counts were recorded as the Flt-4–positive endothelial cell density for each case.

Expression of VEGF-D was also evaluated immunohistochemically. An anti-VEGF-D rabbit polyclonal antibody (H-144, Santa Cruz Biotechnology, San Diego, CA) diluted at 1:100 was used as the primary antibody. VEGF-D expression in tumor cells was classified based on the staining intensity as described in immunohistochemical staining evaluation of Flt-4 expression in tumor cells.

Expression of VEGF-A (25) and VEGF-C (26) was evaluated immunohistochemically as described previously; an anti-VEGF–A goat polyclonal antibody (A-20, Santa Cruz Biotechnology) and an anti-VEGF–C goat polyclonal antibody (N-19; Santa Cruz Biotechnology) were used as the primary antibodies. Angiogenesis was also evaluated immunohistochemically as described in previous study (25); an anti-CD34 mouse monoclonal antibody (QBEnd10, diluted at 1:50; DAKO) was used to highlight endothelial cells.

Statistical Methods. Counts were compared by the χ2 test. Continuous data were compared with Student’s t test if

| Table 1 Characteristics of patients and Flt-4–positive endothelial cell density in resected non-small-cell lung cancer |
|-----------------|-----------------|-------|
| No. of patients | Flt-4-positive   | P     |
| (percentage)    | endothelial cell density |       |
| All patients    | 206 (100%)      | 6.10  |
| Age             |                 |       |
| Lower (<64 y)   | 101 (49.0%)     | 6.52  | 0.836 |
| Higher (≥64 y)  | 105 (51.0%)     | 5.69  |
| Gender          |                 |       |
| Male            | 148 (71.8%)     | 6.50  | 0.057 |
| Female          | 58 (28.2%)      | 5.07  |
| Performance status |             |       |
| 0               | 179 (86.9%)     | 6.19  | 0.496 |
| 1–2             | 27 (13.1%)      | 5.49  |
| Histologic type |                 |       |
| Squamous cell   | 75 (36.4%)      | 5.94  | 0.568 * |
| Adenocarcinoma  | 116 (56.3%)     | 6.37  |
| Large cell      | 10 (4.9%)       | 3.32  |
| Others          | 5               |
| Tumor differentiation †  |               |       |
| Poorly          | 48 (23.9%)      | 6.54  |
| Moderately      | 82 (40.8%)      | 5.38  | 0.855 |
| Well            | 71 (35.3%)      | 6.52  |
| Pathologic stage|                 |       |
| Stage I         | 119 (57.8%)     | 6.38  |
| Stage II        | 25 (12.1%)      | 5.28  | 0.469 |
| Stage IIIA      | 62 (30.1%)      | 5.89  |
| Pathologic T-factor |             |       |
| T1              | 70 (34.0%)      | 5.79  |
| T2              | 108 (52.4%)     | 6.22  | 0.532 |
| T3              | 28 (13.6%)      | 6.39  |
| Pathologic N-factor |             |       |
| N0              | 136 (66.0%)     | 6.22  |
| N1              | 27 (13.1%)      | 5.04  | 0.938 |
| N2              | 43 (20.9%)      | 6.38  |

* Comparison between squamous cell carcinoma and adenocarcinoma.
† Other histologic types were excluded.
the sample distribution was normal or with Mann-Whitney $U$ test if the sample distribution was asymmetrical. The post-operative survival rate was analyzed by the Kaplan-Meier method, and the differences in survival rates were assessed by the log-rank test. Multivariate analysis of prognostic factors was done with Cox’s regression model. Differences were considered significant when $P < 0.05$. All of the statistical manipulations were done with the SPSS for Windows software system (SPSS Inc., Chicago, IL).

RESULTS

Flt-4 Expression in Tumor Cells and Endothelial Cells. Flt-4 expression was found in the cytoplasm of tumor cells and was also found on endothelial cells (Fig. 1). The mean Flt-4–positive endothelial cell density and median Flt-4–positive endothelial cell density were 6.10 and 5.00, respectively. There was no significant difference in the mean Flt-4–positive endothelial cell density according to age, gender, performance status, differentiation of cancer cells, pathological stage, pathological T-factor, or pathological N-factor (Table 1). There were 93 patients (45.1%) with low Flt-4 expression in tumor cells and 113 patients (54.9%) with high Flt-4 expression in tumor cells (Fig. 1C). The mean Flt-4–positive endothelial cell density for patients with high tumoral Flt-4 expression was significantly higher than that for those with low tumoral Flt-4 expression (Fig. 2).

Flt-4–Positive Endothelial Cell Density and Vascular Endothelial Growth Factor-A, -C, -D Expression on Tumor Cells. The mean Flt-4–positive endothelial cell density for tumors showing high expression of VEGF-A, -C, or -D on tumor cells (6.76, 6.41, 6.46, respectively) seemed to be slightly higher than those for tumors showing low expression of VEGF-A, -C, or -D (5.73, 5.73, 5.39, respectively), but the difference did not reach a statistical significance ($P = 0.163$, $P = 0.378$, $P = 0.217$, respectively).

Flt-4–Positive Endothelial Cell Density and CD34–Positive Vessel Density. A correlation between Flt-4–positive endothelial cell density and CD34-positive vessel density was assessed. There was no correlation between Flt-4–positive endothelial cell density and CD34-positive vessel density ($P = 0.281$; $r = 0.076$).

Fig. 1 Immunohistochemical staining for Flt-4 in NSCLC. Flt-4–positive microvessels were present at intratumoral lesion (A, ×100; B, ×200). Intense staining of Flt-4 was found in the cytoplasm of tumor cells (C, ×200).
Flt-4–Positive Endothelial Cell Density and Postoperative Survival. To evaluate prognostic significance of Flt-4–positive endothelial cell density, patients were divided into two groups (lower Flt-4–positive endothelial cell density patients and higher Flt-4–positive endothelial cell density patients) according to the median Flt-4–positive endothelial cell density value (5.00) as a cutoff value. For all of the patients, higher Flt-4–positive endothelial cell density (Flt-4–positive endothelial cell density ≥5.00) patients showed a significantly poor postoperative survival than lower Flt-4–positive endothelial cell density (Flt-4–positive endothelial cell density <5.00) patients (5-year survival rates, 56.4% versus 69.0%, respectively; \( P = 0.046 \)); Table 2). Subset analyses according to TNM classification, Flt-4–positive endothelial cell density was a significant prognostic factor in pathological stage IIIA patients, especially in pN2 patients (Table 2).

In contrast to Flt-4 status in endothelial cells (Flt-4-positive endothelial cell density), Flt-4 status in tumor cells provided no prognostic value (5-year survival rates, 62.0% in low tumoral Flt-4 patients and 63.1% in high tumoral Flt-4 patients, respectively; \( P = 0.945 \)). Next, postoperative survival was analyzed according to Flt-4 status in endothelial cells (Flt-4–positive endothelial cell density) in combination with Flt-4 status in tumor cells; 5-year survival rates of low Flt-4–positive endothelial cell density/low-tumoral Flt-4 (\( n = 67 \)), high Flt-4–positive endothelial cell density/low tumoral Flt-4 (\( n = 26 \)), low Flt-4–positive endothelial cell density/high tumoral Flt-4 (\( n = 35 \)), and high Flt-4–positive endothelial cell density/high tumoral Flt-4 patients (\( n = 78 \)) were 64.7%, 54.9%, 77.3%, and 56.8%, respectively, showing the highest 5-year survival in low Flt-4–positive endothelial cell density/high tumoral Flt-4 tumor.

A multivariate analysis confirmed that Flt-4–positive endothelial cell density, not Flt-4 status in tumor cells, was an independent and significant prognostic factor in resected NSCLC (Table 3).

DISCUSSION

In the present study, we showed for the first time that Flt-4–positive endothelial cell density was a significant and independent prognostic factor in resected NSCLC. Many clinical studies on Flt-4 expression in a variety of malignant tumors (27–36) have been conducted, but only one study on breast cancer have revealed that Flt-4–positive endothelial cell density was a significant prognostic factor (29). We identified 5 clinical studies on Flt-4 expression in NSCLC (18–22); only three studies assessed Flt-4 expression on endothelial cells (19, 20, 22), and no study assessed a prognostic significance of Flt-4 expression on endothelial cells. One study showed that Flt-4 status in tumor cells was a significant prognostic factor (21), which was not consistent with results documented in the present study. The discrepancy might come from antibodies used in these studies, i.e., a rabbit polyclonal antibody purchased from Santa Cruz Biotechnology in the previous study (21) and a goat polyclonal antibody (AF349) from R&D Systems in the present study, and there was a vast difference in the percentage of patients with positive tumoral Flt-4 expression (22.5% and 54.9%, respectively). Anyway, only two studies including the present study assessed Flt-4 status in correlation with postoperative survival, which should be confirmed in future large-scale and prospective studies.

Experimental studies have revealed that VEGF-C and Flt-4 might play important roles in the process of nodal metastasis through a paracrine-signaling mechanism between tumor cells and endothelial cells (32, 37). There has been also documented a possible autocrine loop between VEGF-C and Flt-4 in tumor cells (31). In the present study, we for the first time clinically...
showed a significant positive correlation between Flt-4–positive endothelial cell density and Flt-4 expression; the mean Flt-4–positive endothelial cell density for high tumoral Flt-4 tumor (7.87) was significantly higher than that for low tumoral Flt-4 tumor (3.94). However, there were 26 (12.6%) patients with tumor showing high Flt-4–positive endothelial cell density despite low Flt-4 expression on tumor cells, and there also were 35 (17.0%) patients with tumors showing low Flt-4–positive endothelial cell density despite high Flt-4 expression on tumor cells, which may be because of complicated multiple pathways in regulation of Flt-4 expression on endothelial cells and tumor cells. Flt-4 is a receptor for VEGF-C and -D, and some clinical studies documented a positive correlation between Flt-4–positive endothelial cell density and the status of VEGF-C and/or -D in NSCLC (20, 22). In the present study, however, there was no significant correlation between Flt-4–positive endothelial cell density and VEGF-C or -D status, whereas the mean Flt-4–positive endothelial cell density seemed to be slightly higher in tumor showing high VEGF-C and -D expression, which should be also assessed in large-scale studies.

In contrast to previous studies (19, 22), the present study did not reveal a significant correlation between Flt-4–positive endothelial cell density and nodal metastasis. The exact reason for no correlation between Flt-4–positive endothelial cell density and nodal status is unclear, but it might be partly because Flt-4 is not a specific marker of lymphatic endothelial cells; it should be noted that Flt-4 is present on both blood vascular and lymphatic endothelial cells (19, 37, 38), and that we failed to discern blood vessels and lymphatic vessels in the present study. In addition, we documented no correlation between Flt-4–positive endothelial cell density and CD34-microvessel density. CD34 is usually used as a marker of pan-endothelial cells, but we could not discern blood vessels and lymphatic vessels in the present study. In conclusion, Flt-4 was expressed both on tumor cells and on endothelial cells of microvessels in NSCLC, and there proved to be a significant positive correlation between Flt-4–positive endothelial cell density, a count of Flt-4–positive endothelial cells, and Flt-4 expression in tumor cells. Moreover, Flt-4–positive endothelial cell density, not Flt-4 status in tumor cells, was a significant prognostic factor in NSCLC.

ACKNOWLEDGMENTS

We thank Miss Seiko Sakai for helpful assistance in preparation of the manuscript.

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