Prognostic significance of VEGF-C expression in correlation with COX-2, lymphatic microvessel density, and clinicopathologic characteristics in human non-small cell lung cancer

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Introduction

Non-small cell lung cancer (NSCLC) is one of the most frequent and lethal malignancies worldwide, and the 5-year survival rate is only ~20%. A recent research has shown an increasing trend of NSCLC mortality in China in the past 20 years, especially in urban areas and among aged people. To date, the treatment outcome of this common malignancy is still not satisfactory. One major difficulty in the diagnosis and treatment of NSCLC is that only a few prognostic indicators can predict its clinical behavior. Recently, lymphangiogenesis has been related to metastasis and poor prognosis in NSCLC [1].

Lymphangiogenesis, the process leading to the formation of lymphatic vessels, plays a central role in local tumor growth, and development of distant metastasis [2]. The degree of lymphatic microvessel density (LMVD) intratumoral by immunohistochemistry is thought to influence tumor metastasis and consequently prognosis in various human cancers, including NSCLC [3]. The formation of tumor lymphatic microvessels is dependent on the production of lymphangiogenic growth factors by...
tumor cells. The formation of tumor microvessels is stimulated by lymphangiogenic growth factors, including vessel endothelial growth factor C (VEGF-C), platelet-derived growth factor, and fibroblast growth factor 2 [4–6]. The expression of these factors correlates with tumor lymphangiogenesis, tumor progression, and poor prognosis. Among the known lymphangiogenic factors, VEGF-C has emerged as the central regulator of the lymphangiogenic process in cancer. The biological functions of VEGF-C include selective promotion of mitosis of endothelial cells, stimulation of their proliferation, and lymphangiogenesis [7].

Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin (PG) metabolism. COX has two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues and is thought to be involved in maintaining physiological function. COX-2 is frequently undetectable in normal tissue, but can be induced in response to growth factors, tumor promoters, hormones, and cytokines, thus contributing to the synthesis of PG in inflamed and malignant tissues [8,9]. Over-expression of COX-2 is detectable in various solid malignancies including NSCLC and is thought to be involved in the critical steps in carcinogenesis, as well as a regulator of tumor lymphangiogenesis [10,11]. However, the potential mechanism remains unclear.

To analyze the relationships between LMVD, COX-2, and VEGF-C expression, clinicopathologic parameters, and survival time of patients in NSCLC, 65 specimens were evaluated for COX-2, VEGF-C, and VEGFR-3 polyclonal antibody by immunohistochemical staining of LMVD.

Materials and Methods

Patients and specimens
Sixty-five patients (40 men and 25 women, medium age 58 years) with NSCLC undergone radical lobectomy or pneumonectomy in the Department of Cardiothoracic Surgery, the Second Affiliated Hospital of Soochow University, from October, 2005 to October, 2006, were enrolled in this study. The eligibility criteria were: histologically proven NSCLC, no previous systemic chemotherapy or radiotherapy before operation, and well-documented clinical data. The mean follow-up time was 34 months (from 16 days to 60 months).

All tissues were surgically resected. Each specimen was fixed in 10% phosphate-buffered formalin immediately after resection, embedded in paraffin, and cut into 4 μm-thick sections for immunohistochemical study and routine histological examination.

Immunohistochemistry
The sections were dewaxed and rehydrated by sequential immersion in xylene and graded ethanol and water. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide methanol. Antigen-retrieval treatment was performed in a full pressure cooker for 10–15 min to obtain optimal results. After washed in phosphate-buffered saline (PBS) and exposed to 10% normal horse serum for 10 min to reduce non-specific binding, the sections were incubated with the primary antibody, which reacts specifically with VEGF-C (polyclonal, L2702, 1:50 dilution, overnight at 4°C; Santa Cruz Biotechnology, Santa Cruz, CA, USA), COX-2 (polyclonal, J1602, 1:50 dilution, overnight at 4°C; Santa Cruz Biotechnology), and VEGFR-3 (polyclonal, ZA-0111, overnight at 4°C; Santa Cruz Biotechnology). All sections were incubated with biotinylated IgG for 30 min and then with streptavidin–peroxidase reagent for 20 min. Finally, the sections were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxide for 5 min, counterstained with Mayer’s hematoxylin, and mounted. PBS was substituted for primary antibody as the negative control.

Evaluation of immunostaining and lymphatic microvessel counting
To evaluate COX-2 and VEGF-C expression, a score was established corresponding to the sum of the A group: percentage of positive cells (0, 0% immunopositive cells; 1, 25–50% positive cells; 2, 50–75% positive cells; and 3, >75% positive cells), and the B group: staining intensity (0, negative; 1, weak; 2, mild; 3, moderate; and 4, high). The sum of A þ B reached a maximum score at 6. Scores between 0 and 2 were regarded as negative (–), between 3 and 4 as weak (+), and between 5 and 6 as strongly positive (++), respectively.

Intratumoral lymphatic microvessels were highlighted by immunostaining with VEGFR-3 polyclonal antibody. Any single brownly stained cell or cluster of endothelial cells clearly separated from adjacent lymphatic microvessels, tumor cells, and other connective tissue elements were considered as vessels. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. The stained sections were screened at ×100 magnification under a light microscope to identify the five regions of the section with the highest LMVD. Lymphatic microvessel were counted in the five
regions at ×200 magnification, and the average number of lymphatic microvessels was recorded. Two observers did the counting, and the mean value was used for analysis.

**Statistical analysis**

Data were analyzed by SPSS version 10.0 for windows. The correlations between expression of COX-2, VEGF-C, and clinicopathologic parameters were assessed by the χ² test or the Spearman rank test. The Kaplan–Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. The COX proportional hazard model was used for multivariate analysis of prognostic factors. A value of $P < 0.05$ was considered statistically significant. All $P$ values are represented as two-sided.

**Results**

**Correlations of COX-2 and VEGF-C expression with clinicopathologic parameters**

The associations between COX-2 and VEGF-C expressions and the clinicopathologic parameters are shown in Table 1. There was a significant association between COX-2, VEGF-C expression, and lymph node metastasis, but not with patient gender, age, tumor size, and tumor, nodes, metastasis classification stage.

**Correlation between LMVD and clinicopathologic features**

The LMVD for 65 tumor specimens ranged from 12 to 57 with a mean LMVD of 28.17 ± 7.26. When a mean LMVD value of 28 was chosen as the cut-off point for discrimination of the 65 patients, 35 patients were categorized as low LMVD and 30 as high LMVD. The correlation between LMVD and clinicopathologic features is shown in Table 1. High LMVD was significantly associated with lymph node metastasis ($P = 0.004$).

**Multivariate survival analysis**

Multivariate survival analysis showed that LMVD value and lymph node metastasis were independent prognostic factors (Table 2). No other variables, including COX-2 and VEGF-C expression, were retained in the model or affected the magnitude of the hazard ratios of variables in the final model. Kaplan–Meier curves for patients’ survival are shown in Fig. 1. A significant difference in the overall survival rate was found between patients according to the LMVD value ($P < 0.001$, comparison between low and high LMVD).

**Expression of COX-2 and VEGF-C in NSCLC tissues**

Immunoreactivity of both COX-2 and VEGF-C proteins was found in the cytoplasm of tumor cells (Fig. 2).

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>n</th>
<th>LMVD</th>
<th>COX-2</th>
<th>VEGF-C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.000</td>
<td></td>
<td>0.129</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>15</td>
<td>38</td>
<td>14</td>
</tr>
<tr>
<td>Male</td>
<td>53</td>
<td>17</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td>1.000</td>
<td></td>
<td>0.794</td>
</tr>
<tr>
<td>≤60</td>
<td>30</td>
<td>7</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>&gt;60</td>
<td>35</td>
<td>9</td>
<td>26</td>
<td>9</td>
</tr>
<tr>
<td>Size of tumor (cm)</td>
<td></td>
<td>0.217</td>
<td></td>
<td>0.623</td>
</tr>
<tr>
<td>≤5</td>
<td>29</td>
<td>8</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>&gt;5</td>
<td>36</td>
<td>8</td>
<td>28</td>
<td>9</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td>0.325</td>
<td></td>
<td>0.312</td>
</tr>
<tr>
<td>I &amp; II</td>
<td>57</td>
<td>15</td>
<td>42</td>
<td>8</td>
</tr>
<tr>
<td>III &amp; IV</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td>0.004</td>
<td></td>
<td>0.043</td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>2</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>31</td>
<td>14</td>
<td>17</td>
<td>14</td>
</tr>
</tbody>
</table>

LMVD, lymphatic microvessel density; COX-2, cyclooxygenase-2; VEGF-C, vessel endothelial growth factor C; TNM, tumor, nodes, metastasis classification; −, negative; + to ++, positive to strong positive.
However, occasionally normal cells in adjacent tissues of cancer showed little staining. Among the 65 NSCLC samples, the positive rates of COX-2 and VEGF-C expression were 75.4% and 76.9%, significantly higher than those in the adjacent tissues.

**Correlation between COX-2 or VEGF-C expression and LMVD**

The correlation between COX-2 or VEGF-C expression and LMVD is summarized in Table 3. The mean LMVD value of COX-2- or VEGF-C-positive tumors was higher than that of COX-2- or VEGF-C-negative tumors.

**Association between COX-2 and VEGF-C expression**

A significant correlation was found between the expression levels of COX-2 and VEGF-C (Table 4, $r = 0.385$, $P < 0.001$).

**Discussion**

Jackson [4] initiated a new field of research about tumor lymphangiogenesis in 2001 and found that several factors take part in the process of lymphangiogenesis. Tumor lymphangiogenesis is now believed to be one of

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**Table 2 Multivariate survival analysis of overall survival in NSCLC**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>Standard error (SE)</th>
<th>Odds ratio (95% CI)</th>
<th>$P$</th>
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<tbody>
<tr>
<td>LMVD</td>
<td>1.057</td>
<td>0.501</td>
<td>0.617–0.631</td>
<td>0.044</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>1.146</td>
<td>0.439</td>
<td>1.314–6.548</td>
<td>0.019</td>
</tr>
</tbody>
</table>

LMVD, lymphatic microvessel density.

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**Fig. 1 Kaplan–Meier survival curve correlating disease-specific survival with high or low LMVD**

**Fig. 2 Immunohistochemical stainings of COX-2 (A), VEGF-C (B), and lymphatic microvessel (C and D) in tissue sections obtained from NSCLC**

Both COX-2 and VEGF-C were mainly expressed in the cytoplasm of cancer cells (dark staining; A and B, ×400). Lymphatic microvessels were detected in NSCLC tissues by immunostaining for VEGFR-3 antibody (dark staining; C and D, ×200).

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the most crucial steps in tumor growth and metastasis. Moreover, tumor lymphangiogenesis which can be quantified by measurement of LMVD is a significant negative prognostic factor [12]. In our study, when a mean LMVD value was chosen as the cut-off point for discrimination of the study patients, high LMVD was significantly associated with lymph node metastasis and poor survival. Multivariate survival analysis showed that LMVD value and lymph node metastasis were independent prognostic factors for NSCLC patients. Tumor lymphangiogenesis is controlled by a balance between lymphangiogenic regulators involved in multiple pathways that result in endothelial proliferation, differentiation, and organization into a functional network of lymphatic vessel channels. Among the reported lymphangiogenic factors, VEGF-C is increased in various human tumors, often correlating with higher LMVD [13–15]. In our study, VEGF-C had over-expression in NSCLC tissues. We found that the mean LMVD value of VEGF-C-positive tumors was significantly higher than that of VEGF-C-negative tumors, suggesting that VEGF-C may facilitate tumor progression by promoting tumor lymphangiogenesis.

The expression of COX-2 mRNA and protein is elevated in various human malignancies, which may play a critical role in the development of cancer [16–18]. Our study showed that the positive rate of COX-2 expression in human NSCLC was significantly higher than that in the matched normal NSCLC tissue. COX-2 expression was associated with the degree of tumor cell differentiation and depth of invasion, but not with survival. These results suggest that over-expression of COX-2 plays an important role in the development of human NSCLC. The contributions of COX-2 to tumor lymphangiogenesis include: increasing expression of VEGF-C, producing of prostaglandin E (PGE) 2 and prostaglandin I (PGI) 2 that can directly stimulate endothelial cell migration and growth factor-induced lymphangiogenesis, and inhibiting endothelial cell apoptosis by stimulation of Bcl-2 or Akt activation [19,20].

In our study, COX-2 expression was significantly associated with that of VEGF-C. The mean LMVD value of COX-2- or VEGF-C-positive tumors was higher than that of COX-2- or VEGF-C-negative tumors, which is in agreement with previous reports [21,22]. These data strongly suggest that COX-2 and VEGF-C may be partly responsible for the important process of lymphangiogenesis in the development of human NSCLC, and COX-2 plays the main role in VEGF-C-stimulated lymphangiogenesis. VEGF-C combines VEGFR-3, then stimulates lymphangiogenesis by some pathways in the regulation of lymphatic vessel growth such as MEK/Erk1,2, Akt1, PYK2, NF-κB, JNK1/2, and so on [23]. However, there still exist some other pathways, which also participate in COX-2-induced lymphangiogenesis.

In conclusion, high LMVD is significantly associated with lymph node metastasis and poor survival. LMVD value and lymph node metastasis are independent prognostic factors for NSCLC patients. Expression of COX-2 and VEGF-C is closely correlated to lymph node metastasis and leads to increased lymphangiogenesis, which may be the mechanisms underlying the contribution of COX-2 to the lymph metastasis of NSCLC. VEGF-C might play a main role in the COX-2 lymphangiogenic pathway. According to the significance of lymphangiogenic pathway in tumor metastasis, we may intercept the lymph node metastasis by target gene therapy. A study reported that it can block the VEGF-C/VEGFR-3 lymphangiogenic pathway with resoluble VEGFR-3 fragment mediated by adenovirus or VEGF-C small interference RNA [24]. Some micromolecule inhibitor of VEGFR-3 kinase activity such as BAY 43-9006 (sorafenib), PTK787/ZK222584 (vatalanib), and CEP-7055 are
also in clinical trial [25]. All these studies show that we may have an important therapeutic benefit in the control of NSCLC lymph node metastasis by inhibiting lymphangiogenesis with COX-2 and VEGF-C target gene therapy.

References


