

Original Article

The overexpression of PDGF-BB and its receptor is correlated with lymphatic metastasis in patients with non-small cell lung cancer

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Abstract: The metastasis of tumors through lymphatic vessels is an important adverse prognostic factor for human cancers. Non-small cell lung cancer (NSCLC) is one of the most common types of lung cancers, and usually occurs with metastasis at an early stage and has a poor prognosis. Lymphatic metastasis is the most common route of lung cancer metastasis, and it requires the interaction of multiple growth factors. Platelet-derived growth factor-BB (PDGF-BB) and platelet-derived growth factor receptor- β (PDGFR- β) are related to both angiogenesis and lymphangiogenesis. This study aimed to explore PDGF-BB/PDGFR- β and its correlations with lymphatic microvessel density (LMVD) and lymph node metastasis in patients with non-small cell lung cancer (NSCLC). A total of 127 Chinese patients with NSCLC pathologically diagnosed from 2009 to 2013 were enrolled in the study. The expression levels of PDGF-BB and PDGFR- β were measured in all NSCLC tissues using immunohistochemical staining. Further, LMVD stained by D2-40 was evaluated, and the platelet (PLT) count was determined. The expression levels of PDGF-BB and PDGFR- β in the NSCLC tissues were 73.2% (93/127) and 78.0% (99/127) respectively in 127 patients. The expressions of PDGF-BB and PDGFR- β and LMVD were associated with TNM stage, lymph node metastasis, and PLT ($P < 0.05$ or $P < 0.01$), but not with the patient's age, gender, histological type, histological grade, and tumor size ($P > 0.05$). It was therefore concluded that the expression levels of PDGF-BB and PDGFR- β in tumor cells were correlated with TNM stage and lymph node metastasis in Chinese patients with NSCLC and may play a key role in the development of NSCLC.

Keywords: Lymphatic microvessel density, lymph node metastasis, non-small cell lung cancer, PDGF-BB, PDGFR- β , platelet count

Introduction

Non-small cell lung cancer (NSCLC), one of the most common types of lung cancer among Chinese patients, is the leading cause of cancer-related death, and only 15% of lung cancer patients live more than 5 years [1]. NSCLC usually occurs with metastasis at an early stage and has a poor prognosis [2]. Lymph node metastasis is the most common route of lung cancer metastasis and is an important factor that affects the prognosis of lung cancer.

Lymphatic metastasis is dependent on lymphangiogenesis. Just as in tumor angiogenesis, tumor lymphangiogenesis also requires the interplay of multiple tumor-derived growth fac-

tors. Platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) represent one of the most intensively studied families of growth factors in the last several decades. Recently, the role of PDGF signaling has been extended to pulmonary diseases, including pulmonary hypertension, lung fibrosis, and lung cancer [3]. Members of the PDGF family act as lymphatic angiogenic factors. PDGF has three subforms: PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-BB induced tumor lymphatic angiogenesis, which involves enhanced metastasis in the lymph nodes [4]. So PDGF-BB is a critical growth factor contributing to lymphatic metastasis.

The lymphangiogenesis is usually assessed by lymphatic vessel density (LVD) using specific

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markers for lymphatic endothelial cells. For example, vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin, and LYVE-1 can be detected in the lymphatic vessels [5]. D2-40 is a kind of antibody for podoplanin, a new type of marker for lymphatic endothelial cells which is more specific and sensitive than others. D2-40-labeled lymphatic microvessel density (LMVD), reflecting the activity of lymphangiogenesis [6], was shown to be correlated with the prognosis of breast and lung cancers [7, 8]. However, the impact of PDGF and its receptor on the lymphangiogenesis of lung tumors remains to be elucidated.

In the present study, a total of 127 Chinese patients with NSCLC were selected from among patients at Jinhua Hospital of Zhejiang University. The patients' LMVD levels and the positive expression of PDGF and its receptor in NSCLC tissues were measured by immunohistochemical analysis to investigate their correlation with the lymphatic metastasis of tumors.

Material and methods

Patients

A total of 127 patients with NSCLC were selected from among patients at Jinhua Hospital of Zhejiang University. They underwent surgery, and the tumors were confirmed by pathological examination between January 2009 and December 2013. The patients did not receive chemotherapy or radiotherapy before surgery and did not have other concurrent types of malignancy or severe organ dysfunction. The patients included 79 men and 37 women, with the male-to-female ratio of 2.14:1. Their ages ranged between 36 and 81 years, with the average age of 62.4 years. Further, 66 squamous cell carcinomas and 61 adenocarcinomas were reported, including 62 patients with lymphatic metastasis and 65 patients without lymphatic metastasis. The tumors were classified according to TNM (UICC 2009); 37, 44, 35, and 11 patients had tumors at stage I, stage II, stage III, and stage IV, respectively. The tumor samples were cut into serial sections with a thickness of 4 μm and subjected to immunohistochemical analysis. The platelet (PLT) count was estimated from the peripheral fasting blood obtained at early morning. Meanwhile, the blood from 70 healthy volunteers was col-

lected as a control, including 58 men and 22 women with a male-to-female ratio of 2.64:1. The age of the volunteers ranged between 24 and 77 years, with an average of 55.04 years. The gender and age distribution in the control group was comparable with that in the NSCLC group, and the other clinical features are summarized in **Table 1**. The study was approved by the ethics committee of Zhejiang University Jinhua Hospital (reference number, JHYY-2014-0312), and an informed consent was signed by each participant.

Main instrument and reagents

The rabbit polyclonal antibodies against human PDGF-BB (Cat No. ab21234) and the rabbit polyclonal antibodies against human PDGFR- β were procured from Abcam (Cambridge, United Kingdom). The mouse anti-D2-40 (Podoplanin) lymphatic endothelial marker monoclonal antibody (Cat No. IS072) and EnVision kit were purchased from Dako (Agilent Technologies, USA). The Stago coagulation instrument was obtained from France.

Preparation and detection of blood samples

Fasting blood samples of 3 mL were obtained from each participant in the morning and stored in anticoagulant plastic tubes containing sodium citrate at a concentration of 38 g/L. The ratio between anticoagulant reagent and whole blood was 1:9, and the blood was centrifuged for 10 min at 3000 rpm. The supernatant (plasma) was collected. All the procedures were completed within 4 h. The PLT count was measured using imported reagents.

Immunohistochemical analysis

The expression levels of PDGF-BB and PDGFR- β in the NSCLC tissues were measured using a two-step LMVD detection with an EnVision kit. D2-40 was used as the biomarker for lymphatic endothelium. The paraffin sections were processed sequentially as follows: dewaxing, microwave repairing of antigens, incubation with a 3% H_2O_2 solution at room temperature for 20 min to digest endogenous peroxidase, and blocking with 10% normal goat serum for 30 min. Then, the sections were incubated with rabbit anti-human PDGF-BB polyclonal antibodies (dilution ratio of 1:100), rabbit anti-human PDGFR- β polyclonal antibodies (dilution ratio of

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Table 1. Correlations of the expression levels of PDGF-BB, PDGFR- β , and D2-40 with clinicopathological factors in primary NSCLC

Factor	Case No.	PDGF-BB no. (%)	P value	PDGFR- β no. (%)	P value	LMVD (Mean \pm SD)	P value
Age (year)							
\geq 60	79	58 (73.4)	0.951	71 (78.9)	0.797	12.8 \pm 9.6	0.220
< 60	48	35 (72.9)		28 (75.7)		10.6 \pm 8.7	
Sex							
Male	90	62 (68.9)	0.085	71 (78.9)	0.692	11.3 \pm 8.0	0.922
Female	37	31 (83.8)		28 (75.7)		11.1 \pm 9.9	
Histology							
SCC	66	49 (74.2)	0.788	51 (77.3)	0.848	11.5 \pm 9.8	0.759
ADC	61	44 (72.1)		48 (78.7)		11.0 \pm 8.0	
Differentiation							
WD and MD	94	68 (72.3)	0.703	72 (76.6)	0.533	10.6 \pm 11.4	0.786
PD	33	25 (75.8)		27 (81.8)		10.1 \pm 9.6	
Tumor size							
> 3 cm	64	51 (79.7)	0.098	53 (84.1)	0.096	11.4 \pm 9.0	0.690
\leq 3 cm	63	42 (66.7)		46 (71.9)		10.7 \pm 8.9	
TNM stage							
I/II	81	54 (66.7)	0.027	58 (71.6)	0.022	8.8 \pm 7.0	0.000
III/IV	46	39 (84.8)		41 (89.1)		15.4 \pm 10.5	
Lymph node metastasis							
Yes	62	39 (87.1)	0.0006	45 (69.2)	0.015	13.1 \pm 10.5	0.017
No	65	54 (60.0)		54 (87.1)		9.3 \pm 6.7	
PLT							
High	29	26 (89.7)	0.023	27 (93.1)	0.025	15.8 \pm 8.2	0.002
Normal	98	67 (68.4)		72 (73.5)		9.9 \pm 8.8	

1:100), and mouse anti-human D2-40 monoclonal antibody (dilution ratio of 1:50) at 4°C overnight. The sections were further incubated with the EnVision reagent at room temperature for 1 h. They were rinsed with an 0.01 M phosphate-buffered saline (PBS) buffer for 5 min in each step three times. The sections were developed with DAB-H₂O₂, and the intensity was controlled under microscopy, followed by counterstaining with hematoxylin, dehydration, and mounting with neutral gum. The sections were observed under an Olympus BH-3 microscope and photographed. The colon cancer sections were used as positive control, and PBS instead of first antibody was used as a negative control during staining.

Result interpretation

After immunohistochemical staining, 200 tumor cells were counted under a microscope

(400 \times). The cells with blue or purplish black stains in the cytoplasm were regarded as positive, and their percentage was calculated. The percentage of positively stained cells and the staining intensity were semi-quantitatively analyzed using the image analysis system of the Leica QWin platform. The scoring standard was mildly adjusted [9] as follows: score 0 for a percentage of positively stained cells < 1%; score 1 for a percentage ranging between 1% and 25%; score 2 for a percentage ranging between 26% and 50%; and score 3 for a percentage > 50%. The scoring standard for staining intensity was as follows: score 0 for no staining; score 1 for mild staining; score 2 for moderate staining; and score 3 for strong staining. The sum of the two scores ranging between 0 and 2 was considered as negative, and the sum ranging between 3 and 6 was considered as positive. The positively stained cells with a percentage \geq 10% were regarded as PDGF-BB and PDGFR- β

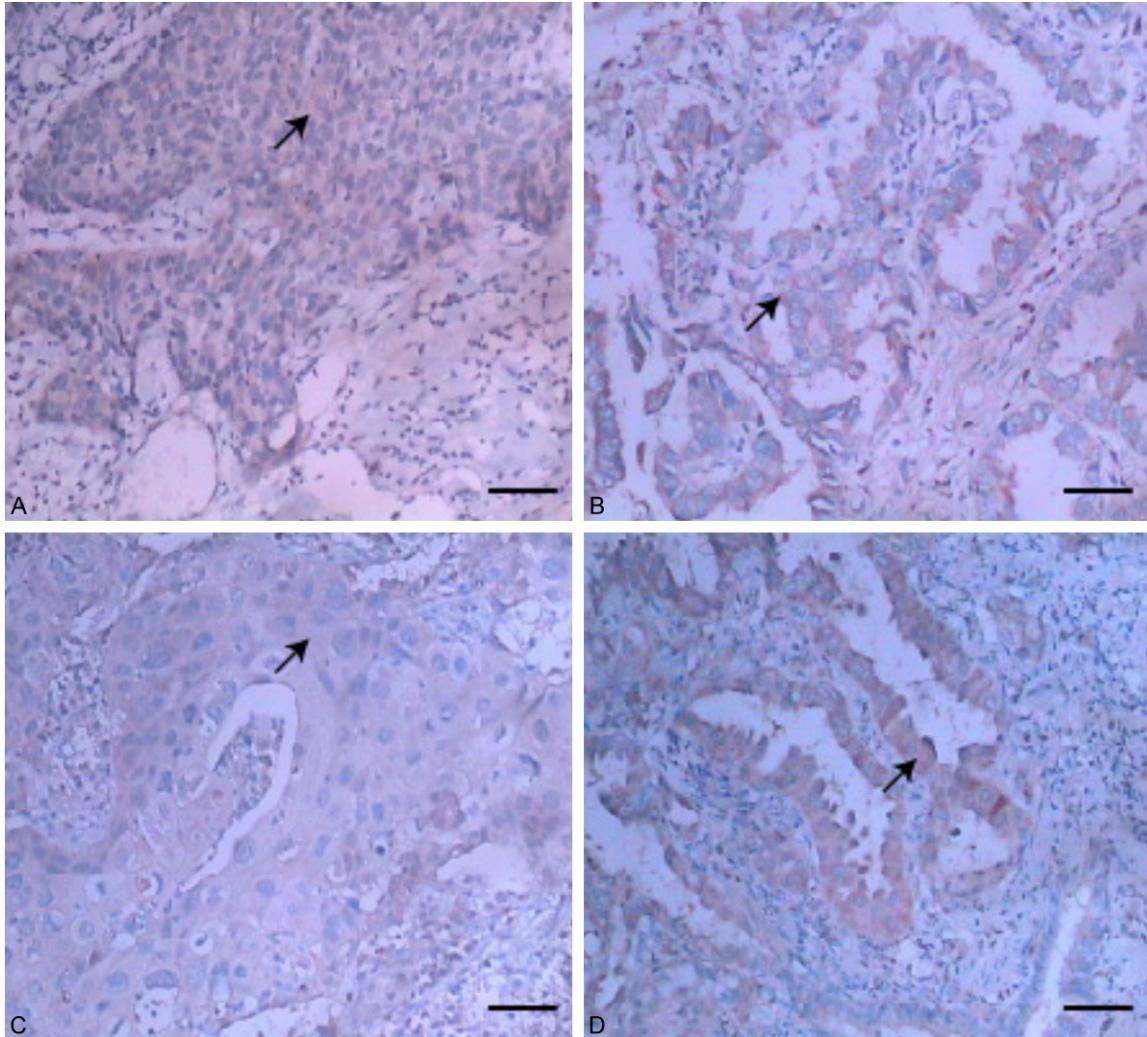


Figure 1. Immunohistochemical staining of primary NSCLC tissues for PDGF-BB and PDGFR- β ($\times 200$), Scale bar = 50 μm . A. Overexpression of PDGF-BB (black arrow) in squamous cell carcinoma. B. Overexpression of PDGF-BB (black arrow) in adenocarcinoma. C. Overexpression of PDGFR- β (black arrow) in squamous cell carcinoma. D. Overexpression of PDGFR- β (black arrow) in adenocarcinoma.

positive; otherwise, they were regarded as negative.

The maximum vascular density counting method reported by Weidner and Vermeulen [10, 11] was used for immunohistochemical staining. Five fields of views ($400 \times$) were randomly selected for each section of hot spots. The average of all five-field counting was used for the LMVD tumor value. The quantitative and semi-quantitative measurements were uploaded to a color image input device (JVC, TK-C1380) for image analysis and processed using Leica QWin software. The personnel responsible for quantitative measurement and counting were unaware of the standard preset for the tumor specimens.

Statistical analysis

The data were presented as the mean \pm standard deviation (SD), processed using Excel, and analyzed using SPSS 22.0 software (SPSS, IL, USA). The intergroup comparison analysis was conducted using a *t* test and an χ^2 test, and a *P* value < 0.05 was considered statistically significant.

Results

Expression of PDGF-BB and PDGFR- β in NSCLC tissues

The PDGF-BB and PDGFR- β positive staining was found mainly in the tumor cells or tumor

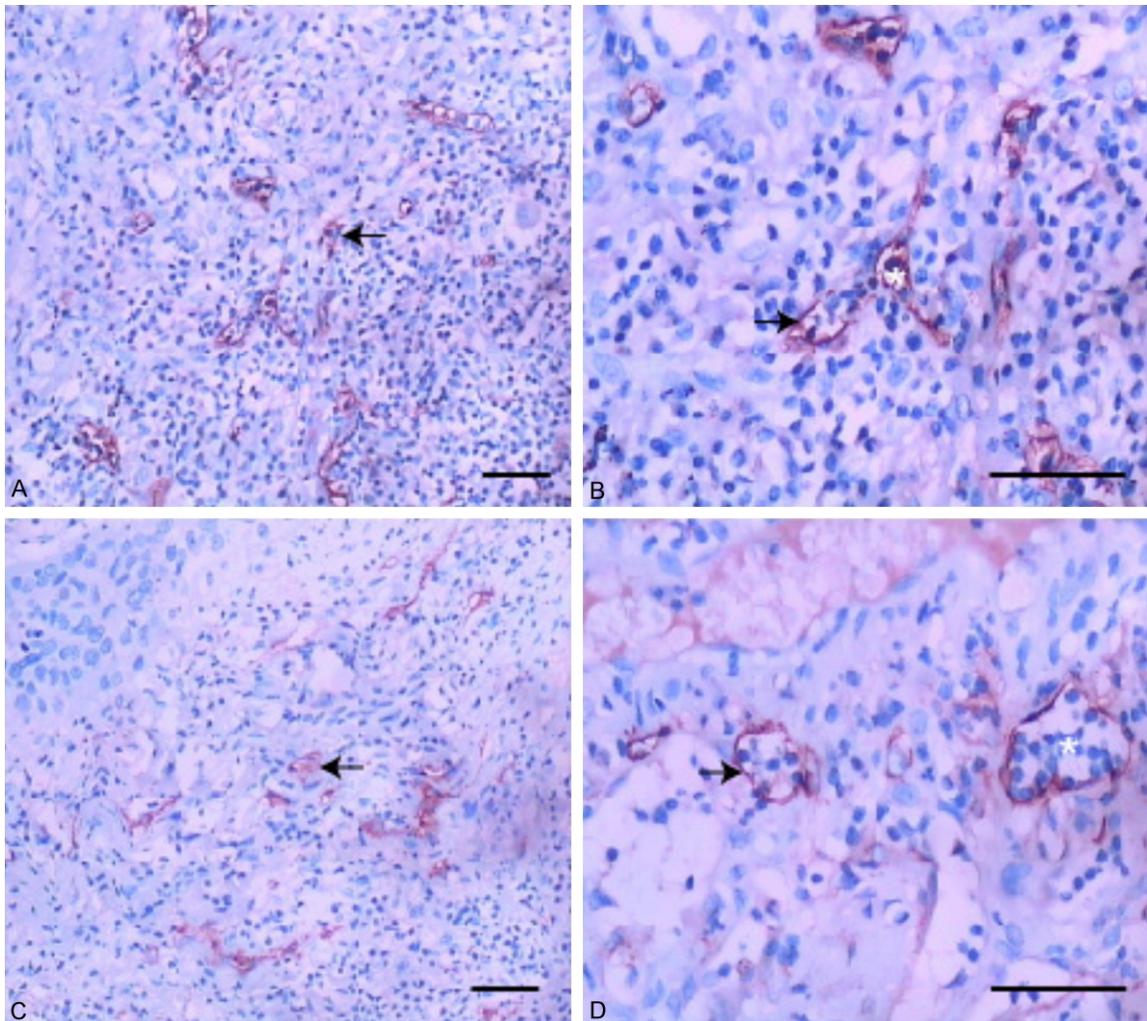


Figure 2. Immunohistochemical staining for D2-40 in primary NSCLC tissues (A and C, $\times 200$; B and D, $\times 400$), Scale bar = 50 μm . (A) The expression of D2-40-positive in the lymphatic endothelial cells (black arrow) of squamous cell carcinoma. (B) D2-40-positive highlighting a lymphatic vessel around a tumor embolus (white asterisk) in squamous cell carcinoma. (C) The expression of D2-40-positive in the lymphatic endothelial cells (black arrow) in adenocarcinoma. (D) D2-40-positive highlighting a lymphatic vessel around a tumor embolus (white asterisk) in adenocarcinoma.

interstitial cells located in the cytoplasm (Figure 1). Of all the 127 NSCLC tumor samples, 93 (73.2%) and 99 (78.0%) samples were positive for the expression of PDGF-BB and PDGFR- β , respectively.

D2-40-positive LMVD in NSCLC tissues

The varied expression of D2-40 in the 127 NSCLC tissue samples was detected. The positive staining of D2-40 was found in the membranes and cytoplasm (Figure 2). Many D2-40-positive lymphatic vessels were found around the tumor tissues, presenting with irregular morphology and a thin wall without smooth

muscle cells. The vessels were expanded with varied lumen sizes; no erythrocytes or neutrophils fulfilled the feature of a microlymphatic vessel. The lymphatic vessel invasion (LVI) was occasionally found in the D2-40-positive lymphatic vessels, and the cancer emboli were observed within the lumen (Figure 2B and 2D).

Association of clinical pathology with the expression levels of PDGF-BB and PDGFR- β and LMVD in NSCLC tissues

The TNM stage and lymphatic metastasis were associated with the expression of PDGF-BB and PDGFR- β in the NSCLC tissues ($P < 0.05$),

but not associated with age, gender, pathological type, histological grade, or the diameter of the tumors ($P > 0.05$) (Table 1). Meanwhile, LMVD in the NSCLC tissues was significantly different in the varied stage of TNM (comparison between stage III/IV and stage I/II), or with and without lymphatic metastasis ($P < 0.05$). However, no difference in LMVD was found in terms of age, gender, pathological type, histological grade, or the diameter of the tumors ($P > 0.05$) (Table 1).

Association of PLT with the expression levels of PDGF-BB and PDGFR- β and LMVD in NSCLC tissues

The average PLT in the 127 NSCLC patients was $240.25 \pm 88.11 \times 10^9/L$, which was significantly higher than the level in the 70 healthy volunteers ($219.86 \pm 49.01 \times 10^9/L$) ($t = 2.087$, $P < 0.05$). Further, 29 (22.8%) patients had an elevated PLT ($> 220 \times 10^9/L$), and 98 (77.2%) patients had a normal range of PLT ($\leq 220 \times 10^9/L$). The expression levels of PDGFR- β and LMVD were significantly higher in patients with an elevated level of PLT compared to those with a normal level of PTL ($P < 0.05$ or $P < 0.01$) (Table 1).

Discussion

The findings of the present study indicated that the expression levels of PDGF-BB and PDGFR- β and LMVD in NSCLC tissues were correlated with the clinical stage and lymphatic metastasis and highly associated with an elevated level of PLT, indicating that the PDGF-BB and PDGFR- β signaling pathways participated in multiple steps of the biological processes of NSCLC, including growth, infiltration, and the migration of tumors, with a significant impact on metastasis and prognosis. Therefore, high expression levels of PDGF-BB and PDGFR- β , as well as high PLT, might be factors predicting a poor prognosis.

Lymphatic metastasis is a key factor for the prognosis of NSCLC. It is dependent on lymphangiogenesis. The lymphangiogenic factors include vascular endothelial growth factor (VEGF) and PDGF families that promote the formation of new lymphatic vessels via activating their corresponding receptors, hence providing a direct channel for the metastasis of tumor cells [12, 13]. PDGF is an alkaline protein synthesized and stored in the α granules of PLTs. It

is composed of two polypeptides and linked through a bisulfate bond to form homo- or heterodimers, including PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD. It regulates the directed migration and wrapping of blood vessels and promotes angiogenesis. PDGFR contains subunits α and β . The subunit β has a higher affinity for B and D chains of PDGF. The overexpression of PDGF-BB could increase the pericyte content via the stromal-derived factor-1 α /CXCR4 axis upon binding to its specific receptor, thus activating endothelial cells as an angiogenesis factor [14]. PDGF-BB, VEGF-C, and VEGFR-3 were shown to promote lymphangiogenesis and lymphatic metastasis in NSCLC, which is consistent with the present observations [6, 9]. However, Liu et al. [9] reported higher expression levels of PDGF-BB and VEGF-C in younger patients, patients with a low histological grade, and in larger diameters of NSCLC tumors. However, high expression levels of PDGF-BB and PDGFR- β were not associated with age, histological grade, or diameters of NSCLC tumors but with clinical stage and lymphatic metastasis. The findings of the present study are similar to the results of Donnem et al. [6], suggesting that the expression level of PDGF-BB could be an independent factor for the prognosis of NSCLC with lymphatic metastasis and could serve as a novel target for therapy in the future.

Tumor-related lymphatic vessels are the main channel for tumor cell invasion into lymph nodes. D2-40-specific LMVD was used to predict the prognosis of lung adenocarcinoma [15]. The association between LMVD and the lymphatic metastasis of NSCLC was further investigated. LMVD was found to be closely associated with lymphatic metastasis and clinical stage, which is consistent with previous findings [7, 15-18]. The microlymphatic vessels were observed around the tumor tissue and believed to increase the possibility of LVI of tumor cells [18]. Tumor-related lymphatic vessels were the major route for lymphatic metastasis. Also, clinical studies indicated that LMVD could be used as an independent factor for tumor metastasis and prognosis [15, 17]. In the present study, the comparison between para- and intertumor LMVD or the LVI analysis was not conducted. Kwak et al. [19] found that the heterogeneity of microlymphatic vessel distribution could be observed in the late stage of colorectal cancer. LMVD was most intensive

around the primary loci; however, the patients with low LMVD had a worse prognosis, and the mechanism still remained unclear. Although the association between LMVD and remote metastasis was reported and LVI was not considered as lymphatic metastasis, LMVD still could be regarded as a pre-stage of metastasis, implying a close correlation between LMVD and lymphatic metastasis. The increase in LVI could elevate the possibility of lymphatic metastasis and influence the treatment and prognosis of tumors.

The findings of the present study also indicated a significant association of PLT with the expression levels of PDGF-BB and PDGFR- β , as well as LMVD, implying that PLTs not only promoted tumor cell proliferation and metastasis but also secreted cytokines such as PDGF to facilitate lymphangiogenesis and lymphatic metastasis. Malignant tumor tissues could activate PLTs and induce thrombotic diseases [20, 21]. Reactive thrombocytosis was often found in the early stage of lung cancer, which might be due to the increased production or a compensatory response to chronic wasting disease. The PLTs could prolong the abnormal aggregation and survival of tumor cells in the peripheral circulation and assist the immune escape of tumor cells. Meanwhile, they could promote the growth of tumors and angiogenesis through cytokine secretions to facilitate the metastasis of tumors and reduce patients' survival times [22, 23]. Therefore, a preoperative PLT count was regarded as an independent prognostic factor for lymphatic metastasis and prognosis [24, 25]. The PLT count was a cost-effective, rapid, and convenient laboratory examination, which could be considered as an extra biomarker of malignant tumors for prognosis. However, studies on PLTs and PDGF, as well as lymphangiogenesis, in lung cancer are rare. Krzystek-Korpaczka et al. [26] reported that the PLT count was positively associated with the serum PDGF-BB level in esophageal cancer, and serum PDGF-BB was suggested to be a potential biomarker for lymphatic metastasis. Therefore, further investigation is needed to explore the mechanism of PLTs and PDGF promoting lymphangiogenesis.

Conclusion

In summary, the expression levels of PDGF-BB and PDGFR- β , as well as LMVD, were associat-

ed with clinical stage and lymphatic metastasis of NSCLC, and highly correlated with the elevated PLT count, suggesting that the involvement of the PDGF-BB and PDGFR- β signaling pathways in the lymphatic metastasis of NSCLC. In addition, PDGF could be regarded as a novel target for lung cancer therapy in the future.

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Disclosure of conflict of interest

None.

Abbreviations

PDGF-BB, Platelet-derived growth factor-BB; PDGFR- β , platelet-derived growth factor receptor- β ; LMVD, lymphatic microvessel density; NSCLC, non-small cell lung cancer; PLT, platelet; PBS, phosphate-buffered saline; SD, standard deviation; LVI, lymphatic vessel invasion.

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