

# A Comparative Analysis of Tumor Angiogenesis and the Clinical Features of Pleomorphic Carcinoma and Adenocarcinoma of the Lung

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Pleomorphic carcinoma (PC) is a rare tumor that usually has an aggressive clinical course and a poor prognosis. We investigated the hypothesis that PC overexpresses angiogenic factors. Furthermore, we compared rates of expression of angiogenic factors in PC and adenocarcinoma (AD) of the lung. We collected each 11 cases of PC and AD in which the patients underwent either a lung resection or at autopsy. We immunohistochemically examined the expression of angiogenic factors. Vascular endothelial growth factor (VEGF) and basic-fibroblast growth factor were expressed in many cases of both PC and AD, and there were no significant differences between the two groups. However, the expression of cyclooxygenase (COX)-2 and hypoxia inducible factor-1 $\alpha$ , and the microvessel density, were significantly greater in PC than in AD. These findings suggest that PC was associated with increased angiogenesis and that the tumor tissue was in a hypoxic state.

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Key words: Pleomorphic carcinoma, angiogenesis, immunohistochemistry, vascular endothelial cell growth factor, cyclooxygenase-2, hypoxia inducible factor-1 $\alpha$

## INTRODUCTION

Pleomorphic carcinoma (PC) of the lung is a

comparatively rare tumor, which constitutes approximately 0.1-0.4% of all lung cancers, and are usually high-grade, aggressive and associated with a poor prognosis [1-3]. Histologically, PCs exhibit characteristic tumor components, i.e., malignant epithelial and homologous sarcomatoid spindle/giant cells. The WHO classification defines pleomorphic carcinoma as “poorly differentiated non-small-cell carcinoma, namely a squamous cell carcinoma, adenocarcinoma or large cell carcinoma, containing spindle cells and giant cells, and the pleomorphic component should comprise at least 10% of the neoplasm” [4]. This subtype consists of large cell lung carcinomas that exhibit a spindle cell component, or both of large cell and spindle cell carcinoma, of 10% or more. In the clinic, PC tends to be negative for tumor markers and difficult to diagnose cytologically or histologically using bronchoscopy and biopsy. Therefore, the clinical features and behavior of PC have been unclear.

On the other hand, it is widely accepted that angiogenesis is essential for both tumor progression and metastasis, as tumors cannot grow beyond 2 mm from vessels [5]. It is also known that many malignancies, including lung cancers, overexpress angiogenic factors [6-12]. One of the major regulators of the neovascularization process is vascular endothelial growth factor (VEGF), which was originally discovered as a vascular permeability factor [13]. Several studies have demonstrated that an increase in microvessel density (MVD) was found to be closely associated with the expression of VEGF, and that MVD and VEGF expression had prognostic value for predicting the metastasis of various malignant solid tumors [6-10].

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While there are a few case reports of an association between a bleeding tendency in tumor tissues and abnormalities of angiogenesis, there have been few studies that have made a comprehensive evaluation of angiogenesis in PC. In addition, distant metastasis is frequently observed in cases of PC, and such metastases have tended to expand into various regions such as the small intestine, peritoneum, skin, and lymph nodes [14]. Moreover, inflammation and hypoxic state are also widely recognized to play an important role in tumor angiogenesis through the upregulation of cyclooxygenase (COX)-2 and hypoxia inducible factor (HIF)-1 $\alpha$ , respectively [15-17]. COX-2 and HIF-1 $\alpha$  are important pro-angiogenic factors that activate the transcription of VEGF. Understanding the clinical features and mechanisms of angiogenesis in PC could lead to the development of new treatments and a more precise diagnosis for non-small-cell lung cancer (NSCLC) patients. Because the majority of the predominant epithelial component of PC is adenocarcinoma (AD) [1, 18-19], we performed a retrospective comparison of the expression of tumor angiogenic factors such as VEGF, basic-fibroblast growth factor (b-FGF), COX-2 and HIF-1 $\alpha$ , and analyzed patient backgrounds and the disease prognosis in PC and AD patients.

## MATERIALS AND METHODS

### *Patients and Samples*

We used formalin-fixed, paraffin-embedded blocks from 11 cases of PC and 11 cases of AD that had undergone surgical resection or autopsy between June 2005 and November 2009, which were retrieved from Shimane University Hospital and Shimane Prefectural Central Hospital. All of the PC cases were diagnosed strictly according to the WHO classification by an experienced pathologist. AD cases were selected that matched the PC cases in terms of patient characteristics such as gender, smoking history and clinical stage. All available clinical information was obtained from the clinical records and reports of the referring physicians. Surgical or pathological staging was performed according to the TNM classification of the International Union Against Cancer criteria.

### *Immunohistochemical Staining*

Formalin-fixed, paraffin-embedded tumor samples were cut into 4 $\mu$ m-thick sections. Tissue samples were routinely deparaffinized in xylene and rehydrated through a series of graded alcohols. Antigen retrieval were carried out using an appropriate heat-induced procedure, and samples were immunostained using a BioGenex AutoStainer i6000<sup>®</sup> automated staining system, with antibodies against VEGF (A-20, Santa Cruz Biotech., CA, USA, dilution 1:200), b-FGF (bFGF88, BioGenex, CA, USA, pre-diluted), COX-2 (CX-294, Dako, CA, USA, pre-diluted), CD31 (JC70A, Dako, CA, USA, pre-diluted) and HIF-1 $\alpha$  (H1 $\alpha$ 67, Santa Cruz, CA, dilution 1:50). After buffer washes, samples were visualized using a Dako Envision System using horseradish peroxidase (Dako, CA). Finally, the sections were lightly counterstained with Mayer's haematoxylin, dehydrated through alcohols of increasing concentration, and coverslipped using permount. The positive control slides for VEGF, b-FGF and HIF-1 $\alpha$  were also stained at same procedure.

### *Evaluation of Immunostaining*

All of the immunostained sections were evaluated by three authors (Y.T, A.S and T.I) who had no knowledge of the clinical diagnosis. The staining of VEGF, b-FGF and HIF-1 $\alpha$  was assessed semiquantitatively according to three indices: (1) the percentage of area stained (<10%, 25%, 50%, 75% and 100%), (2) intensity of staining (none, 0; weak, +1; moderate, +2; and strong, +3), and (3) the final score (product of area and intensity, named the histological (H) score). H score was shown as a total of (1) and (2). In the evaluation of the staining for COX-2, the reactions in vascular endothelial cells, which were present in all specimens, were used as internal built-in controls, and cases with tumor cells showing significantly more intense staining than the internal control cells were recorded as positive. The intensity of staining was graded as follows: weak, +1; moderate, +2; and strong, +3. The intensity score was shown as H score.

### *Assessment of Tumor Vascular Density*

Vascularity was measured by the average of the

MVD. The MVD as measured by assessing CD31 immunostaining according to the international consensus report [20]. The tumor areas with the three highest densities of distinctly highlighted “hot spots” were selected by light microscopy under low power magnification. The count of CD31 positive vessels was determined in three separate fields in each of these areas, and a 200x field (0.785 mm<sup>2</sup> per field) was used for all evaluations. The counting score was represented by the sum of the vessel counts of nine of these fields. The MVD was expressed as the number of microvessels/field.

### **Statistical Analysis**

The Statcel2 statistical software program (OMS, Japan) was used for all analyses. Student’s *t*-test or the Mann Whitney U test was applied to assess the immunoreactivity and patient characteristics. The Kaplan-Meier method was used to analyze patient survival. A *p*-value < 0.05 was considered to indicate statistical significance.

## **RESULTS**

### **Immunohistochemical Staining of VEGF, b-FGF, COX-2 and HIF-1 $\alpha$**

Immunohistochemical staining was performed on tumor samples from 22 patients, with 11 cases of PC and 11 cases of AD, who underwent surgical resection or autopsy. A cytoplasmic staining pattern was found for VEGF, b-FGF and COX-2. On the other hand, HIF-1 $\alpha$  exhibited a nuclear staining pattern. Fig. 1 shows an example of the staining for tumor angiogenesis factors in a representative sample. To determine whether there was a statistically significant difference between PC and AD, we assessed the H score and calculated *p*-values using Student’s *t*-test or the Mann Whitney U test. The results of the immunohistochemical analysis are summarized in Fig. 2.

Many cases of PC and AD were positive for both VEGF and b-FGF expression, so the H scores of both groups were high, and there were no significant differences between the two groups. However, in the PC samples, the expression of COX-2 (*p*=0.005) and HIF-1 $\alpha$  (*p*=0.03) were significantly higher than in AD. There were no significant cor-

relations between the VEGF, b-FGF, COX-2 and HIF-1 $\alpha$  expression status and patient prognostic factors, such as the pathological or clinical stage, existence of symptoms, smoking habits or tumor markers (data not shown).

### **Analysis of MVD**

The tumor MVD was identified by immunohistochemical reaction to an anti-CD31 antibody. The mean of all MVDs was 11.5 microvessels/field, and the range was 1.1-28.7 microvessels/field. The MVD of PC was significantly higher than that of AD (13.8 versus 9.1, *p*=0.005) (Fig. 3). However, there were no significant correlations between the MVD and other patient prognostic factors, such as the pathological or clinical stage, existence of symptoms, smoking habits and tumor markers (data not shown).

### **Clinical Findings**

As summarized in Table 1, a total of 22 patients were included in the study. In the PC group, which included 9 males and 2 females ranging in age from 54 to 83 years (median age; 74 years), ten (90.9%) of them were smokers, and 6 of them were heavy smokers (over BI 600). No history of symptoms was available for 3 patients, but the other 8 patients experienced one or more of the following symptoms: hemoptysis (25%), cough (25%), chest or upper back pain (25%), and dyspnea (25%). The locations of the primary tumors were in the right upper lobe (45.5%), the left upper lobe (18.2%), and other locations (36.4%). The clinical or pathological staging was stage IA in 2 cases, stage IB in 3 cases, stage IIIA in 2 cases and stage IV in 4 cases. A comparison of the clinical findings with those of 11 cases of AD indicated that there were no statistically significant differences between the two groups in terms of these parameters. In the PC and AD groups, three and seven patients were still alive at the time of the most recent follow-up, and their average follow-up time was 14 months. The median survival time of the PC group was 5.9 months. However, the median survival of AD had not yet reached 50%. Therefore, a longer follow-up will be necessary (Fig. 4).

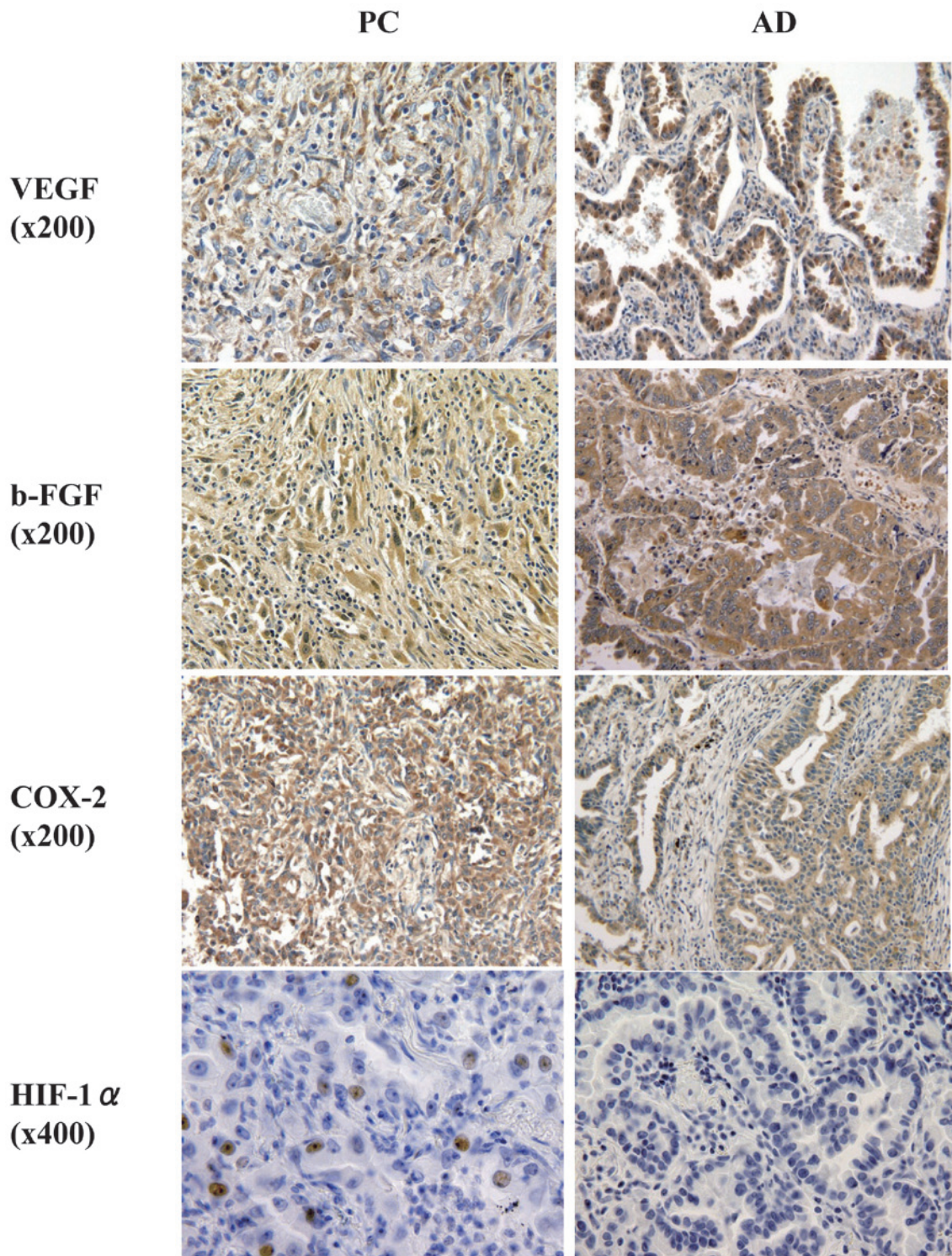


Fig. 1. Immunohistochemical staining for angiogenic factors in pleomorphic carcinoma and adenocarcinoma. VEGF, b-FGF and COX-2 were localized to cytoplasm, and HIF-1 $\alpha$  was localized to nuclei. PC: pleomorphic carcinoma; AD, adenocarcinoma; VEGF, vascular endothelial growth factor; b-FGF, basic-fibroblast growth factor; COX-2, cyclooxygenase-2; HIF-1 $\alpha$ , hypoxia inducible factor-1 $\alpha$ .

Table 1. The demographic and clinical characteristics of patients with pleomorphic carcinoma and adenocarcinoma

Characteristic		PC (N=11)	AD (N=11)	<i>p</i> value <sup>a</sup>
Gender (%)	Male	9 (81.8)	10 (90.9)	
	Female	2 (18.2)	1 (9.1)	
Pathological stage (%)	I	5 (45.4)	5 (45.4)	
	II	2 (18.2)	2 (18.2)	
	III	4 (36.4)	4 (36.4)	
Smoking status (%)	Yes	10 (90.9)	10 (90.9)	
	No	1 (9.1)	1 (9.1)	
(Average B.I.)		736	1149	0.14
Age, median (range)		74	69	0.31
		54-83	50-81	
Site of primary tumor (%)	Upper	7 (63.6)	3 (27.3)	
	Middle	1 (9.1)	-	0.054
	Lower	3 (27.3)	8 (72.7)	
Positive markers (%) (CEA, CYFRA, SLX, SCC)	Yes	4 (36.4)	6 (54.5)	0.40
	No	7 (63.6)	5 (45.5)	
Symptoms at the time of diagnosis (%)	Yes	8 (72.7)	6 (54.5)	0.28
	No	3 (27.3)	5 (45.5)	
Hemoptysis or bloody pleural effusion		3 (27.3)	1 (9.1)	0.28

PC indicates pleomorphic carcinoma; AD, adenocarcinoma; B.I., Brinkman index; CEA, carcinoembryonic antigen; CYFRA, cytokeratin 19 fragment; SLX, sialyl lewis x-i antigen; SCC, squamous cell carcinoma antigen.

<sup>a</sup> Student's *t*-test or Mann Whitney U-test

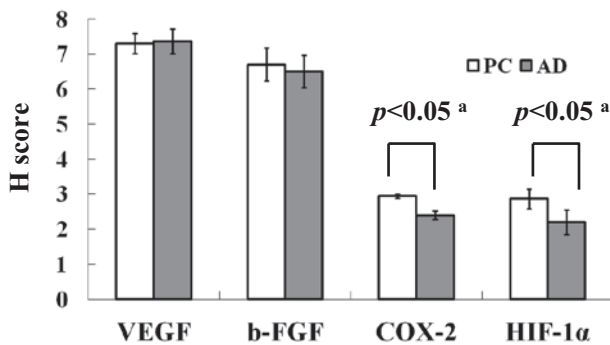


Fig. 2. The results of immunohistochemical staining. Many cases of both PC and AD were positive for VEGF and b-FGF, there were no significantly differences between the groups. The expression of COX-2 and HIF-1α in PC was significantly higher than that of AD. PC: pleomorphic carcinoma; AD, adenocarcinoma; VEGF, vascular endothelial growth factor, b-FGF; basic-fibroblast growth factor, COX-2, cyclooxygenase-2; HIF-1α; hypoxia inducible factor-1α.

<sup>a</sup> Student's *t*-test or the Mann Whitney U test

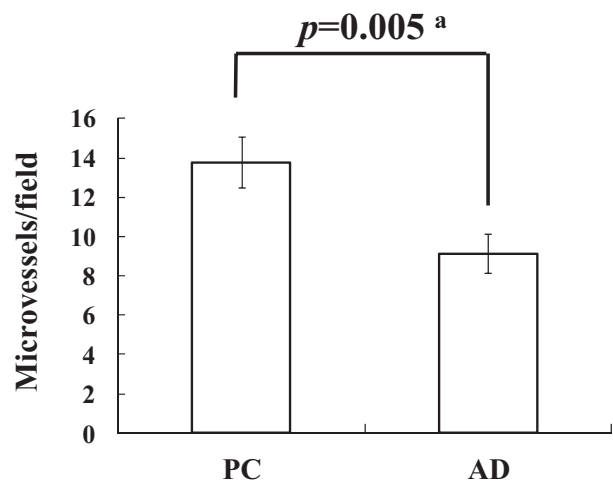


Fig. 3. The microvessel density of PC was significantly greater than that of AD. PC: pleomorphic carcinoma; AD, adenocarcinoma.

<sup>a</sup> Student's *t*-test.

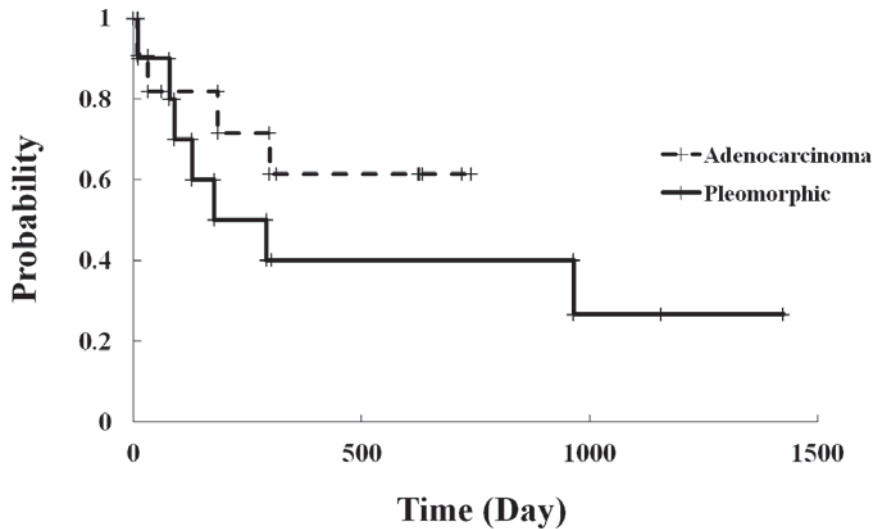


Fig. 4. The Kaplan-Meier curve is shown for the overall survival of pleomorphic carcinoma and adenocarcinoma patients.

## DISCUSSION

The present study is the first report that focused specifically on the angiogenesis of PC and its relation to the clinical features of the patients and tumors. We have shown that various angiogenic factors are upregulated in PC, and that the MVD is significantly greater in PC than in AD. Furthermore, because COX-2 and HIF-1 $\alpha$  are overexpressed in PC, the results of this study suggest that there is prolonged inflammation and hypoxic state of the PC tumor cells, and that this strongly affects angiogenesis through upregulation of VEGF.

Angiogenesis is the formation of new blood vessels from pre-existing vessels, and is essential for tumor growth, development and metastasis [21-22]. Several studies have found a correlation between the MVD and the expression of angiogenic factors, especially VEGF, as well as with a poor prognosis and the incidence of metastasis in NSCLC [23-24]. The MVD is thought to closely reflect the intratumoral angiogenesis and to be a prognostic factor for NSCLC. The present study shows that the MVD of PC is significantly higher than that of AD, suggesting that the poor prognosis and frequency of distant metastasis in PC is likely influenced by the increased angiogenesis.

Although the VEGF family plays roles in the physiological and pathological regulation of angiogenesis in NSCLC [25], we found no association

between the MVD and VEGF expression, which is in contrast to other recent studies [12, 26]. This discrepancy is probably attributable to the differences in the expression of other angiogenic and anti-angiogenic factors. Tumor angiogenesis is a multistep process controlled by various factors, and is thought to be due to the balance between pro-angiogenic factors such as interleukin-8 [27] and reduction of anti-angiogenic factors such as angiotensin and thrombospondin-1 [28]. The specific angiogenic factors and pathways activated in PC will need to be investigated in further clinical studies evaluating the expression of various factors and the MVD in a larger number of PC patients.

The present study indicates that COX-2 and HIF-1 $\alpha$  are strongly upregulated in PC. The prognostic significance of elevated COX-2 expression in lung cancers has been evaluated in several previous studies [29-30]. COX-2-mediated production of PGE<sub>2</sub> and IL-1 $\alpha$  appears to play an important role in tumor angiogenesis through the induction of VEGF [13]. Some reports have indicated the possibility that VEGF expression may be regulated by the COX-2 pathway [31-32]. On the other hand, intratumoral hypoxia and genetic alterations can lead to HIF-1 $\alpha$  overexpression. This phenomenon is associated with a poor prognosis, resistance to chemotherapy and radiotherapy, and increasing potential for invasion, metastasis and patient mortality [16, 33-

34]. Many researchers have reviewed the importance of intratumoral hypoxia in the regulation of tumor angiogenesis [16-17]. Recent preclinical studies have provided evidence indicating that inhibition of HIF-1 $\alpha$  activity has marked effects on tumor growth [35-36]. Efforts are underway to identify inhibitors of HIF-1 $\alpha$  and to test their efficacy as anticancer therapeutics [37-39].

In the clinic, PC predominantly occurs in male heavy smokers, with an average age at diagnosis of 60 years, and a very poor prognosis. Moreover, several unique clinical features of PC have been demonstrated in past studies, including that the majority of tumors arise in the right upper lobe, that they are frequently large peripheral tumors, with chest wall invasion and distant metastasis to unusual organs [1, 3, 18]. Our observations are compatible with these studies, in that our patients with PC frequently presented with symptoms such as hemoptysis, chest pain and bloody pleural effusion at the time of diagnosis, and the site of the primary tumor was most often the right upper lobe. In addition, the results of the present study also showed that PC has a poor prognosis. This study demonstrated that tumor angiogenesis provides significant prognostic information about the clinical outcome of PC patients. However, because the population of the present study was small, a comprehensive pathological and clinical study with a large number of cases is needed to provide an improved understanding of the clinical characteristics and the behavior of PC of the lung.

In conclusion, the results of the current study showed that the expression of VEGF, COX-2 and HIF-1 $\alpha$  was associated with angiogenesis in PC. These findings suggest that prolonged inflammation and the hypoxic state of tumor cells is strongly related to angiogenesis, and that angiogenesis is related to the progression of PC. We speculate that there is a correlation between the upregulation of angiogenesis and the clinical features in PC. The use of anti-angiogenic agents, such as an anti-VEGF antibody or VEGF receptor blocker, and COX-2 inhibitors, may therefore provide more successful treatments for PC patients.

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