Comparative Analysis of Tumor Angiogenesis and Clinical Features of 55 Cases of Pleomorphic Carcinoma and Adenocarcinoma of the Lung

YUKARI TSUBATA[1], AKIHISA SUTANI[1], TAMIO OKIMOTO[1], ISAO MURAKAMI[2], RYOSUKE USUDA[3], TSUNEO OKUMICHI[4], SEI-ICHI KAKEGAWA[5], KEN-ICHI TOGASHI[6], SHINJI KOSAKA[7], YOSHINORI YAMASHITA[8], KOJI KISHIMOTO[9], TAKASHIGE KURAKI[1] and TAKESHI ISOBE[1]

Departments of [1]Internal Medicine-Division of Medical Oncology and Respiratory Medicine and [9]Thoracic Surgery, Shimane University Faculty of Medicine, Izumo, Shimane, Japan;
[2]Department of Respiratory Medicine, Higashi-hiroshima Medical Center, Hiroshima, Japan;
[3]Department of Thoracic Surgery, Showa University Fujigaoka Hospital, Yokohama, Kanagawa, Japan;
[4]Department of Surgery, Yoshijima Hospital, Hiroshima, Japan;
[5]Department of Thoracic Surgery, Nishigunma National Hospital, Shibukawa, Gunma, Japan;
[6]Department of Thoracic Surgery, Japanese Red Cross Nagaoka Hospital, Nagaoka, Niigata, Japan;
[7]Department of Thoracic Surgery, Shimane Prefectural Central Hospital, Izumo, Shimane, Japan;
[8]Department of Thoracic Surgery, Kure Medical Center/Chugoku Cancer Center, Kure, Hiroshima, Japan

Abstract. *Background/Aim: Pleomorphic carcinoma (PC) of the lung is a rare tumor that usually has an aggressive clinical course and a poor prognosis. Clinical and pathological features remain unclear. The aim of this study was to determine whether tumor angiogenesis of PC is up-regulated compared to that in adenocarcinoma (AD). Materials and Methods: We collected 55 cases of PC and AD in which the patients had undergone either lung resection or autopsy and immunohistochemically examined the expression of vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1α and microvessel density (MVD) in tissue specimens. Results: VEGF was expressed in many cases of both PC and AD with no significant differences between the groups. In contrast, the expression of HIF-1α and MVD were significantly greater in PC than AD. Median survival time of the PC group was 14.7 months and significantly shorter than that of the AD group. Conclusion: MVD and expression of HIF-1α are associated with angiogenesis in PC and confer a poorer prognosis. Tumor angiogenesis provides significant prognostic information regarding clinical outcome in patients with PC.*

Correspondence to: Professor Takeshi Isobe, Department of Internal Medicine, Division of Medical Oncology and Respiratory Medicine, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane, 693-8501 Japan. Tel: +81 853202580, Fax: +81 853202580, e-mail: isobeti@med.shimane-u.ac.jp

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Pleomorphic carcinoma (PC) of the lung is histologically defined as either non-small-cell carcinoma combined with neoplastic spindle and/or giant cells or a carcinoma that consists of spindle cells and giant cells only (1). The pleomorphic component should comprise of at least 10% of the neoplasm. Although the confirmation of diagnostic criteria has seen an increase in diagnoses, PC of the lung is nevertheless a rare tumor that accounts for only about 0.1-0.4% of all lung cancers. Most cases are high-grade, aggressive and associated with a poor prognosis (2-5), highlighting the need to elucidate the clinical features and behavior of this carcinoma. Common findings in patients with PC are hemoptysis and bloody pleural effusion (5), which are thought to be related to abnormalities of angiogenesis. However, the angiogenic status of this condition remains poorly understood.

It is widely accepted that angiogenesis is essential for both tumor progression and metastasis, as tumors cannot grow beyond 0.2 mm from vessels (6). It is also known that many malignancies, including lung cancers, over-express angiogenic factors (7-13). One major regulator of the neovascularization process is vascular endothelial growth factor (VEGF), which was originally discovered as a vascular permeability factor (14). Several studies have demonstrated that the expression of VEGF is closely associated with an increase in microvessel density (MVD) and VEGF expression have prognostic value in predicting the metastasis of various malignant solid tumors (7-11).

Previously, we showed that the expression of tumor angiogenic factors in PC was significantly associated with a poorer prognosis (5). Herein, because the majority of the predominant epithelial component of PC is adenocarcinoma (AD) (2, 15-16), we compared expression of tumor angiogenic factors, including VEGF, cyclooxygenase-2 (COX-2) and hypoxia-inducible factor (HIF)-1α and analyzed patient background and prognosis in 110 patients with PC or AD.

Materials and Methods

Patients and samples. We used formalin-fixed paraffin-embedded (FFPE) blocks from 55 cases of PC and 55 of AD that had undergone surgical resection or autopsy between August 2001 and May 2010. Samples were those in which PC had been initially diagnosed in all cases and were retrieved from the Higashi Hiroshima Medical Center, Showa University Fujigaoka Hospital, Yoshijima Hospital, Nishigunma National Hospital, Japanese Red Cross Nagaoka Hospital, Shimane Prefectural Central Hospital, Kure Medical Center and Shimane University Hospital. Diagnosis of all cases as PC for the present study was confirmed strictly in accordance with the WHO classification by an experienced pathologist. AD cases that matched the PC cases in terms of patient characteristics, such as gender, smoking history and clinical stage, were selected. All available clinical information was obtained from clinical records and reports of the attending physicians and e-mails or letters were sent to contributors for additional information. These records were reviewed for patient age, gender, smoking status, presenting symptoms, location of the primary tumor, follow-up tumor markers, stage at the time of diagnosis and the duration of follow-up. Surgical, pathological or clinical staging was performed in accordance with the TNM classification of the International Union against Cancer criteria.

Immunohistochemical staining. FFPE samples were cut into 4-μm-thick sections. Tissue samples were routinely de-paraffinized in xylene and rehydrated through a graded series of alcohols. Antigen retrieval were carried out using an appropriate heat-induced procedure and samples were immunostained using a BioGenex AutoStainer i6000® automated staining system, with antibodies against VEGF (A-20, dilution 1:200; Santa Cruz Biotech., Dallas, Texas, USA), COX-2 (CX-294, pre-diluted; Dako Japan, Tokyo, Japan), CD31 (JC70A, pre-diluted; Dako Japan, Tokyo, Japan) and HIF-1α (H1α67, dilution 1:50; Santa Cruz Biotech., Dallas, Texas, USA). After buffer washes, samples were visualized using a Dako Envision System (Dako Japan, Tokyo, Japan). Finally, the sections were lightly counterstained with Mayer's hematoxylin.

Evaluation of immunostaining. All immunostained sections were independently evaluated by four authors (Y.T., T.O., A.S. and T.I.). When evaluations differed by more than two points, scores were discussed again among all members. Staining of VEGF and HIF-1α was assessed semi-quantitatively according to three indices: (i) percentage of area stained (<10%, 25%, 50%, 75% and 100%), (ii) intensity of staining (none, 0; weak, +1; moderate, +2; and strong, +3) and (iii) final score (product of the area and intensity, called the histological (H) score). In the evaluation of staining for COX-2, reactions in vascular endothelial cells, which were present in all specimens, were used as internal 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.

Vascularity was measured by the average of the MVD, which was measured by assessing CD31 immunostaining according to an international consensus report (17). 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 using a 200x field (0.785 mm^2 per field) for all evaluations. Count score was represented by the sum of the vessel counts of nine of these fields. MVD was expressed as the number of microvessels/field.

Statistical analysis. Immunoreactivity and patients' characteristics were assessed using the Student's *t*-test or the Mann Whitney *U*-test. Median survival time (MST) was calculated using the Kaplan-Meier method and significance was determined using the log-rank test. Multivariate analyses were conducted using the Cox proportional regression model. All analyses were conducted using the SPSS software program version 19 (IBM, Okinawa, Japan).

Results

Clinical findings. A total of 110 patients were included in the study (Table I). AD cases that matched the PC cases in terms of patient characteristics, such as gender, smoking history and clinical stage, were selected. About 85% of subjects in both groups were male and about 80% were current or former smokers. Although smoking history was closely matched between the two groups, the Brinkman index (BI) was significantly lower in the PC group than in the AD group.

With regard to pathological or surgical stage, distributions of stage IA to IV cases were approximately equal in both groups, while advanced-stage IIIB and IV cases represented about 35% in both. Median age in the PC and AD groups was 69 (range=39-85) and 72 years (range=36-89), respectively. There was a significant difference between groups in primary site, with the right upper lobe (RUL) being the predominant primary site in the PC group (47.3%, p=0.003). On inclusion of the left upper lobe (LUL), 69.1% of PC developed from an upper lobe. In contrast, the left lower lobe (LLL) was a less frequent primary site in the PC than the AD group (p=0.048). In the PC group, the history of symptoms at diagnosis was not available for 21 patients but the other 34 patients experienced one or more of the following symptoms: hemoptysis (32.4%), cough (26.5%), chest or upper back pain (14.7%) and dyspnea (5.9%). Having a history of symptoms at diagnosis was significantly more frequent in the PC group than the AD group (p=0.007).

The median follow-up time was also similar between the groups; at 19 months in the PC group and 20 months in the AD group. Median survival time was 14.7 months in the PC group and significantly shorter than that in the AD group (p=0.001) (Figure 1). Median survival in the AD group had not yet reached 50% and, thus, longer follow-up is necessary.

Table I. *Demographic and clinical characteristics of patients with pleomorphic carcinoma and adenocarcinoma.*

Characteristics		PC (N=55)	AD (N=55)	*p*-Value*
Age, median (range)		69	72	0.13
		39-85	36-89	
Gender (%)	Male	48 (87.3)	45 (81.8)	-
	Female	7 (12.7)	10 (18.2)	
Pathological stage (%)	IA	6 (10.9)	6 (10.9)	-
	IB	10 (18.2)	11 (20.0)	
	IIA	0	2 (3.6)	
	IIB	12 (21.8)	6 (10.9)	
	IIIA	9 (16.4)	10 (18.2)	
	IIIB	8 (14.5)	10 (18.2)	
	IV	10 (18.2)	10 (18.2)	
Smoking status (%)	Yes	48 (87.3)	42 (76.4)	-
Average B.I.	No	7 (12.7)	13 (23.6)	
		729	1135	0.09
Site of primary tumor (%)	RUL	26 (47.3)	12 (21.8)	0.003
	RML	3 (5.5)	3 (5.5)	-
	RLL	7 (12.7)	14 (25.5)	0.11
	LUL	12 (21.8)	13 (23.6)	-
	LLL	5 (9.1)	13 (23.6)	0.048
	unknown	2 (3.6)	0	-
Positive markers (%)	Yes	23 (41.8)	31 (56.4)	0.13
(CEA, CYFRA, SLX, SCC)	No	32 (58.2)	24 (43.6)	
Symptoms at the diagnostic time (%)	Yes	34 (61.8)	20 (36.4)	0.008
	No	21 (38.2)	35 (63.6)	

PC: 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; RUL: right upper lobe; RML; right middle lobe; RLL: right lower robe; LUL: left upper lobe; LLL: left lower lobe. *Student's *t*-test or Mann Whitney *U*-test.

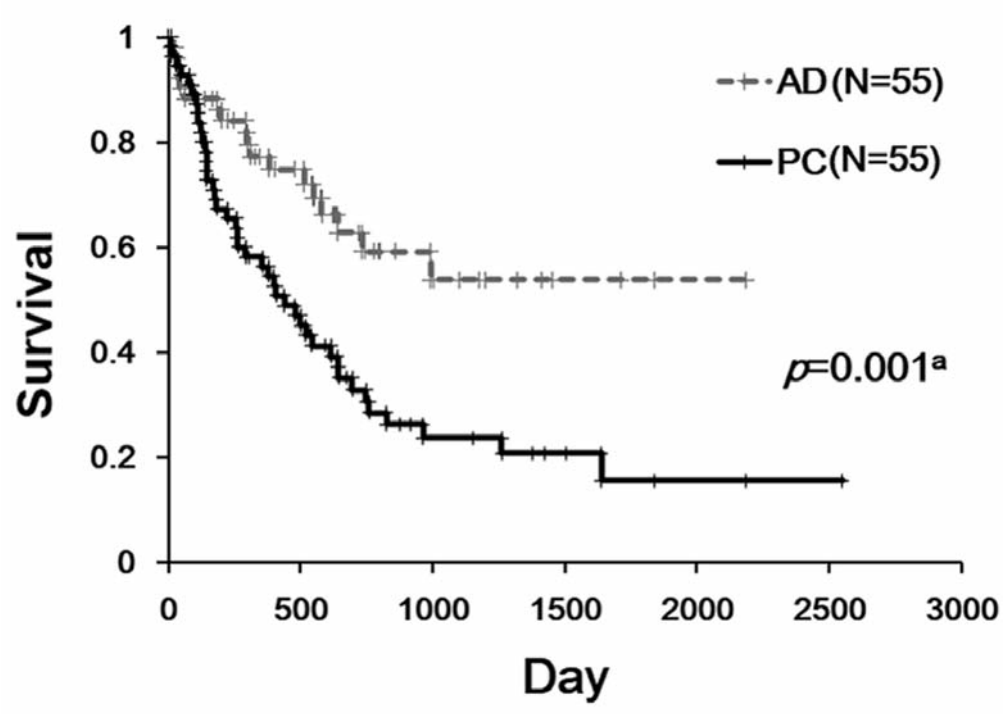

Figure 1. *Kaplan-Meier curve for overall survival of patients with pleomorphic carcinoma and adenocarcinoma. PC, Pleomorphic carcinoma; AD, adenocarcinoma, [a]Log-rank test.*

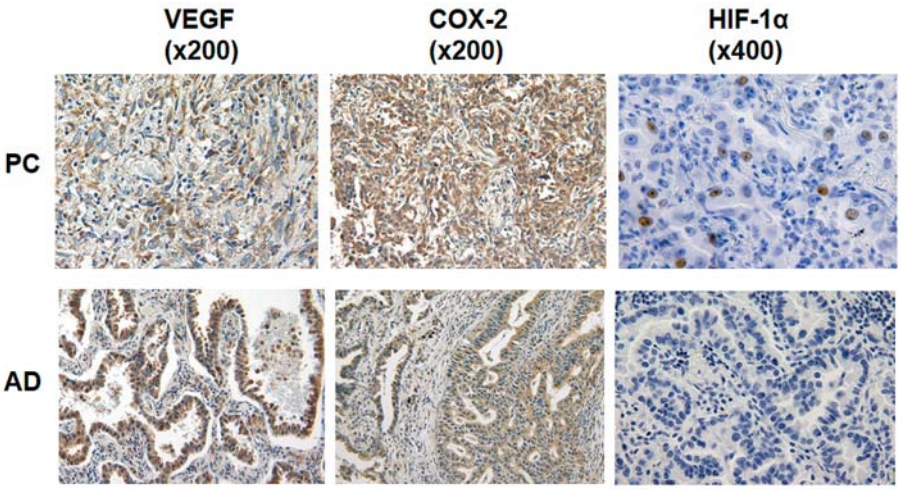

Figure 2. *Immunohistochemical staining for angiogenic factors in pleomorphic carcinoma and adenocarcinoma. PC, Pleomorphic carcinoma; AD, adenocarcinoma; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; HIF-1α; hypoxia-inducible factor-1α.*

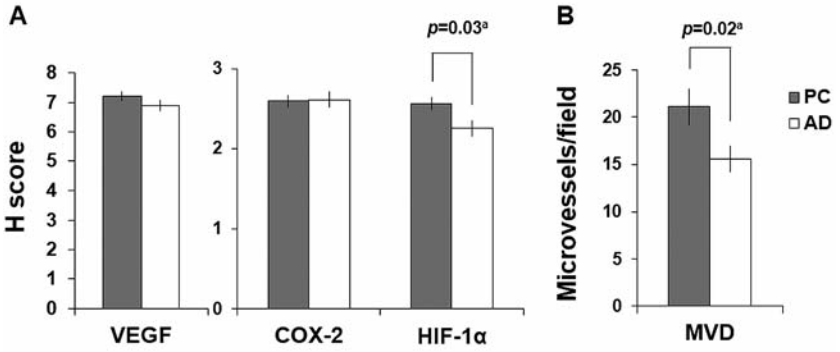

Figure 3. *Results of immunohistochemical staining. A, Expression of VEGF, COX-2 and HIF-1α in PC and AD. B, Microvessel density was significantly higher in PC than in AD. PC, Pleomorphic carcinoma; AD, adenocarcinoma; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; HIF-1α, hypoxia-inducible factor-1α; MVD, microvessel density. [a]Student's t-test or Mann Whitney U-test.*

Immunohistochemical staining of VEGF, COX-2 and HIF-1α. VEGF and COX-2 exhibited a cytoplasmic staining pattern, whereas HIF-1α exhibited a nuclear staining pattern. Figure 2 shows an example of staining for tumor angiogenesis factors in a representative sample. To determine the presence of a statistically significant difference between PC and AD, we assessed the H score and calculated *p*-values using the Student's *t*-test or the Mann Whitney *U*-test. Results of the immunohistochemical analysis are summarized in Figure 3.

Many cases of PC and AD were positive for VEGF expression and the H scores of both groups were accordingly high with no significant difference between them. In contrast, expression of HIF-1α was significantly higher in the PC group than in the AD group (p=0.03). No significant correlations were seen between VEGF, COX-2 and HIF-1α expression status and patient prognostic factors, including pathological or clinical stage, existence of symptoms at diagnosis, smoking habit or tumor markers (data not shown).

Analysis of MVD. Tumor MVD was identified by immunohistochemical reaction to an anti-CD31 antibody. MVD of the PC groups was significantly higher than that of the AD groups (22.8 microvessels/field versus 15.6 microvessels/field, p=0.02) (Figure 3). However, no significant correlations were seen between MVD and other patient prognostic factors, such as pathological or clinical stage, existence of symptoms at diagnosis, smoking habit and tumor markers (data not shown).

Discussion

The present study is the first report to specifically focus on angiogenesis in PC and its relation to the clinical features of patients and tumors. We have shown that various angiogenic factors are up-regulated in PC and MVD and expression of HIF-1α are significantly greater in PC than in AD. The results of the present study suggest that the hypoxic state of tumor cells correlates with the up-regulation of angiogenesis and that this is one prognostic factor of PC.

Angiogenesis, formation of new blood vessels from pre-existing vessels, is essential for tumor growth, development and metastasis (18, 19). It is frequently assessed in tumors by the evaluation of MVD. Several studies have found a correlation between MVD and the expression of angiogenic factors, particularly VEGF, as well as with a poor prognosis and the incidence of metastasis in NSCLC (20-21). MVD is thought to closely reflect intratumoral angiogenesis and be a prognostic factor for NSCLC. The present study shows that the MVD of PC is significantly higher than that of AD, which may suggest that the poor prognosis and frequency of distant metastasis in PC is likely influenced by this increased angiogenesis.

Although the VEGF family plays roles in the physiological and pathological regulation of angiogenesis in NSCLC (22), we found no association between MVD and VEGF expression, in contrast to other recent studies (13, 23). This discrepancy is probably attributable to differences between the present and these previous studies in the expression of other angiogenic and anti-angiogenic factors. Tumor angiogenesis is a multi-step process controlled by various factors and is thought to represent a balance between pro-angiogenic factors, such as interleukin-8 (24), and a reduction of anti-angiogenic factors, such as angiostatin and thrombospondin-1 (25). Identification of the specific angiogenic factors and pathways activated in PC requires further clinical investigation.

We showed that the expression of HIF-1α in PC is significantly higher than that of AD. Many researchers have reviewed the importance of intratumoral hypoxia in the regulation of tumor angiogenesis (26-29). Intratumoral hypoxia and genetic alterations can lead to HIF-1α over-expression; HIF-1α is an important pro-angiogenic factor that activates the transcription of VEGF. Several analyses have shown that HIF-1α over-expression is associated with a worse prognosis and resistance to chemotherapy (26, 28). Although approximately 65% of PC patients in our study had early-stage disease, prognosis was nevertheless markedly poor. One reason for this might be resistance to adjuvant or initial chemotherapy and an increased potential for invasion and metastasis.

Clinically, PC occurs predominantly in male heavy smokers with an average age at diagnosis of 60 years and a very poor prognosis. Our investigation of 75 cases of PC has revealed several unique clinical features, including that the majority of tumors arise in the right upper lobe and that patients frequently experience symptoms, such as hemoptysis at the time of diagnosis, chest pain and bloody pleural effusion (5). Moreover, we previously showed that the presence of symptoms at diagnosis and surgical and pathological stage were independent prognostic factors of poor patient survival. Here, although we selected AD cases that matched the PC cases with regard to patients' characteristics, such as gender, smoking history and clinical stage, the MST of PC was significantly shorter than that of AD. Thus, PC should be recognized as a high-grade malignancy. Our data may indicate that the aggressiveness of this tumor is due to its strong up-regulation of angiogenesis, which is in turn likely associated with the poor prognosis and frequency of distant metastasis.

This study demonstrated that tumor angiogenesis provides significant prognostic information about clinical outcome in patients with PC. Furthermore, agents inhibiting angiogenesis may represent a new treatment strategy for this condition. Several agents are already available, including bevacizumab (anti-human VEGF-A monoclonal antibody) and everolimus (mTOR inhibitor). Given the small population of PC patients, prospective clinical trials of new treatments will need to be conducted on a global scale.

Several limitations of the study worth mentioning. First, because of the heterogeneity of PC, it is not clear whether it is an element of NSCLC or spindle and/or giant cells that induce the up-regulation of angiogenesis. To clarify this issue, we are now planning the use of a micro-dissection method to distinguish NSCLC or spindle and/or giant cells. Second, we did not evaluate other factors, which may affect cell growth or metastasis. Finally, our study did not analyze the chemotherapy regimens of patients and, therefore, the relationship between the up-regulation of HIF-1α and resistance to chemotherapy remains unclear. To clarify these issues, we are planning to carry out an additional study that will include a comprehensive gene mutation analysis of PC.

In conclusion, our study indicates that MVD and the expression of HIF-1α are associated with angiogenesis in PC and points to a poorer prognosis. Given the current lack of understanding of this condition, our findings may suggest that chemotherapy with anti-angiogenic agents, such as an anti-VEGF antibody or VEGF receptor blocker and HIF-1α inhibitors, represents, at present, the best available management for PC.

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