Frequent Expression of the Vascular Endothelial Growth Factor in Human Non-small-cell Lung Cancers

Makoto Takahama¹, Masahiro Tsutsumi¹, Toshifumi Tsujiuchi¹, Akira Kido¹, Eijiro Okajima¹, Kunimoto Nezu², Takashi Tojo², Keiji Kushibe², Soichiro Kitamura² and Yoichi Konishi¹

¹Department of Oncological Pathology, Cancer Center, and ²Third Department of Surgery, Nara Medical University, Kashihara, Nara, Japan

Background: Angiogenesis is an essential factor for progression and metastases in solid tumors. It has been reported that several angiogenic factors play a role in the regulation of angiogenesis. Vascular endothelial growth factor (VEGF) is one of the most important molecules in angiogenesis. We investigated expressions of VEGF in a series of lung carcinomas with regard to clinicopathological factors.

Method: VEGF expression was investigated by use of immunohistochemical studies and Northern blot analysis, using 155 primary and 26 metastatic lung carcinomas for the immunohistochemical studies and 10 primary and two metastatic lung carcinomas for the Northern blot analysis. All lesions were resected at surgery.

Results: The frequencies for positive VEGF expression were 64 of 74 (86.5%) adenocarcinomas, 38 of 67 (56.7%) squamous cell carcinomas, four of four (100%) large cell carcinomas, two of three (66.7%) adenosquamous carcinomas and one of five (20%) small-cell carcinomas, the degree of positivity generally being greater in well differentiated tumors. The majority of metastatic foci from adenocarcinomas and squamous cell carcinomas at other sites were also positive (76.5 and 66.7%, respectively). VEGF expression did not correlate with clinicopathological factors such as tumor size or pathological stage, but pathological stage I adenocarcinoma cases positive for VEGF demonstrated a shorter disease-free period when followed up for 48 months than those cases expressing VEGF negatively.

Conclusions: The results indicated that VEGF expression was frequently detected in non-small-cell lung cancers and suggested that VEGF might relate to the disease-free period of the patients with early adenocarcinomas.

Key words: vascular endothelial growth factor – immunohistochemistry – non-small-cell lung cancers – Northern blotting

INTRODUCTION

It is well accepted that carcinogenesis is a multistep process involving various gene alterations and expression of various factors and their receptors (1-3). The genetic and molecular mechanisms that control angiogenesis have yet to be fully elucidated, but the vascular endothelial growth factor (VEGF) is known to be important for vessel development (1-5).

It is now clear that angiogenesis can be positively regulated by several angiogenic molecules (4,5) which are essential for the late stage of carcinogenesis and especially the progression phase (1,2). VEGF acts directly on endothelial cells through specific receptors (6-11) and it has been demonstrated that VEGF mRNA and protein are expressed in colon cancer cell lines (12). Further, recent studies have shown a positive correlation between VEGF expression and high metastatic potential of breast (13-15), prostate (16), colon (17,18) and other cancers (19-22). These results indicate that VEGF expression might be an indicator of a poor prognosis but this is still controversial and more studies of different human neoplasms are needed. In the respiratory system, it has been reported that VEGF overexpression can be detected in endothelial cells under hypoxic conditions and in ischemic regions (23). With regard to lung cancers, it has been reported that VEGF mRNAs in secretory forms (VEGF121 and VEGF165) are predominantly transcribed in both resected lung cancer tissues and in human lung cancer cell lines and suggested that VEGF121 expression, which is efficiently secreted and promotes mitogenesis.

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For reprints and all correspondence: Yoichi Konishi, Department of Oncological Pathology, Cancer Center, Nara Medical University, 840 Shijocho, Kashihara, Nara 634, Japan.

E-mail: ykonishi@nmu-gw.cc.naramed-u.ac.jp

Abbreviations: VEGF, vascular endothelial growth factor; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer; RT-PCR, reverse transcription polymerase chain reaction; cDNA, complementary DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase
of endothelial cells, in particular might be a prognostic marker for lung cancer patients after operation (24). In the present investigation, we studied the frequency of VEGF expression in resected lesions in relation to clinicopathological factors.

**MATERIALS AND METHODS**

**PATIENTS AND TISSUE SAMPLES**

Paraffin-embedded tumor specimens taken from lung cancer patients who underwent operations at the Third Department of Surgery, Nara Medical University, from January 1993 to December 1995 were used. The histological types, numbers, gender of patients and pathological stages of the primary lung and metastatic cancers are shown in Table 1. The histology and pathological stage were classified according to General Rules for Clinical and Pathological Recording of Lung Cancers (25). The metastatic lung tumors were from nine rectal adenocarcinomas, four colon adenocarcinomas, three osteosarcomas, two squamous cell carcinomas of the uterus, two transitional cell carcinomas of the urinary bladder, two breast adenocarcinomas, one renal cell carcinoma, one laryngeal squamous cell carcinoma, one thyroid adenocarcinoma and one hepatocellular carcinoma. Two representative serial 5 μm sections from each case were made for the investigations from paraffin-embedded samples. One section was stained with hematoxylin and eosin for histological examination and the other section was stained with VEGF for further immunohistochemical investigation. In addition to these, fresh tissues from five lung adenocarcinomas, three squamous cell carcinomas and two adenosquamous carcinomas, in addition to two metastatic colon adenocarcinomas and the respective non-cancerous tissues adjacent to tumor areas were frozen immediately after surgical removal in liquid nitrogen and stored at −80°C for subsequent Northern blot analysis.

**IMMUNOHISTOCHEMICAL STUDIES**

Deparaffinized sections were incubated with normal horse serum diluted with ‘Iris-buffered saline (TBS) at I:50 for 30 min and then the rabbit polyclonal anti-VEGF antibody (SantaCruz Biotechnology, Santa Cruz, CA, USA) at 1:200 in TBS containing 2% bovine serum albumin for 2 h at room temperature. Visualization of binding was performed with an LSAB 2 kit (DAKO Japan, Kyoto, Japan) and 0.03% 3,3-diaminobenzidine tetrahydrochloride in TBS for 5 min. We defined it as immunohistochemically positive when the cytosolic area of the cells diffusely exhibited the anti-VEGF binding. In any of the other cases, the results were treated as negative. Expression levels of VEGF were characterized as follows: not stained at all, negative (A−); less than 50% of the tumor tissue stained, slightly positive (A+); over 50% of the tumor tissue stained, positive (+); and almost all the tumor tissue stained, strongly positive (++). As the negative control, rabbit non-immune serum was used instead of the primary antibody.

**PREPARATION OF cDNA PROBES**

It has been reported that the rat form 3 VEGF splicing variant is 100% homologous to human VEGF exon 6 (Yakovlev AG, Faden AD, uploaded with RNVEGF3H as an identification number on EMBL, Nucleotide Sequence Data Base). A 0.432 kilobase fragment of its cDNA sequence was therefore obtained by RT-PCR amplification and used as a probe. Total RNAs were derived from 4-hydroxyaminoquinoline 1-oxide-induced osteosarcomas (26) using an RNazol kit (TEL-TFST, Friendwood, TX, USA), reverse transcribed to cDNA with an oligodeoxythymidylic acid primer and then poly-A selected. The cDNA obtained was subjected to PCR to amplify the 0.432 kilobase fragment using a protocol modified from that recommended for the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT, USA) with 20 μl of PCR mixture containing 0.2 mM of each dNTP, 0.25 mM of each primer and 0.5 units of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden) in PCR buffer (50 mM KCl-10 mM Tris, pH 8.0–1.5 mM MgCl2–0.01% gelatin). The primer sequences were 5′-GGT GAA GTI CAT GGACGT CT-3′ for the forward primer and 5′-GTCTGCGGA TCT TGG ACA A-3′ for the reverse primer. The PCR conditions were 1 min at 94°C for preheating; 30 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 57°C, 90 s of extension at 72°C; and 8 min post-extension at 72°C. The amplified segments were verified by electrophoresis in 1% agarose gels with ethidium bromide and extracted with a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). Direct sequencing was then carried out to confirm the human VEGF cDNA sequence and the amplified cDNA was used as a probe.

**Table 1. Histological tumor types and clinical features for patients with lung cancers**

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of primary lung cancer cases</th>
<th>Stage of primary tumors</th>
<th>No. of metastatic lung tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>I</td>
</tr>
<tr>
<td>NSCLC</td>
<td>95</td>
<td>53</td>
<td>86</td>
</tr>
<tr>
<td>Ad</td>
<td>35</td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>Sq</td>
<td>54</td>
<td>13</td>
<td>40</td>
</tr>
<tr>
<td>Large</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Adsq</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>SCLC</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NSCLC, non-small-cell lung carcinoma; SCLC, small-cell lung carcinoma; Ad, adenocarcinoma; Sq, squamous cell carcinoma; Large, large cell carcinoma; Adsq, adenosquamous carcinoma. *Three osteosarcomas, two transitional cell carcinomas and one clear cell carcinoma.
Table 2. VEGF expression a pathological stage

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of positive cases per no. of cases examined (%)</th>
<th>No. of positive cases per no. of cases examined relative to pathological stage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>NSCLC</td>
<td>108/148 (73.0)</td>
<td>67/86 (77.9)</td>
</tr>
<tr>
<td>Ad</td>
<td>64/74 (86.5)</td>
<td>39/42 (92.9)</td>
</tr>
<tr>
<td>Sq</td>
<td>38/67 (56.7)</td>
<td>24/40 (60.0)</td>
</tr>
<tr>
<td>Large</td>
<td>4/4 (100)</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>Adsq</td>
<td>2/3 (66.7)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>SCLC</td>
<td>1/5 (20.0)</td>
<td>1/3 (33.3)</td>
</tr>
</tbody>
</table>

VEGF, vascular endothelial growth factor; NSCLC, non-small-cell lung carcinoma; SCLC, small-cell lung carcinoma; Ad, adenocarcinoma; Sq, squamous cell carcinoma; Large, large cell carcinoma, Adsq, adenosquamous carcinoma. Percentage values are given in parentheses.

NORTHERN BLOT ANALYSIS

Total RNA was extracted from cancerous and non-cancerous lung tissues using an ISOGENE kit (Nippon Gene, Toyama, Japan) and 20 μg aliquots were electrophoresed in 1% agarose-formaldehyde gels, transferred to Biodyne A nylon membranes (Pall Biosupport, East Hills, NY, USA) and hybridized with 1 × 10^6 c.p.m./ml of the 32P-radiolabeled cDNA probe prepared using a DNA Labeling Kit (d-CTP) (Pharmacia Biotech) in a mixture of 50% formamide, 5× standard saline citrate (SSC), 0.1 M phosphate buffer (pH 7.4), 5× Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA and 100 μg/ml salmon testis DNA at 45°C overnight. Blots were washed in 2× SSC and 0.1% SDS at room temperature for 15 min and twice in 2× SSC and 0.1% SDS at 55°C for 15 min; then overnight they were exposed to Hyper-film-MP (Amersham Japan, Tokyo, Japan) using an intensifying screen, at −80°C. After the exposure, the Northern blots were reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe to confirm that similar amounts of RNA were loaded and transferred from each sample.

STATISTICAL ANALYSES

VEGF expression in relation to pathological stage, histological type and cancer cell differentiation was analyzed for significance using Student’s t-test. Disease-free periods were calculated by the Kaplan–Meier method and the data were evaluated with the log rank test.

RESULTS

VEGF EXPRESSION AND PATHOLOGICAL FINDINGS

Data for VEGF expression in comparison with pathological stage of primary lung cancers are shown in Table 2. VEGF expression was seen only in cancer cells and not in non-neoplastic areas immunohistochemically. A total of 148 non-small-cell lung cancers (NSCLCs) including 74 adenocarcinomas, 67 squamous cell carcinomas, four large cell carcinomas and three adenosquamous carcinomas were examined, along with five small-cell lung cancers (SCLCs). VEGF expression of the NSCLC was found in 73.0% with no differences in frequency among the histological types, while only one of the SCLC was positive (20.0%). All positive cases were (+) or (++). The cases of (±) were classified as being negative.

Figure 1. Immunohistochemical staining of a well differentiated adenocarcinoma with the anti-VEGF antibody. The epithelial but not the stromal cells are stained strongly positive.
Table 3. VEGF expression, differentiation and pathological stage of adenocarcinomas and squamous cell carcinomas

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>No. of positive cases per no. of cases examined (%)</th>
<th>No. of positive cases per no. of cases examined relative to pathological stage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Well</td>
<td>30/32 (93.8)*</td>
<td>24/25 (96.0)</td>
</tr>
<tr>
<td>Moderately</td>
<td>25/27 (92.6)*</td>
<td>11/12 (91.7)</td>
</tr>
<tr>
<td>Poorly</td>
<td>9/15 (60.0)†</td>
<td>4/5 (80.0)</td>
</tr>
<tr>
<td>Sq</td>
<td></td>
<td>12/15 (80.0)</td>
</tr>
<tr>
<td>Well</td>
<td>14/29 (48.3)†</td>
<td>9/17 (52.9)</td>
</tr>
<tr>
<td>Moderately</td>
<td>12/23 (52.2)†</td>
<td>8/15 (53.3)</td>
</tr>
</tbody>
</table>

VEGF, vascular endothelial growth factor; Ad, adenocarcinoma; Sq, squamous cell carcinoma. *p < 0.05 as compared with †; and ‡, p < 0.05 as compared with §. Percentage values are given in parentheses.

VEGF EXPRESSION IN METASTATIC LUNG TUMORS

The majority of the metastatic foci from adenocarcinomas showed positive staining (Fig. 3) and the frequency was 13 out of 17 (76.5%) cases. Negative cases were two rectal adenocarcinomas, one colon adenocarcinoma and one thyroid adenocarcinoma. For squamous cell carcinomas, the frequency was two out of three (66.7%) cases. Metastatic foci from transitional cell carcinomas of the urinary bladder and clear cell carcinomas of the kidney proved negative, while three cases of metastatic osteosarcomas were positive where present, the staining intensity of the metastatic tumors being (+) or (++) positive appearances.

NORTHERN BLOT ANALYSIS

In recent cases, paired samples of tumor and non-cancerous lung tissue from 12 patients were subjected to Northern blot analysis, using cDNA probe for VEGF. Representative results of Northern blot analysis are shown in Fig. 4. VEGF mRNA was expressed in five primary adenocarcinomas, three squamous cell carcinomas, two adenosquamous carcinomas and two metastatic carcinomas from the colon compared with non-cancerous lung tissue in each. All cases which expressed VEGF mRNA in Northern blot analysis were stained with anti-VEGF antibody positively or strongly positively, respectively.

VEGF EXPRESSION AND THE DISEASE-FREE PERIOD FOR PATHOLOGICAL STAGE I CASES WITH NSCLC

Results for the analysis of data for VEGF expression and the disease-free period after 48 months follow-up for pathological stage I cases are shown in Fig. 5. With the adenocarcinomas, the disease-free period was shorter for VEGF positive than for negative cases (p < 0.05). However, with the squamous cell carcinomas, no difference between the VEGF positive and negative cases was found.

DISCUSSION

VEGF was first detected as an important angiogenic factor inducing microvascular hyperpermeability (27,28) and extra-
Expression of VEGF in NSCLCs

Figure 4. Northern blot analysis for the vascular endothelial growth factor (VEGF) mRNA in representative cases. Cases 1–3 are primary squamous cell carcinomas, cases 4–6 are primary adenocarcinomas and case 7 is a metastatic adenocarcinoma from the colon. mRNA overexpression is evident for all tumors as compared with non-cancerous lung tissue. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; N, non-cancerous tissue; T, tumor tissue.

![Northern blot analysis for VEGF mRNA expression](image)

Figure 5. Vascular endothelial growth factor (VEGF) α-small-cell lung cancers. With the adenocarcinoma cases, the disease-free period was longer for the VEGF negative than for the VEGF positive group (*, p < 0.05, log rank test). On the other hand, no correlation was observed between VEGF expression and the disease-free period in the squamous cell carcinoma cases.

![Graph showing VEGF expression and disease-free period](image)

vascation of various proteins related to metastases, which also acts as an endothelial cell mitogen (8,29). In recent studies, it has been shown that various malignant tumors exhibit up-regulation of VEGF expression (13-22) and that this is associated with a high probability of metastasis and a resultant poor prognosis (12,13).

In the present study, VEGF expression was investigated in a large series of resected primary and metastatic lung carcinomas. NSCLCs demonstrated greater positivity than SCLCs. Our results also indicate that overexpression of VEGF is more frequent in primary adenocarcinomas than in squamous cell carcinomas. Ohta et al. (24) reported that there was no positive correlation between expression of the VEGF receptor, flt-1 and VEGF itself, but further studies are needed to clarify this and the role of another VEGF receptor, flk-1 (KDR), in NSCLCs. Rak et al. (30) reported that the expression of a mutant ras oncogene is associated with marked up-regulation of VEGF mRNA and found functional protein to be secreted by human and rodent tumor cell lines expressing mutant K-ras and H-ras oncogenes, respectively. Further, they showed that genetic disruption of mutant K-ras allele in human colon carcinoma cells was associated with a reduction in VEGF activity. The present findings of a high frequency of VEGF expression might therefore suggest a background of ras mutations, although generally low mutation frequency of this oncogene in NSCLCs could not account for all cases (31,32). Up-regulation of VEGF was detected in almost all well differentiated carcinomas in the present series but was unexpectedly less common in poorly differentiated carcinomas.

VEGF expression did not correlate with the pathological stage in our cases, but follow-up of stage I adenocarcinoma patients for 48 months after operation revealed a significantly longer disease-free period for patients with negative compared with those with positive tumors. Our results therefore are in line with the report of Ohta et al. (24) that survival of VEGF121 mRNA high-expressing cases is less than that for the low-expressing group of stage I primary lung cancer patients.

Recently, the potential importance of VEGF for diagnosis and treatment has been emphasized. Salven et al. (33) reported patients with locoregional or disseminated cancer often to have elevated serum VEGF levels. Yuan et al. (34) described time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-VEGF antibody. The present finding of frequent expression of VEGF in NSCLCs suggests that further research in this area is warranted with a view to future applications in lung cancer clinics.

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