Prognostic value of tumor-associated CD177+ neutrophils in lung adenocarcinoma

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Abstract. The aim of the present study was to detect CD177+ neutrophils in tumor tissues and analyze their association with the clinical characteristics and prognosis of patients with lung adenocarcinoma (LUAD). Immunohistochemistry was used to detect CD177+ neutrophils in tumors and adjacent tissues of 16 patients with LUAD who underwent curative surgical resection. A total of 120 patients with LUAD were recruited, and their clinical data were collected; survival follow-up was performed. CD177+ neutrophils in tumor tissues were detected via immunohistochemistry, and the association between CD177+ neutrophils and clinical characteristics was analyzed. The density of CD177+ neutrophils in tumor tissues and adjacent tissues of patients with LUAD was analyzed using t-test, and the association between CD177+ neutrophils and clinical characteristics was analyzed through the Chi-square test. Survival was calculated using the Kaplan-Meier survival rate curve. Finally, the association between these indicators and the survival of LUAD patients was evaluated using Cox regression analysis. CD177+ neutrophil infiltration was significantly higher in LUAD tumor tissues, and the high density of CD177+ neutrophils was associated with the clinical characteristics of TNM stage, tumor differentiation and poor progression-free and overall survival in LUAD. In conclusion, tumor-associated CD177+ neutrophils associated with malignant progression and poor prognosis may be independent and unfavorable prognostic biomarkers for LUAD.

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Abbreviations: LUAD, lung adenocarcinoma; NSCLC, non-small cell lung cancer; TANs, tumor-associated neutrophils; TME, tumor microenvironment; OS, overall survival; PFS, progression-free survival; NLR, neutrophil-to-lymphocyte ratio; LUSC, lung squamous carcinoma

Key words: LUAD, CD177, neutrophils, clinical characteristics, prognostic

Introduction

Lung cancer, characterized by increased morbidity and mortality among malignant tumors, is predominantly represented by non-small cell lung cancer (NSCLC), accounting for 85% of all lung cancer cases. Among NSCLC subtypes, lung adenocarcinoma (LUAD) has the highest incidence (1). The tumor microenvironment (TME) plays an important role in tumorigenesis, development and prognosis of lung cancer. Neutrophils are an important component of the TME and affect the growth, progression and prognosis of almost all solid tumors (2).

Neutrophils account for 50-70% of the total circulating leukocytes. Increasing evidence suggests that tumor-associated neutrophils (TANs) are involved in the malignant behavior of tumors, including recurrence and metastasis (3). TANs exhibit high functional plasticity. A previous study revealed that TANs resemble the two-sided phenotype of tumor-associated macrophages: the 'N1' type, which inhibits tumor growth, and the 'N2' type, which promotes tumor growth and malignant metastasis (4). Most studies have revealed a significant association between TANs and poor prognosis in various tumors, such as liver, head and neck, and kidney cancers; however, higher TAN infiltration is significantly associated with longer survival in gastric and colon cancer (5-9). CD177 is a surface protein unique to neutrophils with stable expression and an adhesion molecule involved in the adhesion between neutrophils and endothelial cells and the migration of neutrophils, can represent neutrophils that exert activity in TME (10).

As the role of CD177+ neutrophils in LUAD has not been previously elucidated, in the present study, CD177+ neutrophils were detected in LUAD using immunohistochemistry and their association with clinical characteristics was analyzed.

Additionally, the prognostic value in the survival of patients with LUAD was evaluated.

Materials and methods

Reagent. The CD177+ polyclonal antibody was purchased from Wuhan Boster Biological Technology, Ltd (cat. no. PA5-83575).

Patients and clinical tissue specimens. Between January 2021 and August 2021, at the Department of Thoracic Surgery, tumor (T) and adjacent normal (DN) tissues were collected from 16 patients with LUAD who underwent curative surgery at Jining First People's Hospital (Jining, China). These tissue samples were used for immunohistochemical analyses. Moreover, 120 qualified formalin-fixed paraffin-embedded tissue samples were obtained from patients with LUAD at the Department of Pathology in Jining First People's Hospital (Jining, China) from January 2013 to June 2017 and subjected to immunohistochemical analysis. Tumor tissues were collected from LUADs pathologically confirmed after thoracic surgery. The samples were obtained from respiratory or oncology biopsies conducted via bronchoscopy or percutaneous lung puncture. Notably, all patients included in the present study had not undergone any prior antitumor therapy and had no comorbidities related to other tumors, autoimmune diseases, or infectious diseases. Therapeutic choices were made in accordance with the prevailing yearly Chinese Society of Clinical Oncology guidelines on the diagnosis and treatment of NSCLCs based on stage, condition and family selection. All patients were followed-up over telephone, outpatient follow-up, and case data review; the date of diagnosis was marked as the starting time of follow-up. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of tumor progression (in any aspect). Overall survival (OS) was estimated from the date of diagnosis to the date of death or last follow-up. The follow-up period was up to June 2022. The present study followed the principles of the Declaration of Helsinki (2013) and was approved by the Ethics Committee of Jining First People's Hospital (approval no. 2020-035). Written informed consent was provided from all prospective patients for participation in the study. The retrospective study was approved a request to waive the documentation of informed consent.

Hematoxylin and eosin (H&E) staining. Tissue sections (4-μm thick) were heated at 65˚C for 30 min and were deparaffinized by conventional xylene, and dehydrated with gradient alcohol. Further, the sections were stained with hematoxylin for 3-5 min at room temperature and washed. The sections were then reblued with rebluing solution for 45-60 sec at room temperature and washed, followed by which they were immersed in 95% alcohol for 30-45 sec and stained with drops of eosin staining solution for ~30 sec. The sections were then dehydrated with gradient alcohol, made transparent with xylene and sealed with neutral gum.

Immunohistochemistry. Tissue sections (4-μm thick) were heated at 65˚C for 1 h and were deparaffinized by conventional xylene and dehydrated with gradient alcohol. To facilitate antigen retrieval, the sections were heated in sodium chloride/sodium citrate cleaning solution at 95˚C for 30 min. After achieving antigen retrieval, the sections were treated with 3% H_2O_2 at 37˚C for 15 min to inactivate endogenous peroxidase. The sections were rinsed with phosphate-buffered saline and incubated with CD177+ polyclonal antibody diluted at 1:200 overnight at 4˚C. After which the IHC-specific secondary antibody was added to the tissue and incubated for 30 min. The sections were washed, subjected to DAB color development, re-stained with hematoxylin, re-blued with reblueing solution, and separated with a color separation solution. The sections were then dehydrated with gradient alcohol, made transparent with xylene and sealed with neutral gum. Finally, the sections were viewed under light microscope by two independent pathologists blinded to the patients' clinical data. When each tissue section was reviewed, stromal and epithelial cells were randomly selected from six representative regions at x400 magnification. Moreover, the number of CD177+ neutrophils was analyzed, and the mean of the six counts was calculated. The number of CD177+ neutrophils in the tumor and paracancerous tissues was determined as the mean number of positively stained cells per high-power microscopic field [high-power field (HPF), x400). The patients were divided into high- and low-density groups according to the median number of CD177+ neutrophils (11).

Statistical analysis. All data are expressed as the mean ± SEM and were analyzed using SPSS V.22.0 (IBM Corp.). An unpaired two-tailed Student's t test was performed for statistical comparisons, and a chi-square test was used to evaluate the association between CD177+ neutrophil density and the clinicopathological characteristics of patients with LUAD. Based on the median density (6 cells/HPF) of CD177+ neutrophils, patients with LUAD were divided into the high-density group and low-density group, and PFS and OS curves were plotted using the Kaplan-Meier method and compared utilizing the log-rank test. Univariate and multivariate COX risk regression analysis were employed to detect prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased infiltration of CD177+ neutrophils in tumor tissues of LUAD. CD177+ neutrophils may signify active neutrophils within the TME. The potential increase of the infiltration of CD177+ neutrophil in the tumor tissues of patients with LUAD was investigated. Neutrophils were detected in tumor (T) tissues and adjacent normal (DN) tissues. It was observed through H&E staining that neutrophils infiltrated higher in 'T' compared with 'DN' of LUAD (Fig. 1A and B); then it was also observed through immunohistochemistry that the CD177+ neutrophils infiltrated higher in 'T' compared with 'DN' of LUAD (Fig. 1C-E).

Association of CD177+ neutrophils with clinical characteristics of LUAD. To further examine whether CD177+ neutrophil infiltration is associated with the clinical characteristics of LUAD, 120 paraffin-embedded LUAD tissue specimens were collected and used for immunohistochemical analysis. The clinical characteristics of the 120 LUAD patients (including sex and age distribution) enrolled in the present study are summarized in Table I. There was no statistical difference in treatment regimen

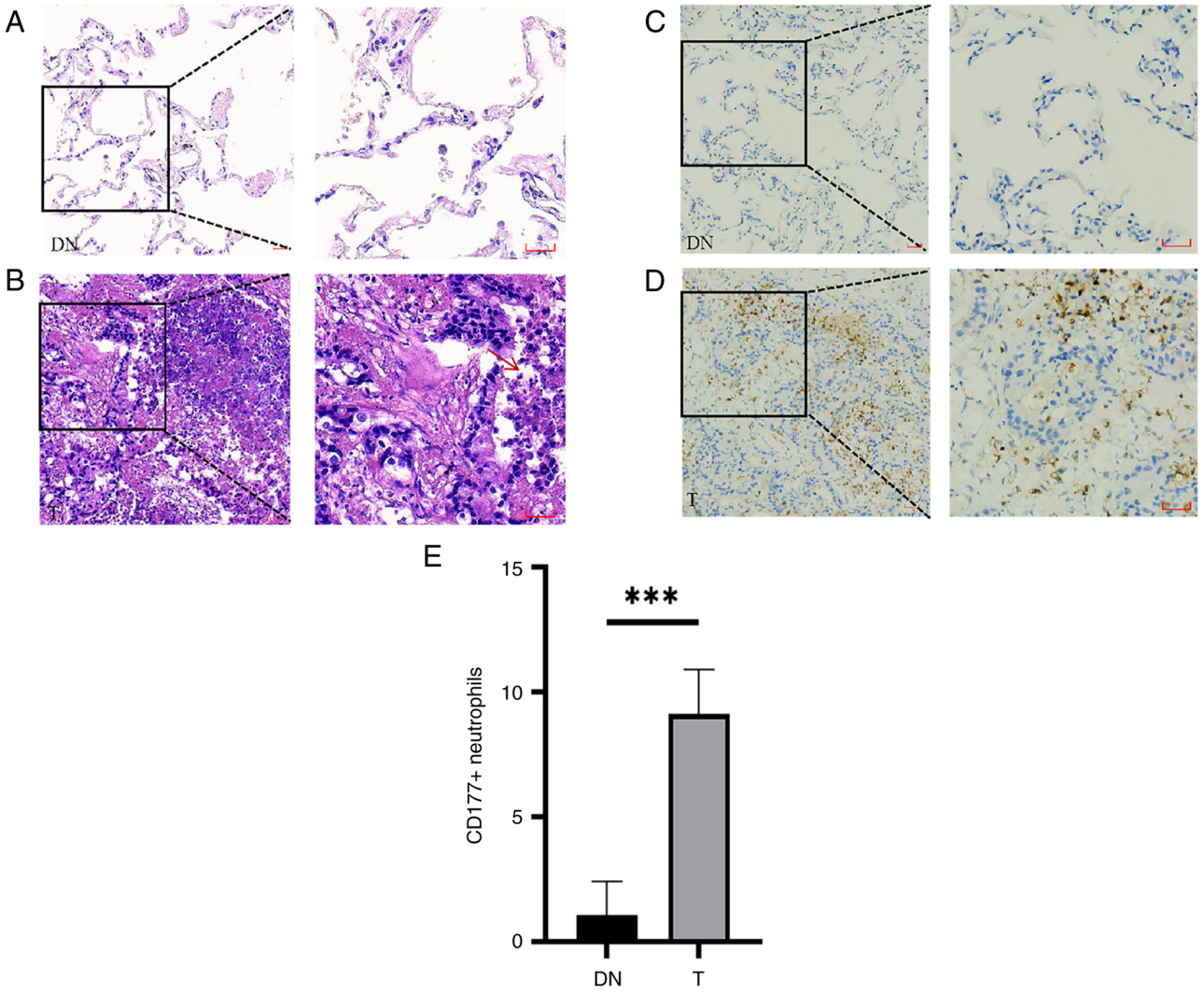

Figure 1. CD177⁺ neutrophils significantly infiltrating in tumor tissues compared with distant normal tissues of lung adenocarcinoma. (A and B) Representative images of H&E staining in T and DN tissues. (C and D) Representative images of immunohistochemical staining of CD177⁺ neutrophils' infiltration in T and DN tissues. (E) CD177⁺ neutrophils infiltration infiltrating differently in the T and DN. The arow indicate neutrophil in the figure. ***P<0.001. Scale bar, 50 μm. T, tumor; DN, distant normal.

selection between the two populations. Through H&E staining, the variations in neutrophils infiltration among different patients with LUAD were observed (Fig. 2A and B). Additionally, immunohistochemistry revealed distinct patterns of CD177⁺ neutrophil infiltration in different patients with LUAD (Fig. 2C and D). Based on the median density (6 cells/HPF) of CD177⁺ neutrophils, 120 patients with LUAD were divided into the high-density group (≥6 cells/HPF) and low-density group (<6 cells/HPF) (7). A significant association between the density of CD177⁺ neutrophil infiltration, TNM stage and tumor differentiation (P<0.05) was observed; however, no significant association was observed between age, sex, tumor size, smoking index, and lymph node metastasis (P>0.05) (Table II). These differences indicated that increased CD177⁺ neutrophil infiltration in tumor tissues was associated with the malignant progression of LUAD.

Association of CD177⁺ neutrophils with prognosis of LUAD. During a 96 months follow-up period (ranging from 2.5 to 96 months), the association between CD177⁺ neutrophil infiltration and PFS and OS of LUAD patients was further examined using Kaplan-Meier survival curve analysis. It was revealed that patients with high-density of CD177⁺ neutrophils had shorter PFS (25.48±2.18 months vs. 43.56±4.56 months) and OS (38.16±2.63 months vs. 55.65±4.09 months) than those with low-density in LUAD (Fig. 3A and B, respectively). These data indicated that a higher density of CD177⁺ neutrophils was associated with poor PFS and OS in patients with LUAD.

Univariate and multivariate analyses were performed to assess whether CD177⁺ neutrophils served as independent indicators of PFS and OS. Univariate analysis revealed that the CD177⁺ neutrophils [PFS: Hazard Ratio (HR), 2.063; 95% CI, 1.367-3.113; P=0.001. OS: HR, 2.184; 95% CI, 1.446-3.298; P<0.001], TNM stage (PFS: HR, 3.269; 95% CI, 2.098-5.095; P<0.001. OS: HR, 3.290; 95% CI: 2.122-5.101; P<0.001), lymph node metastasis (PFS: HR, 2.592; 95% CI: 1.723-3.901; P<0.001. OS: HR, 2.813; 95% CI, 1.878-4.212; P=0.001) and poor tumor differentiation (PFS: HR, 6.779; 95% CI, 2.378-19.321; P<0.001. OS: HR, 6.732; 95% CI, 2.364-19.174; P<0.001) were the major factors affecting the OS and PFS of LUAD patients (Tables III and IV). Moreover, following

Table I. Clinical characteristics of patients with lung adenocarcinoma.

Clinicopathological characteristics (n=120)	N (%)
Age, years	
<60	58 (48.3)
≥60	62 (51.7)
Sex	
Male	59 (49.2)
Female	61 (50.8)
Smoking index	
0	72 (60.0)
>0	48 (40.0)
TNM stage	
I	41 (34.2)
II	27 (22.5)
III	27 (22.5)
IV	25 (20.8)
T stage	
T1	48 (40.0)
T2	45 (37.5)
T3	22 (18.3)
T4	5 (4.2)
Lymph node metastasis	
Positive	59 (50.0)
Negative	61 (50.0)
Differentiation	
Poor	36 (25.0)
Moderate	76 (37.5)
Well	8 (37.5)

adjustment for all potential clinical factors that may influence PFS and OS through multivariate Cox regression analyses, the density of CD177+ neutrophils (PFS: HR, 1.759; 95% CI, 1.162-2.661; P=0.008. OS: HR, 1.987; 95% CI, 1.304-3.026; P=0.001), TNM stage (PFS: HR, 2.519; 95% CI, 1.525-4.162; P<0.001. OS: HR, 2.384; 95% CI, 1.461-3.891; P=0.001) and lymph node metastasis (PFS: HR, 1.691; 95% CI, 1.065-2.685; P=0.026. OS: HR, 2.054; 95% CI, 1.326-3.181; P=0.001) along with poor tumor differentiation (PFS: HR, 3.620; 95% CI, 1.182-11.090; P=0.024. OS: HR, 3.602; 95% CI, 1.208-10.743; P=0.022) were independent indicators for predicting the PFS and OS in LUAD patients (Tables III and IV).

In summary, these data indicated that tumor associated CD177+ neutrophils may be independent and unfavorable prognostic biomarkers for LUAD.

Discussion

Lung cancer is a global health concern with the highest rates of morbidity and mortality. Among its subtypes, LUAD holds the highest proportion, significantly impacting human health (12). Chronic inflammation is closely related to lung cancer (13). Neutrophils are the most abundant immune cells, serving as the organism's first line of defense against infection and responding to various inflammatory signals, even in cancer (14). In previous years, the correlations between the neutrophil-to-lymphocyte ratio (NLR) in the peripheral blood and the treatment effectiveness and prognosis of malignant tumors have been extensively studied. Most of the studies have found that a higher NLR is associated with poorer survival and tumor recurrence (15-17); therefore, concern is growing regarding neutrophil infiltration in tumors.

In malignant tumors, an increase in neutrophils or NLR usually leads to a systemic inflammatory reaction. This can lead to a poor prognosis by inhibiting tumor cell apoptosis, promoting angiogenesis, inducing DNA damage, and promoting tumor cell proliferation and metastasis (18-20). Studies identified NLR as a potential prognostic marker strongly associated with outcomes and responsiveness to various treatments, including anti-angiogenic therapy such as bevacizumab, chemotherapy, radiotherapy and immunotherapy in patients with lung cancer (21-25). However, limited studies have investigated the relationship between TAN and the therapeutic efficacy and prognosis of different lung cancer treatment regimens.

TANs can be detected in most intratumoral stroma; they play important roles in tumor progression, angiogenesis, cell migration, extracellular matrix modification and immunosuppression, and can also affect the growth and progression of almost all solid tumors (26,27). In the present study, a higher infiltration of CD177+ neutrophils in LUAD tumor tissues was discovered. An association between the density of CD177+ neutrophils and clinical characteristics of LUAD was also revealed. CD177+ neutrophils were significantly associated with the TNM stage and tumor differentiation, indicating that CD177+ neutrophil infiltration in LUAD was associated with malignant progression. During the collection of cases from January 2013 to June 2017, immunotherapy had not been implemented in China. Nevertheless, additional fresh lung tissues for the detection of TAN using flow cytometry, western blotting, or multiple immunofluorescence techniques can be acquired. Future studies will delve into the association of tumor-associated CD177+ neutrophil infiltration status with tumor immune status, pathological phenotype, microvessel density, and LUAD locus size to provide further insights into how TANs regulate the TME.

The association between TANs and the prognosis of lung cancer patients is debatable. Eruslanov *et al* (28) reported that TANs inhibit the malignant progression of lung cancer. A previous study has reported that CD66b+ neutrophils are an adverse prognostic factor in NSCLC (29). Another study found that high CD66b neutrophil density had a small effect on OS but was correlated with the incidence of recurrence after surgical resection in early stage (stage I-III) in patients with NSCLC (30). Previous studies showed that the expression of CD177+ can be used to represent the infiltration of neutrophils. It was demonstrated that high expression of CD177+ was associated with poor prognosis in pancreatic ductal adenocarcinoma (28) and that CD177+ tumor neutrophil infiltration was an adverse prognostic factor for bevacizumab treatment in colorectal cancer (31). However, CD177+ neutrophils were highly expressed in gastric and colorectal cancers. Furthermore, a high density of CD177+ neutrophils predicts a favorable prognosis in these patients and is an independent prognostic factor for OS in gastric and colorectal cancers, as

Table II. Association between CD177+ neutrophils and clinical characteristics of lung adenocarcinoma.

Clinicopathological characteristics	Density of CD177+ neutrophils		P-value
	High-density, n (%)	Low-density, n (%)	
Age, years			0.29
<60	29 (43.9)	29 (53.7)	
≥60	37 (56.1)	25 (46.3)	
Sex			0.1
Male	37 (56.1)	22 (40.7)	
Female	29 (43.9)	32 (59.3)	
Smoking index			0.55
No	38 (57.6)	34 (63.0)	
Yes	28 (42.4)	20 (37.0)	
TNM stage			0.002
I	19 (28.8)	22 (40.7)	
II	17 (25.8)	10 (18.5)	
III	22 (33.3)	5 (9.3)	
IV	8 (12.1)	17 (31.5)	
T stage			0.56
T1	25 (37.9)	23 (42.6)	
T2	23 (34.8)	22 (40.7)	
T3	15 (22.7)	7 (13.0)	
T4	3 (4.5)	2 (3.7)	
Lymph node metastasis			0.57
Positive	34 (51.5)	25 (46.3)	
Negative	32 (48.5)	29 (53.7)	
Differentiation			0.023
Poor	23 (34.8)	13 (24.1)	
Moderate	42 (63.6)	34 (63.0)	
Well	1 (1.5)	7 (13.0)	

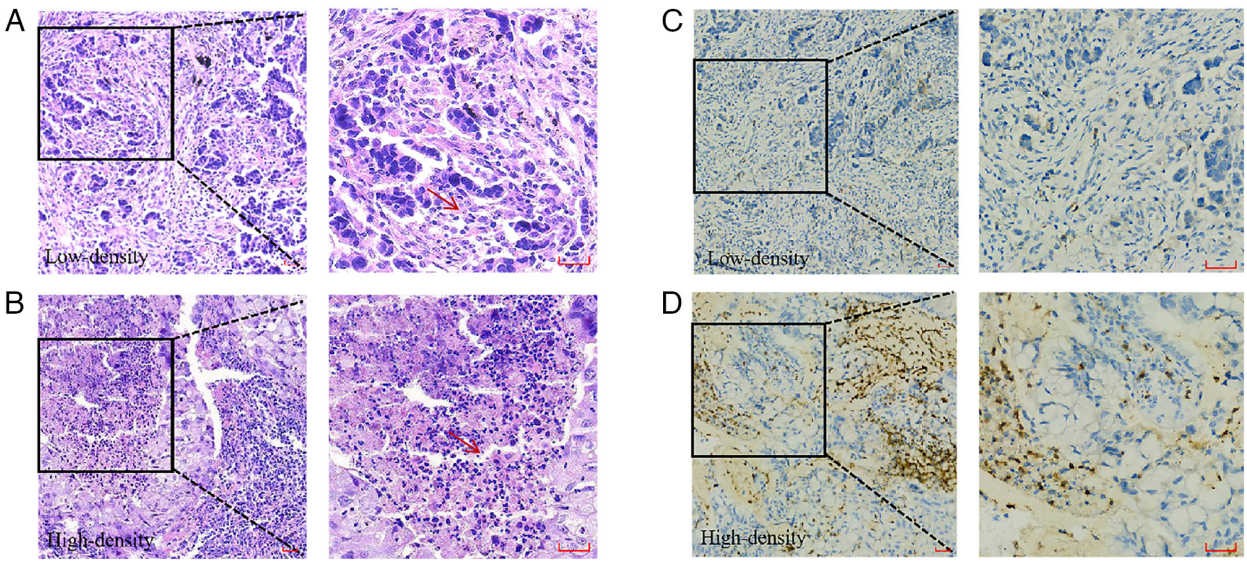

Figure 2. Tumor-associated CD177+ neutrophils infiltrating differently in different patients with LUAD. (A) Representative images of H&E staining of low-density CD177+ neutrophils infiltration in LUAD. (B) Representative images of H&E staining of high-density CD177+ neutrophils infiltration in LUAD. (C) Representative images of immunohistochemical staining of low-density CD177+ neutrophils infiltration in LUAD. (D) Representative images of immunohistochemical staining of high-density CD177+ neutrophils infiltration in LUAD. The arrow indicates neutrophil in the figure. Scale bar, 50 μm. LUAD, lung adenocarcinoma.

Table III. Univariate and multivariate analyses of progression-free survival in patients with lung adenocarcinoma.

	Univariate analysis			Multivariate analysis		
Factors	HR	95% CI	P-value	HR	95% CI	P-value
Density of CD177+ neutrophils (high vs. low)	2.063	1.367-3.113	0.001	1.759	1.162-2.661	0.008
TNM stage (IIIB + IV vs. I-IIIA)	3.269	2.098-5.095	<0.001	2.519	1.525-4.162	<0.001
Differentiation						
Poor	6.779	2.378-19.321	<0.001	3.620	1.182-11.090	0.024
Moderate	2.861	1.035-7.908	0.043	2.331	0.810-6.714	0.117
High	Ref.					
Lymph node metastasis (Positive vs. Negative)	2.592	1.723-3.901	<0.001	1.691	1.065-2.685	0.026
Smoking index (Yes vs. No)	1.654	1.114-2.458	0.013	1.212	0.787-1.864	0.383

HR, hazard ratio; CI, confidence interval.

Table IV. Univariate and multivariate analyses of overall survival in patients with lung adenocarcinoma.

	Univariate analysis			Multivariate analysis		
Factors	HR	95% CI	P-value	HR	95% CI	P-value
Density of CD177+ neutrophils (high vs. low)	2.184	1.446-3.298	<0.001	1.987	1.304-3.026	0.001
TNM stage (IIIB + IV vs. I-IIIA)	3.290	2.122-5.101	<0.001	2.384	1.461-3.891	0.001
Differentiation						
Poor	6.732	2.364-19.174	<0.001	3.602	1.208-10.743	0.022
Moderate	2.630	0.954-7.254	0.062	2.055	0.724-5.833	0.176
High	Ref.					
Lymph node metastasis (Positive vs. Negative)	2.813	1.878-4.212	<0.001	2.054	1.326-3.181	0.001
Smoking index (Yes vs. No)	1.487	1.003-2.204	0.048	1.025	0.668-1.573	0.911

HR, hazard ratio; CI, confidence interval.

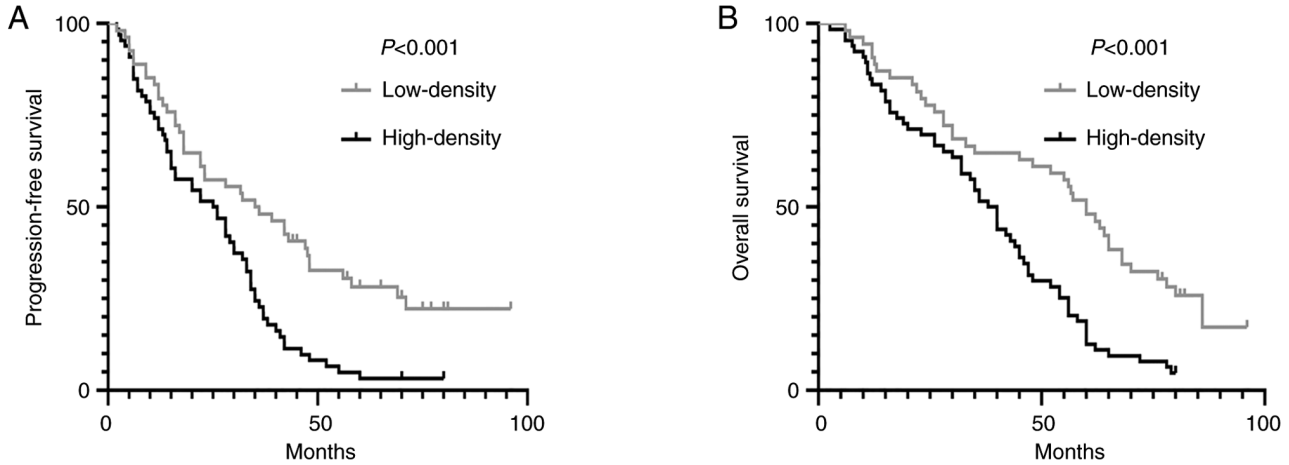

Figure 3. Kaplan-Meier analysis for the association between tumor-associated CD177+ neutrophils density and PFS and OS in LUAD patients. (A) The association between tumor-associated CD177+ neutrophils density and PFS in patients with LUAD. (B) The association between tumor associated CD177+ neutrophils density and OS in patients with LUAD. PFS, progression-free survival; OS, overall survival; LUAD, lung adenocarcinoma.

indicated by multivariate analysis (8,11). Therefore, the association between CD177+ neutrophils and the overall prognosis of LUAD was further explored. The analysis of the present study confirmed that patients with high-density of CD177+ neutrophils

had shorter PFS (25.48±2.18 months vs. 43.56±4.56 months) and OS (38.16±2.63 months vs. 55.65±4.09 months) than those with low-density in LUAD. The preliminary experiment of the present study also identified that the density of CD177$^+$ neutrophils in lung squamous carcinoma (LUSC) tumor tissues was significantly higher than that in paracancerous tissues, and a large amount of neutrophil infiltration was observed when focal necrosis was detected in LUSC. However, Rakaee *et al* (32) revealed an association between TAN infiltration in lung cancer and patient survival; high intratumoral CD66b$^+$ TANs density in the LUSC subgroup was an independent positive prognostic factor for disease-specific survival, whereas high intratumoral TANs density was an independent negative prognostic factor in the LUAD subgroup. Therefore, CD177$^+$ neutrophils in LUSC can be considered as a potential target for future studies.

The treatment plans collected included postoperative sequential chemoradiotherapy, chemotherapy combined with or without radiotherapy, and targeted therapy. It is not yet possible to analyze the predictive effect of tumor-associated CD177$^+$ neutrophil infiltration on the treatment course of a single regimen. Additionally, immunotherapy had not yet been performed in China during the period in which the cases were collected. In the future, relevant research to determine whether tumor-associated CD177$^+$ neutrophil infiltration can predict the clinical outcomes of surgery, chemotherapy, radiotherapy, or immunotherapy will be conducted. Univariate and multivariate analyses revealed that PFS and OS were significantly associated with CD177$^+$ neutrophils, TNM stage, lymph node metastasis and tumor differentiation. These findings suggested that tumor-associated CD177$^+$ neutrophils may be independent and unfavorable prognostic biomarkers for LUAD. Smoking can cause chronic inflammation in the lungs and induce lung cancer, which is a risk factor for poor prognosis of lung cancer (33,34). To the best of the authors' knowledge, this is the first study demonstrating the prognostic value of CD177$^+$ neutrophil infiltration in LUAD.

In conclusion, the present study revealed that CD177$^+$ neutrophils are functionally activated neutrophil populations that infiltrate LUAD. Tumor-associated CD177$^+$ neutrophils are highly associated with malignant progression and poor survival and may be used as an independent and unfavorable prognostic biomarker of LUAD. Therefore, tumor-associated CD177$^+$ neutrophils may serve as novel therapeutic targets for the treatment and prognosis of LUAD.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to privacy or ethical restrictions but are available from the corresponding author on reasonable request.

Authors' contributions

JZ, SJ and WW contributed to conception and design of the study. JM, CB and YW acquired and interpreted the data. QX performed the experiments. JZ and HL performed the statistical analysis. JZ wrote the first draft of the manuscript. SJ supervised the project and provided funds for the whole project. JZ and SJ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study followed the principles of the Declaration of Helsinki and was approved by the ethics committees of Jining First People's Hospital (approval no. 2020-035; Jining, China). Written informed consent was provided from all prospective patients for participation in the study. The retrospective study was approved a request to waive the documentation of informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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