Original Article
Vasculogenic mimicry and cancer stem-like cell markers are associated with poor prognosis of non-small cell lung cancer

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Abstract: Vasculogenic mimicry (VM) is a tumor microcirculation pattern, and it is related to cancer stem-like cell (CSC). However, the clinical significance and prognosis of CSC markers, and VM is not still clear in non-small cell lung cancer (NSCLC). In this study, immunohistochemistry and CD31/PAS double staining were performed to investigate the relationship between epithelial cell adhesion molecule [EpCAM], nestin, CD44, CD34, and VM in 205 patients with NSCLC. The positive expressions of VM, EpCAM, nestin, CD44, and CD34 were 13.66%, 36.59%, 18.05%, 57.07% and 2.93%, respectively. VM was more often detected in large cell lung cancer, poor differentiation, advanced stage, or distant metastasis samples. The VM-positive NSCLC specimens also showed increased expression of nestin, CD44, and CD34, as well as higher MVD, compared with the VM-negative samples. CD34-MVD was significantly different among the three subtypes and was related to VM and nestin. EpCAM was significantly down-expressed in squamous cell carcinoma, non-distant metastasis and good-prognosis specimens. The positive expression of CD44 increased in squamous cell carcinoma, advanced stage, central lung, and male samples. Minimal correlation was found among nestin, CD34, and clinicopathological parameters, except prognosis. The positive expression of both CD34 and nestin showed marked increase in patients who had shorter survival periods. In a conclusion, higher VM, CSCs, and MVD were associated with more aggressive NSCLC. VM, MVD, and some CSC markers were independent unfavorable prognostic factors of NSCLC. CSCs possibly participate in neovascularization in NSCLC.

Keywords: Vasculogenic mimicry, microvessel density, cancer stem-like cell, non-small cell lung cancer

Introduction

Primary lung cancer is the leading cause of cancer mortality in the world [1]. Non-small cell lung cancer (NSCLC), a subtype of lung cancer, accounts for about 80% of all lung cancer cases [1, 2]. Although recent advancements in early tumor detection, surgical resection, radio chemotherapy, targeted therapy, and anti-angiogenesis therapy have improved the survival rate of NSCLC patients, the high mortality related to NSCLC remains a daunting challenge [3]. Vasculogenic mimicry (VM) theory and cancer stem-like cell (CSC) theory have contributed significantly to tumor research.

In 1999, Maniotis detected special blood vessels in highly aggressive uveal melanomas and named them VM [4]. Two kinds of distinct vessels reportedly contribute to the blood supply of malignant tumors: (1) VM, which refers to vessels exclusively surrounded with tumor cells, and (2) endothelium vessel, which refers to vessels covered by endothelial cells [4]. Numerous studies have reported that VM has been found in various malignant tumors, such as gastric adenocarcinoma, tongue carcinoma, hepatocellular carcinoma, colorectal cancer and renal cell carcinoma [5-8]. In view of the potential of VM theory to improve anti-angiogenesis therapy for malignant tumors, close attention has been given to the molecular mechanism of VM, which is further explained by CSC theory.

CSC theory has explained the biological heterogeneity of solid tumors [2]. Similar to normal

adult stem cells, CSCs possess the capacity for self-renewal and pluripotency, so they are capable of differentiating into different cell types [3, 9, 10]. CSCs also exhibit high tumorigenic ability when implanted into immunodeficient mice [4]. This theory may explain a question why pernicious tumors are difficult to heal. In the process of studying its mechanism, CSCs have been proven to be capable of differentiating into vascular endothelial cells, which contribute to tumor neovascularization [11, 12]. Hitherto, several CSC markers have been identified in numerous cancers. However, to our knowledge, special CSC markers for lung cancer have seldom been examined, and only limited reports study the clinical value and relationship between CSC markers and VM in NSCLC. The present study aims to explain the role of common CSC markers and VM in NSCLC and to demonstrate the relationship between CSC markers and VM in NSCLC.

Materials and methods

Patients

For this study, the subjects recruited included 205 Chinese patients who had undergone surgical resection for lung cancer in Tianjin Medical University Cancer Institute and Hospital from October 1990 to November 2010. All human studies were approved by the Tianjin Medical University Ethics Committee. Information on the aims, methods, and other details regarding the medical research were given to the patients involved. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The average age of the patients at the time of diagnosis was 59.1 years (30 years to 88 years). The 205 NSCLC samples were composed of 79 cases of squamous cell carcinoma, 75 cases of adenocarcinoma, and 51 cases of large cell lung cancer. The diagnoses of these samples were confirmed by two pathologists according to the standard of classification [2, 13]. The data for the clinicopathological parameters were collected from the patients' clinical records and pathological reports. Time of death, final follow-up examination, and diagnosis of metastasis were recorded from the date of surgery. This study was approved by the Ethical Committee of Tianjin Medical University prior to its initiation.

Immunohistochemistry and CD31/periodic acid Schiff (PAS) double staining

This assay was conducted following the methods described by Sun *et al.* [14, 15] and Zhang *et al.* [5]. The tissues were 10% formalin fixed, paraffin embedded, and cut into 4 μm thickness. All slides were then deparaffinized in xylene and dehydrated with descending-grade alcohol. Endogenous peroxidase activity was quenched by brooding in methanol containing 3% hydrogen peroxide for 30 min at room temperature. After blocking with goat serum (serum to Oct 3/4 primary antibody was 5% fetal bovine serum) for 20 min at room temperature, the slides were incubated with a primary antibody overnight at 4°C in a humidified box. Next morning, the sections were incubated with a homologous secondary antibody for 1 h at room temperature and stained with freshly dispensed diaminobenzedine solution (DAB) for observation under a microscope. The sections were all washed three times in phosphate-buffered saline (PBS) (pH 7.2) before each step, except for the procedure of serum blocking to incubation with the primary antibody. The slides were then counterstained with hematoxylin, dehydrated with ascending-grade ethanol, air dried, cleared with xylene, and mounted. The CD31/PAS double staining was performed between DAB staining and hematoxylin counterstaining, and the sections were still incubated with 1% heptaiodic acid for 15 min and Schiff reagent for observation under a microscope at 37°C. In this process, distilled water instead of PBS was used for washing.

In our study, the primary antibodies to CD44 and Oct3/4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies to nestin, epithelial cell adhesion molecule (EpCAM), CD31, and CD34 were from Zhongshan Biotechnology (Zhongshan Chemical Co., Beijing). The antibody to CD133 was from Miltenyi Biotechnology (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Positive control and negative control were performed for each batch. For the negative control, PBS was used instead of the primary antibody. For the positive control, a foregone positively expressed tissue section was used. The results were evaluated following the method described by Bittner *et al.* [16]. The intensity and the percentage of the positive cells were both mea-
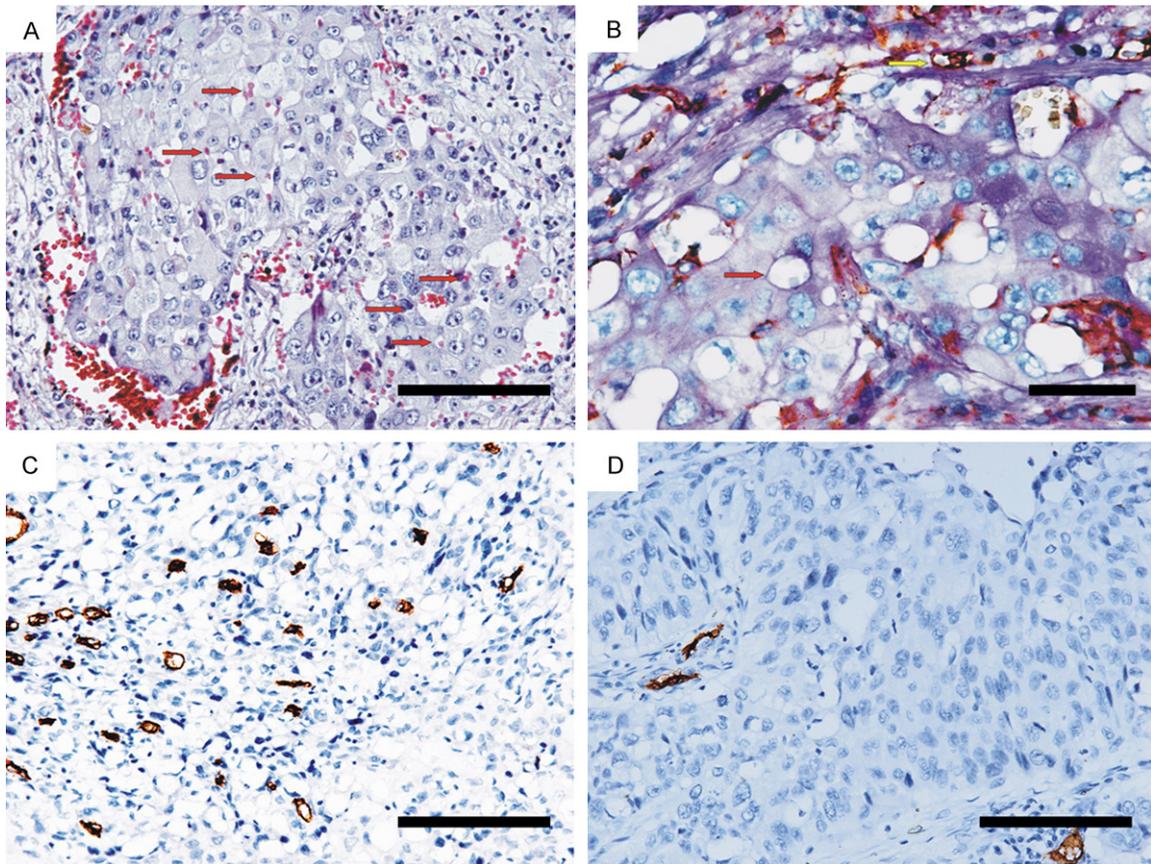
Figure 1. Histological and double immunohistochemical staining of NSCLC. A. Morphological appearance of VM with H&E staining. The VM channel was surrounded only by tumor cells and RBC (red arrow). Absence of necrosis and phlogocyte was observed in the vicinity (Scale bar = 100 μm). B. Results of CD31/PAS double staining (Scale bar = 50 μm). The VM channel showed a positive expression for PAS but a negative expression for CD31 (red arrow). The endothelial channel showed positive expressions for both CD31 and PAS (yellow arrow). C. MVD staining for CD34 in NSCLC. A hotspot with high angiogenesis was positively stained (Scale bar = 100 μm). D. Those with values < 29 were regarded as low MVD (Scale bar = 100 μm).

sured. The intensity was classified as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The percentage was also stratified as follows: 0 for less than 5% positive cells, 1 for less than 30% positive cells, 2 for less than 60% positive cells, and 3 for more than 60% positive cells. The sum of staining intensity and positive cell scores, which was more than 3 for the final result, was considered as the positive sample for each slide. MVD was determined from CD34-stained sections at the hot spot through light microscopy examination. The fields with the greatest neovascularization were examined by scanning tumor sections at low power (×100). The average vessel count of the five fields (×200) was regarded as the MVD.

Statistical analysis

All the data in the study were evaluated using SPSS17.0 software (SPSS, Chicago, IL, USA).

The survival data were analyzed using Kaplan-Meier analysis. Crosstabs, Pearson χ^2 test, Spearman correlation analysis, and one-way analysis of variance (ANOVA) were used as needed. All *P* values were two-sided, and *P* < 0.05 was considered statistically significant.

Results

Association of VM and CD34-MVD with clinico-pathological features in human NSCLC tissue

In the CD31/PAS double staining, VM was identified through the detection of PAS-positive and CD31-negative channels surrounded by tumor cells, with red blood cells. Among the 205 NSCLC tissues, VM was found in 28 (13.66%) specimens (**Figure 1A** and **1B**). Thus, all samples were divided into VM and non-VM groups. Further analysis showed that the presence of VM was significantly associated with histologi-

Table 1. Correlation between VM, CD34-MVD, CD34 and clinicopathological features in NSCLC

	N	VM		P-value	CD34-MVD	P-value*	CD34		P-value
		Negative	Positive		Median (25%, 75%)		Negative	Positive	
Gender									
Male	145	122	23	0.184	26.67 (20.84, 37.17)	0.194	140	5	0.673
Female	60	55	5		29.50 (23.00, 39.25)		59	1	
Age (yr)									
< 60	101	86	15	0.687	28.67 (21.33, 37.33)	0.737	96	5	0.115
≥ 60	104	91	13		28.17 (21.17, 38.25)		103	1	
Size (cm)									
< 5	107	92	15	1.000	28.67 (21.00, 38.33)	0.647	104	3	1.000
≥ 5	98	85	13		28.34 (21.33, 35.75)		95	3	
Location									
Center	106	91	15	0.842	29.33 (21.67, 38.00)	0.486	104	2	0.432
Peripheral	99	86	13		27.34 (20.83, 35.83)		95	4	
Histological classification									
SCC	79	75	4	< 0.001	24.00 (19.33, 33.67)	0.002	78	1	0.308
AC	75	68	7		29.67 (25.33, 38.33)		73	2	
LCC	51	34	17		31.33 (21.67, 47.67)		48	3	
Differentiation									
Well	35	35	0	< 0.001	25.67 (20.33, 30.67)	0.147	35	0	0.090
Moderate	87	81	6		29.33 (22.67, 38.00)		86	1	
Poor	83	61	22		29.00 (20.67, 42.33)		78	5	
Pleura invasion									
No	113	96	17	0.547	29.00 (22.33, 38.50)	0.149	112	1	0.092
Yes	92	81	11		27.34 (20.33, 34.92)		87	5	
Lymph node metastasis									
No	117	104	13	0.227	27.67 (21.50, 37.84)	0.369	114	3	1.000
Yes	88	73	15		28.84 (21.08, 36.50)		85	3	
T stage									
T1+T2	149	134	15	0.021	29.00 (21.84, 37.17)	0.853	146	3	0.348
T3+T4	56	43	13		27.34 (20.33, 39.34)		53	3	
Clinical stage									
I+II	158	144	14	0.001	27.67 (21.59, 35.58)	0.078	155	3	0.135
III+IV	47	33	14		31.67 (21.00, 44.33)		44	3	
Distant metastasis									
No	147	136	11	<0.001	28.67 (20.33, 37.00)	0.086	145	2	0.055
Yes	58	41	17		27.67 (22.67, 42.33)		54	4	
Therapy before surgery									
No	186	160	26	1.000	29.00 (21.67, 38.00)	0.421	180	6	1.000
Yes	19	17	2		25.00 (19.33, 32.00)		19	0	
Therapy after surgery									
No	98	83	15	0.547	29.00 (21.67, 37.08)	0.607	95	3	1.000
Yes	107	94	13		27.00 (20.33, 38.00)		104	3	

*Independent t-test was used after logarithmic transformation.

cal classification, differentiation, T stage, clinical stage, and distant metastasis ($P < 0.05$). By contrast, little correlation was found between VM and other clinicopathological parameters, such as age, gender, tumor size, tumor location, pleural invasion, lymph node metastasis and whether or not therapy was administered ($P > 0.05$). VM was found more frequently in samples with poor differentiation, advanced stages, and distant metastasis. For the pathologi-
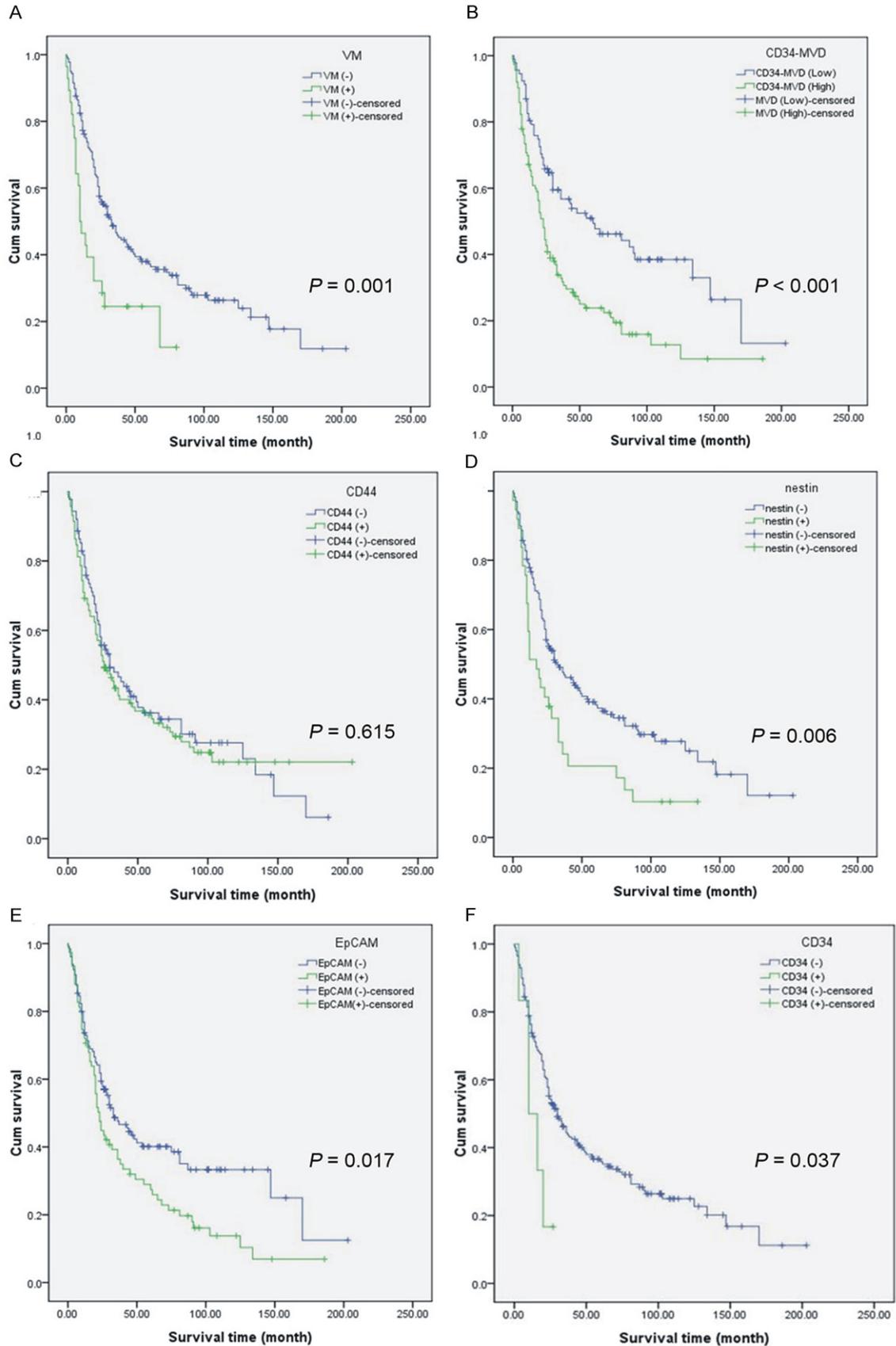
Figure 2. Results of the Kaplan-Meier survival analysis. A. Kaplan-Meier survival analysis showing that VM-positive patients have shorter survival periods than VM-negative patients (*P* = 0.001). B. Kaplan-Meier survival analysis showing that CD34-MVD-high patients have shorter survival periods than CD34-MVD-low patients (*P* < 0.001). C. Kaplan-Meier survival analysis showing that CD44-positive patients have shorter survival periods than CD44-negative patients (*P* = 0.615). D. Kaplan-Meier survival analysis showing that nestin-positive patients have shorter survival periods than nestin-negative patients (*P* = 0.006). E. Kaplan-Meier survival analysis showing that EpCAM-positive patients have shorter survival periods than EpCAM-negative patients (*P* = 0.017). F. Kaplan-Meier survival analysis showing that CD34-positive patients have shorter survival periods than CD34-negative patients (*P* = 0.037).

cal types, VM was found most frequently in large cell lung cancer (17/51, 33.33%), adenocarcinoma (7/75, 9.33%), and squamous cell carcinoma (4/79, 5.06%). All the data pertaining to the presence of VM and clinicopathological characteristics in the 205 NSCLC cases are summarized in **Table 1**.

CD34 was stained to calculate the MVD. By one-way ANOVA, significant discrepancies of CD34-MVD were found in three types of lung cancer (*P* < 0.05). The MVD was largest in large cell lung cancer (31.33), followed by adenocarcinoma (29.67) and squamous cell carcinoma (24.00). However, little difference was detected between MVD and other clinicopathological features (**Table 1**).

To validate the clinical significance of VM and CD34-MVD, all 205 patients were followed up. The relation between their outcomes and VM formation or CD34-MVD was examined. In our study, the median value of MVD was 29, and all tissues were classified as high ($\geq$ 29) or low (< 29). As shown by the results, the total survival period of patients with VM or high MVD was significantly shorter than that of the patients without VM or with low MVD (P < 0.05). The average survival period for VM-positive patients was 26 months, whereas that for VM-negative patients was 68 months (**Figure 2A**). The overall mean survival period for high-MVD patients was 27 months, whereas that for low-MVD patients was 55 months (**Figure 2B**).

Association of CSC markers with clinicopathological features in human NSCLC tissue

The positive expression of nestin was identified through brown stains in the cytoplasm of the tumor cells. The positive expressions of CD44 and EpCAM were both detected in the membrane of the tumor cells. The positive expression of CD34 was found in the cytoplasm or membrane of the tumor cells. Among the 205 specimens, the positive expression of nestin

was detected in 37 cases (37/205, 18.05%), whereas that of CD44, EpCAM, and CD34 was found in 117 (117/205, 57.07%), 75 cases (75/205, 36.59%), and 6 cases (6/205, 2.93%), respectively (**Figure 3**). The correlation between the clinicopathological features and CSC markers are summarized in **Tables 1** and **2**.

The positive expression of EpCAM was significantly related to histological classification, distant metastasis, and survival period (*P* < 0.05, **Table 2**). EpCAM was positively expressed in 28 cases of patients with distant metastasis (28/58, 48.28%) and in 47 samples without distant metastasis (47/147, 31.97%). However, little association between it and other clinicopathological variables (*P* > 0.05) was found. Moreover, the total average survival period of EpCAM-positive patients was 39 months, which is shorter than that of EpCAM-negative patients (42 months, *P* < 0.05, **Figure 2E**).

The positive expression of nestin had a significant correlation with the outcomes (*P* < 0.05). The average survival periods of nestin-positive and nestin-negative patients were 30 and 43 months, respectively (**Figure 2D**). Nevertheless, little correlation was found between nestin expression and clinicopathological parameters (*P* > 0.05, **Table 2**). The positive expression of nestin was also observed in new blood vessels.

Similarly, no remarkable gap was found between CD34 and clinicopathologic factors, expect prognosis (**Figure 2F**; **Table 1**). The overall survival periods of CD34-positive and CD34-negative patients were 14 and 42 months, respectively, and this difference was statistically significant (*P* < 0.05). Nevertheless, CD34 tended to be associated with distant metastasis.

A difference was also obviously observed between the positive expression of CD44 and pathological categories, such as T stage, clini-
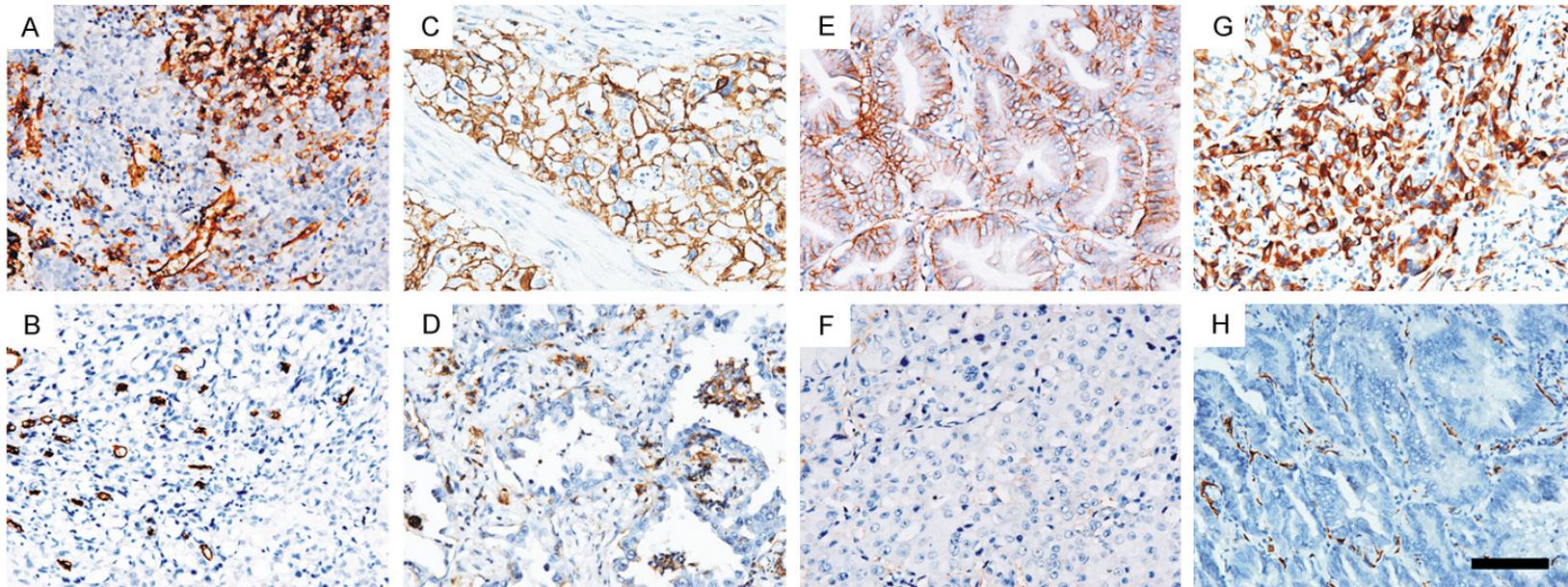
Figure 3. Expression of CSC-related markers in NSCLC (immunohistochemical staining). A. The positive expression of CD34 was located in tumor cells and endothelial cells. B. CD34-negative samples were regarded as negative expression in tumor cells and positive expression in endothelial cells as internal controls. C. CD44-positive expression was located in the membrane of tumor cells. D. Tumor cells showed negative expression for CD44, whereas lymphocytes showed positive expression for the internal control. E. Tumor cells expressing EpCAM were considered as EpCAM positive. F. The expression of EpCAM failed in tumor cells. G. Nestin was expressed in the cytoplasm of tumor cells. H. Nestin was negatively expressed in tumor cells and positively expressed in new blood vessels. (Scale bar = 100 μm).

Table 2. Correlation between CD44, nestin, EpCAM and clinicopathological features in NSCLC

	N	EpCAM		P-value	Nestin		P-value	CD44		P-value
		Negative	Positive		Negative	Positive		Negative	Positive	
Gender										
Male	145	89	56	0.426	120	25	0.691	54	91	0.013
Female	60	41	19		48	12		34	26	
Age (yr)										
< 60	101	63	38	0.774	81	20	0.588	48	53	0.206
≥ 60	104	67	37		87	17		40	64	
Size (cm)										
< 5	107	65	42	0.469	91	16	0.276	47	60	0.779
≥ 5	98	65	33		77	21		41	57	
Location										
Center	106	73	33	0.111	88	18	0.719	35	71	0.005
Peripheral	99	57	42		80	19		53	46	
Histological classification										
SCC	79	60	19	0.012	66	13	0.745	18	61	<0.001
AC	75	41	34		62	13		50	25	
LCC	51	29	22		40	11		20	31	
Differentiation										
Well	35	24	11	0.078	31	4	0.535	16	19	0.634
Moderate	87	61	26		70	17		34	53	
Poor	83	45	38		67	16		38	45	
Pleura invasion										
No	113	71	42	0.085	94	19	0.715	74	39	1.000
Yes	92	59	33		74	18		61	31	
Lymph node metastasis										
No	117	74	43	1.000	96	21	1.000	51	66	0.887
Yes	88	56	32		72	16		37	51	
T stage										
T1+T2	149	95	54	0.872	125	24	0.308	73	76	0.004
T3+T4	56	35	21		43	13		15	41	
Clinical stage										
I+II	158	101	57	0.863	131	27	0.521	76	82	0.007
III+IV	47	29	18		37	10		12	35	
Distant metastasis										
No	147	100	47	0.036	125	22	0.073	60	87	0.351
Yes	58	30	28		43	15		28	30	
Therapy before surgery										
No	186	120	66	0.325	152	34	1.000	77	109	0.224
Yes	19	10	9		16	3		11	8	
Therapy after surgery										
No	98	57	41	0.149	77	21	0.276	38	60	0.262
Yes	107	73	34		91	16		50	57	

cal stage, tumor location and gender ($P < 0.05$, **Table 2**). CD44 was more frequently expressed in specimens in an advanced T stage (T3+T4) and advanced clinical stage (III+IV) than those in the early stage (T1+T2, I+II). In addition, the expression of CD44 was also found more often

positive group (42 months) and CD44-negative group (40 months) ($P > 0.05$, **Figure 2C**).

Furthermore, CD133 and Oct3/4, known as classical CSC markers, were detected in the NSCLC tissues. The positive expression of Oct3/4 was detected in the nucleus of the tumor cells, and that of CD133 was found in both membrane and cytoplasm of the cells. Unfortunately, although the positive control was strongly and properly expressed in each assay, the positive expression of neither CD133

Table 3. Relationship between VM, CD34-MVD and expression of CSC related markers in NSCLC

	VM		P-value	r	CD34-MVD	P-value*	r
	Negative	Positive			Median (25%, 75%)		
EpCAM							
Negative	115	15	0.292	——	26.84 (20.83, 36.50)	0.410	——
Positive	62	13			29.33 (21.67, 39.00)		
Nestin							
Negative	150	18	0.015	0.183	27.00 (21.08, 34.59)	0.002	0.190
Positive	27	10			36.33 (22.50, 46.50)		
CD44							
Negative	83	5	0.004	0.201	28.17 (21.75, 37.08)	0.235	——
Positive	94	23			28.67 (21.00, 38.17)		
CD34							
Negative	174	25	0.035	0.008	31.12 (21.67, 38.00)	0.203	——
Positive	3	3			23.89 (15.17, 34.75)		

*Independent t-test was used after logarithmic transformation.

nor Oct3/4 was detected in any neoplastic cell in the 49 NSCLC tissues. Thus, the attempt at immunohistochemical staining of CD133 and Oct3/4 was stopped.

Relationship between CSC markers and angiogenic parameters

The relationship between CSC markers and tumor vascularization was also examined in the 205 specimens, as summarized in **Table 3**. VM was remarkably associated with CD44, CD34, and nestin (*P* < 0.05, **Table 3**), but not with EpCAM (*P* > 0.05, **Table 3**). CD34-MVD was closely correlated with the positive expression of nestin, whereas its relation with CD44, CD34, EpCAM, and MVD had no statistical significance (*P* > 0.05, **Table 3**).

Further study demonstrated that nestin was positively correlated with CD44 (*P* = 0.016, *r* = 0.176), whereas EpCAM had little correlation with CD44 (*P* = 0.306) and nestin (*P* = 0.258). Moreover, no intergroup difference was found between CD34 and EpCAM (*P* = 0.671), nestin (*P* = 1.000), and CD44 (*P* = 0.240). The relationship between VM and CD34-MVD was conspicuous (*P* < 0.001, *r* = 0.383).

Discussion

VM is the vessel surrounded by tumor cells that are PAS-positive but CD31-negative during staining. Moreover, VM is also the channel with erythrocytes but without necrosis and inflammatory cell infiltration. In this study, VM was more commonly found in the progressive stage of NSCLC than in the primary stages of both T

stage and clinical stage, which is in accordance with previous studies on renal cell cancer and melanoma [5, 17]. VM increases as histological differentiation decreases, a correlation corroborated by our previous study [5]. In addition, VM is closely related to distant metastasis, a finding consistent with that of Liu TJ *et al.* [18]. For the subtypes, the positive rate of VM was detected mostly in large cell lung cancer, then in adenocarcinoma and squamous cell carcinoma, a finding that is not similar to the result obtained by Wu *et al.* [19]. We speculate that this result is due to the difference in the scope of their study and ours. Their study covers only lung adenocarcinoma and squamous cell carcinoma, whereas the present study covers adenocarcinoma, squamous cell carcinoma, and large cell lung cancer. Moreover, VM was an independent unfavorable prognostic factor in NSCLC patients, a finding consistent with previous results [5, 8, 19]. Thus, we presumed that VM has an important function in the aggressive behavior of NSCLC by assisting in the invasion and metastasis of tumor cells.

This study also examined MVD in NSCLC tissues. MVD is the standard method of measuring tumor angiogenesis, and is closely related to tumor growth and postoperative prognosis [7, 20]. The patients with high scores of MVD indicated early metastasis and short survival [21], a result in accord with cited studies. Unfortunately, patients with high MVD died earlier than those with low MVD. Moreover, MVD tended to be associated with distant metastasis and clinical stage. Our data showed that MVD was significantly different across these

three subtypes, which is in agreement with a previous report [22].

Numerous studies have shown that CSCs have an important function in tumorigenesis and even act as vascular progenitors, directly forming the wall of tumor vessels [23, 24]. Numerous CSC markers are observed in several cancers. In this study, we also attempted to detect some stem cell markers in NSCLC tissues because no special CSC marker of lung cancer has been identified to date. EpCAM, a transmembrane glycoprotein, has been suggested to be involved in early-stage embryogenesis because of its expression in fertilized oocytes, embryonic stem cells, and embryoid bodies during embryogenesis [25]. EpCAM overexpression is directly linked to the stimulation of the cell cycle and proliferation by up-regulating c-myc and cyclin A/E [26]. EpCAM appears to be an epithelial tumorigenic CSC marker, which has been confirmed in the CSCs of colorectal cancer and hepatocellular carcinoma [27-30]. In this study, EpCAM was correlated with histological classification, distant metastasis, and overall survival period. Our data are in agreement with previous reports [31, 32]. Nestin is a class 6 intermediate filament protein that is especially expressed in stem/progenitor cells of the developing central nervous system [33]. Janikova *et al.* successfully identified nestin+/CD133+ putative cancer stem cells in NSCLC [34]. Similar to a previous report, nestin-positive patients were correlated with poor prognosis in our study [35]. In addition, the expression of nestin was also observed in new vascular endothelial cells; they participated in neovascularization, as supported by previous reports [35-37]. Unfortunately, our examination failed to correlate the positive expression of nestin with clinicopathological features. Ryuge et al. examined nestin in 171 NSCLC cases, which included 131 cases of adenocarcinoma, 31 cases of squamous cell carcinoma, and 9 cases of others [38]. Our study included 79 cases of squamous cell carcinoma, 75 cases of adenocarcinoma, and 51 cases of large cell lung cancer. Furthermore, no significant association between nestin expression and clinicopathological parameters in large cell neuroendocrine carcinoma of the lung was reported by Ryuge *et al.* [35]. Thus, we analyzed that the variously contained subtypes in each individual trial may have contributed to the different results. CD44, CD34, CD133, and Oct3/4 as common CSC biomarkers were detected in this study. CD44

and CD34 were associated with related clinicopathological factors, a finding also reported by Leung *et al.* [39]. Unpredictably, CD44 was more often expressed in male and center lung cancer cases. We suspect that other factors, such as androgen, may have taken part in this result. After failing to find a positive expression of CD133 and Oct3/4 in 49 NSCLC tissues, we abandoned the two markers.

In addition, the relationship among VM, CD34-MVD, and CSC markers in NSCLC tissues was reviewed. To our knowledge, this study is the first systematic assessment of the relationship between CSCs and vasculature in NSCLC. In our study, CSC biomarkers were found to be closely related with VM and MVD, indicating that CSCs have an important function in tumor angiogenesis. Tumors need blood and nutrient supply for their growth and metastasis, especially when their volumes reach more than 1 mm^3. Thus, they attempt to search for and stimulate neovascularization to acquire adequate oxygen and nutrition. Under the influence of some biological factors, CSCs mimic endothelial cells and form VM, and then mosaic and endothelium vessels, a finding suggested in our previous article [40]. Zhang and his colleagues proposed that CSCs can even generate erythroid cells [41]. These pieces of evidence can be used to explain the ineffectiveness of conventional anti-angiogenesis therapies in neoplasms.

In conclusion, the present results disclose a definite relationship among CSCs, VM, and MVD. Thus, we can reasonably deduce that CSCs, VM, and MVD have significant functions in tumorigenesis, and that CSCs are possibly related to angiogenesis and VM. Although determining the precise mechanism continues, this study provides a new way of inspecting and administering therapies for tumors.

In conclusion, Higher VM, CSCs, and MVD were associated with more aggressive NSCLC. VM, MVD, and some CSC markers were independent unfavorable prognostic factors of NSCLC. CSCs possibly participate in neovascularization.

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