Detection of PD-L1 expression and epithelial-mesenchymal transition of circulating tumor cells in non-small cell lung cancer

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Abstract. The present study aimed to assess the roles of peripheral circulating tumor cell (CTC) count, CTC subtypes and programmed death ligand 1 (PD-L1) expression in the clinical staging and prognosis of patients with non-small cell lung cancer (NSCLC). A total of 100 patients with NSCLC with available tumor tissues were enrolled in the present study, and 7.5 ml peripheral blood was collected. Patients were divided into PD-L1-positive and PD-L1-negative groups according to PD-L1 immunohistochemical staining. Peripheral blood samples from both groups were analyzed to determine the CTC count, epithelial-type CTCs (E-CTCs), mesenchymal-type CTCs (M-CTCs) and PD-L1 expression. Clinical data were collected, and patients were followed up for a maximum of 36 months, with patient death as the endpoint event. Patients with PD-L1-positive tumors had a worse prognosis compared with those with PD-L1-negative tumors (P=0.045). The PD-L1-positive group exhibited significantly higher numbers of CTCs and M-CTCs compared with the PD-L1-negative group (P≤0.05). However, the number of E-CTCs did not differ significantly between the two groups (P>0.05). PD-L1-positive patients with higher CTC and M-CTC counts had relatively poorer prognoses (P≤0.05), while the number of E-CTCs had no significant effect on prognosis (P>0.05). Compared with the early-stage NSCLC group, the late-stage NSCLC group exhibited a significant increase in the CTC count (P≤0.05), while E-CTC and M-CTC counts did not significantly differ between the two groups (P>0.05). The PD-L1-positive group exhibited a significant increase in the number of PD-L1$^+$ CTCs and PD-L1$^+$ M-CTCs compared with the PD-L1-negative group (P≤0.05), while PD-L1$^+$ E-CTC counts did not differ significantly between the two groups (P>0.05). The PD-L1-positive patients with a higher number of PD-L1$^+$ CTCs and PD-L1$^+$ M-CTCs had relatively poorer prognoses (P≤0.05), while the PD-L1$^+$ E-CTC count had no significant effect on prognosis (P>0.05). Compared with the early-stage NSCLC group, the late-stage NSCLC group exhibited a significant increase in the number of PD-L1$^+$ CTCs and PD-L1$^+$ M-CTCs (P≤0.05), while PD-L1$^+$ E-CTC counts did not significantly differ between the two groups (P>0.05). Based on univariate and multivariate analyses, the number of PD-L1$^+$ M-CTCs was identified as an independent prognostic factor for NSCLC. In conclusion, the presence of CTCs in peripheral blood, particularly PD-L1$^+$ M-CTC subtype, indicated poorer clinical staging and prognosis in patients with NSCLC. These findings suggested that CTCs, specifically the PD-L1$^+$ M-CTC subtype, could serve as a monitoring indicator for the clinical staging and prognosis of patients with NSCLC.

Introduction

Lung cancer is the most common type of cancer worldwide, ranking second in incidence and first in mortality among malignant tumors (1). Non-small cell lung cancer (NSCLC) accounts for ~85% of lung cancer (2). Despite advancements in the diagnosis and treatment of lung cancer in recent years, the 5-year survival rate of patients with lung cancer remains <20% (3). The 5-year survival rate is closely related to tumor recurrence and metastasis following treatment (4). Liquid biopsy, a convenient and non-invasive method that dynamically reflects the genetic profile of tumors, serves a crucial role in lung cancer therapy and prognostic monitoring (5). Liquid biopsy is a minimally invasive and easily repeatable test for the cytological and molecular analysis of cancer markers that are secreted from the tumor cells into the blood. The detection of circulating tumor cells (CTCs) in blood, as the earliest liquid biopsy technique, has been widely used in early tumor diagnosis, prognostic assessment, disease monitoring and treatment management (6). CTCs are rare and have a limited survival time in the bloodstream, and thus, their identification

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and isolation is challenging (7). Lung cancer is a type of epithelial cancer, and thus, the current detection method of CTCs is mainly through enriching and selecting EpCAM antigen positive cells in the blood (8,9).

CTCs are a highly heterogeneous cell population, and most CTCs entering the bloodstream undergo apoptosis due to immune recognition, mechanical damage and tumor cell-intrinsic factors, which prevent the formation of metastatic lesions. This phenomenon is referred to as 'ineffective metastasis' (10). Only a small number of CTCs with high metastatic potential survive in the circulatory system. These CTCs then aggregate to form microemboli and, under certain conditions, develop into metastatic tumors (11). It is these cells with metastatic potential that require further study. CTC-subsets retain a mesenchymal-like phenotype to adapt EMT, characterized by an up-regulation of Vimentin and N-cadherin genes (12). Because of EMT-process, tumor cells and derived-CTCs can undergo to various alterations during the early stages of carcinogenesis, leading to cancer cell dissemination and micrometast (13). Studies have reported that the EMT phenotype of CTCs in the peripheral blood of patients with NSCLC is associated with the distant metastasis of tumors (14,15). CTCs with epithelial cell markers can undergo EMT and enter nearby blood vessels for distant dissemination. During EMT, cancer cells lose their epithelial characteristics due to the downregulation of epithelial genes, including E-cadherin, EpCAM and β-catenin, and obtain mesenchymal properties due to the upregulation of mesenchymal genes, including N-cadherin, vimentin and fibronectin. Different types of CTCs have been identified by combining epithelial [EpCAM and cytokeratin (CK)8/18/19] and mesenchymal (vimentin) markers (16).

In the past few years, immune checkpoint inhibitors targeting programmed cell death-1 (PD-1) or programmed death ligand 1 (PD-L1) have revolutionized the treatment of advanced NSCLC (17). The PD-L1/PD-1 system regulates the immune response mainly through the intracellular inhibitory signal transduction mechanism of effector T cells and regulatory T cells (18). In the tumor microenvironment, combination of PD-L1 and PD-1 can inhibit the initiation and activation of T cells and promotes cancer progression (19). Therefore, PD-L1 serves a key role in the immune escape of cancer cells. By blocking the interaction between PD-1 and PD-L1, these inhibitors enable the immune system to eliminate tumors. Notably, PD-L1 is also the only approved predictive biomarker in clinical practice for monitoring the application of anti-PD-1 drugs in NSCLC (20). Currently, researchers primarily detect PD-L1 expression in tumor tissues (21), whereas the expression status of PD-L1 on CTCs remains unclear.

Therefore, in addition to the expression of PD-L1 in tumor tissues, the present study aimed to assess the expression of PD-L1 and EMT markers on CTCs to evaluate the feasibility of detecting these as indicators for clinical staging of NSCLC and patient prognosis, and to identify suitable biomarkers for improved assessment of NSCLC occurrence and progression, as well as patient prognosis.

Patients and methods

Subjects. The present study included 100 patients with NSCLC enrolled at the Department of Respiratory Medicine, The Second Affiliated Hospital of Jiaxing University (Jiaxing, China) between January 2021 and June 2023. The inclusion criteria were as follows: i) Pathologically confirmed diagnosis of NSCLC; ii) availability of suitable tissue samples for immunohistochemical testing; and iii) collection of 7.5 ml peripheral blood from patients before treatment. The exclusion criteria were as follows: i) Age <18 or >85 years; ii) history of other malignant tumors; iii) severe impairment of liver or kidney function or severe congestive heart failure, as ascites or edema can cause peripheral blood to be diluted; iv) active infection defined as a sharp increase in the number of white blood cells, which leads to a decrease in the detection rate of CTCs; v) irreversible coagulation disorders and marked hemogram abnormalities or evident bleeding tendencies; vi) a history of cranial or brain injury or trauma; and vii) patients lost to follow-up during the study.

Sample collection. Tumor tissue samples were obtained from patients who were pathologically diagnosed with lung cancer and treated with chemotherapy. The samples were selected for immunohistochemical testing of PD-L1 expression. Peripheral blood samples (7.5 ml) were collected from 100 patients with NSCLC before chemotherapy and placed in EDTA-K_2 solution. The blood samples were obtained in the middle of the venipuncture procedure after the first 5 ml of blood was discarded, to avoid contamination by epithelial cells from the skin. A total of 7.5 ml peripheral blood was prepared for CTC detection.

Immunohistochemical staining. The fresh tissues of NSCLC patients were fixed with 10% neutral formalin (pH 7.0) for 24 h at room temperature. Paraffin-embedded tissue was performed on 3-μm-thick sections and dewaxing. The tissues were then treated with 3% H_2O_2 at room temperature for 5-10 min and washed with PBS. Sections were placed in citrate buffer (pH 6.0) at 95˚C for 15 min, cooled at room temperature and washed with PBS. The sections were blocked with 10% normal goat serum (Balb, WK300, China) at room temperature for 10 min, and the serum was discarded without washing. Subsequently, the PD-L1 antibody (1:100; cat. no. 15165; Cell Signaling Technology, Inc.) was added, and the sections were incubated overnight at 4˚C. Sections were rinsed with PBS for 5 min, after which 50 μl biotin-conjugated goat anti-rabbit IgG (cat. no. as-7002, 1:1,000, Guangzhou Ascend Biotechnology Co., Ltd.) was added and the sections were incubated at 37˚C for 30 min. After rinsing with PBS for 5 min, the sections were stained with 3,3'-diaminobenzidine for 10 min. Sections were then thoroughly rinsed with PBS, counterstained with hematoxylin at room temperature for 3 min and rehydration in descending alcohol series for 5 min. Finally, the sections were cleared in xylene and observed under a light microscope.

The IHC sections were analyzed by three independent investigators and determined manually. PD-L1 expression in tumor tissue was classified according to the percentage of cells with a positive score for staining. The sum of intensity (0: negative; 1: weak; 2: clear; 3: strong) and percentage (0: 0-1%; 1: 1-10%; 2: 10-25%; 3: 25-50%; 4: 50-75%; 5: 75-100%). The positivity rate of PD-L1 staining was defined as 1% (22). According to IHC, patients were divided into two groups: PD $L1^+$ group (≧1%) and PD $L1^-$ group (<1%).

H&E staining. Paraffin sections were deparaffinized with xylene and rehydration in descending alcohol series for 5 min. They were washed with distilled water once, then placed in hematoxylin for 5 min and washed with distilled water again after staining. Paraffin sections were soaked in hydrochloric acid alcohol (1%) for 30 sec to differentiate, and in ammonia water (1%) for 30 sec back to blue after washed with clean water, and in eosin alcohol for 1 min to stain after washed with clean water. Paraffin sections were dehydrated with 75, 90 and 100% ethanol for 10 sec, respectively. Then washed with clean water, and soaked in xylene for 1 min. Finally, sections sealed and observed by a light microscope.

Tumor cells were identified using hematoxylin and eosin staining. Tumor cells are generally large, more than three times the size of normal cells. The nuclear chromatin is rough, the nucleolus is clear, the cytoplasm is rich and mucus vacuoles can be seen (23).

CTC detection and immunofluorescence staining. CTC detection experiments were performed using blood samples and the EpCAM/Vimentin/EGFR/Folic Acid magnetic bead system (cat. no. 2001, Hangzhou Fanglue Biotechnology Co., Ltd.) according to the product instructions. Magnetically labeled EpCAM or Vimentin or EGFR or Folic Acid WBCs are retained within the column, while unlabeled cells pass through the column. The isolated cell population was fixed using 4% neutral buffered formalin at room temperature for 15 min followed by permeabilization with 0.4% Triton X-100 for 10 min. The cells were blocked by 5% BSA) at room temperature for 30 min. The cells were then incubated with CK-FITC (1:50, Cat No. FITC-66187, Proteintech Group, Inc.), CD45-phycoerythrin (1:100, Cat No. PE-65082, Proteintech, China) and PD-L1-Alexa Fluor 647 (1:50, Cat No. CL647-65082, Proteintech, China) antibodies at room temperature in the dark for 2 h, followed by washing with PBS and DAPI staining at room temperature for 10 min. Subsequently, the coverslip was added, and excess liquid was removed. Finally, the cells were scanned using a Fluorescence microscope (LEICA DMi8, Germany) and images were collected and observed using Leica Application Suite X (Leica Germany).

The CTC interpretation criteria were as follows: i) Clear cell morphology under white light; ii) CD45 staining negative in CTCs; iii) separation of cells using EpCAM/Vimentin/EGFR/Folic Acid magnetic beads; iv) positive cells were CK^+ and $DAPI^+$. Cells with $EpCAM^+$, CK^+ and $DAPI^+$ staining were categorized as epithelial-type CTCs (E-CTCs). Cells with $Vimentin^+$, CK^+ and $DAPI^+$ staining were categorized as mesenchymal-type CTCs (M-CTCs). Cells with CK^+, $DAPI^+$ and $PD\text{-}L1^+$ staining were classified as $PD\text{-}L1^+$ CTC.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7.0 software (Dotmatics). The χ^2 test or Fisher's exact test was used to analyze the relationship between PD-L1 expression and clinical information. Data are presented as the mean ± standard deviation. Statistically significant differences between two groups were evaluated using unpaired Student's t-test. Survival analysis was performed using the log-rank test to compare Kaplan-Meier curves. Univariate and multivariate Cox regression analyses were used to identify independent prognostic factors. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Expression and clinical significance of PD-L1 in the tumor tissues of patients with NSCLC. Blood samples from 100 patients with NSCLC were used in the present study. The mean age of the patients was 62.8±10.3 years (range, 39-82), 43 patients were male and 57 patients were female. The histological types included adenocarcinoma (78%), squamous cell carcinoma (21%) and other NSCLC (1%). The longest follow-up time of the two groups was 36 months, the median follow-up time was 20 months and the shortest follow-up time was 4 months.

Immunohistochemical staining was performed to detect PD-L1 protein expression in the pathological sections from 100 patients with NSCLC. PD-L1 was demonstrated to be mainly expressed on the cell membrane of tumor cells (Fig. 1A-D). Based on the intensity of PD-L1 protein expression, patients with NSCLC were divided into the PD-L1-positive group and the PD-L1-negative group to assess the difference in survival time between the patient groups. There were 60 samples in the PD-L1-positive group and 40 samples in the PD-L1-negative group. Patients in the PD-L1-positive group had a significantly shorter overall survival (OS) time compared with those in the PD-L1-negative group ($P \leq 0.05$; Fig. 1E). Furthermore, in the analysis of clinical characteristics of patients with NSCLC, there were statistically significant differences in TNM stage, tumor differentiation and diameter and lymph node metastasis between the PD-L1-positive group and the PD-L1-negative group ($P \leq 0.05$). However, there were no statistically significant differences between the two groups in terms of sex, age, smoking history, pathological type and tumor location ($P > 0.05$; Table I).

Number of CTCs and CTC subtypes in patients with NSCLC and their relationship with prognosis. The EpCAM/Vimentin/EGFR/Folic Acid magnetic bead system was used to isolate the positive cell population ($EpCAM^+$ cells, $Vimentin^+$ cells, $EGFR^+$ cells, $Folic\ Acid^+$ cells) from peripheral blood samples. Cell morphology was observed under light microscope, immunofluorescence staining with CK, CD45 and DAPI was then used to detect $CK^+/DAPI^+/CD45^-$ cells, which were defined as CTCs (Fig. 2A). Furthermore, E-CTCs and M-CTCs were identified by isolating $EpCAM^+$ and $Vimentin^+$ cell populations using the magnetic bead system, and then detecting CK, CD45 and DAPI using immunofluorescence staining.

Peripheral blood samples from 100 patients with NSCLC demonstrated that the presence of CTCs per 7.5 ml peripheral blood were 9.92±0.42 in the PD-L1-negative group and 13.42±0.58 in the PD-L1-positive group. The quantity of CTCs in the PD-L1-positive group was significantly higher compared with the PD-L1-negative group ($P < 0.0001$). The quantity of E-CTCs in the PD-L1-negative group was 6.02±0.37 and the quantity of E-CTCs in the PD-L1-positive group was 6.64±0.31. There was no statistically significant difference between the two groups in terms of E-CTC counts ($P = 0.20$). The quantity

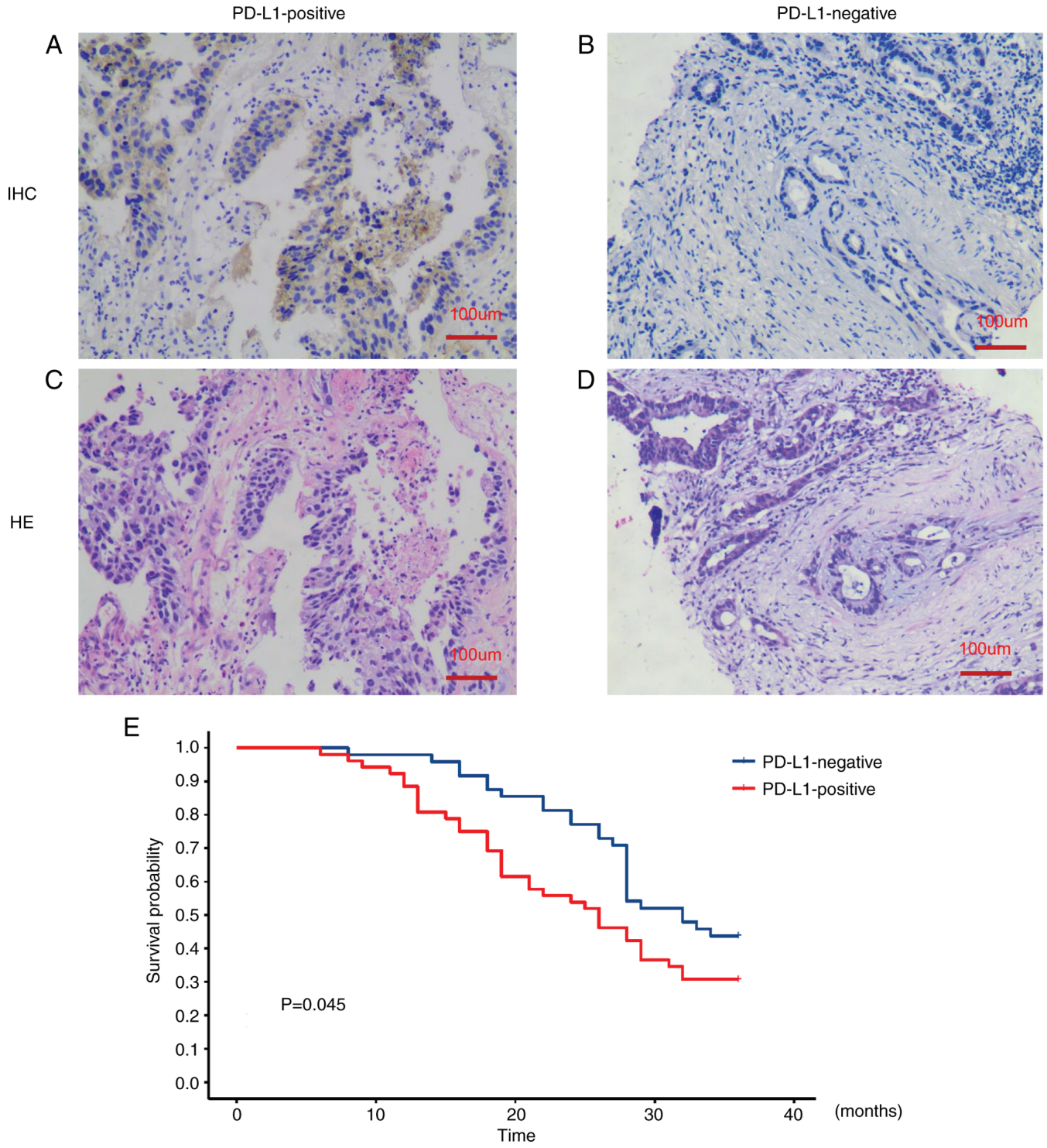

Figure 1. Expression of PD-L1 in tumor tissues and prognosis of patients with NSCLC. (A) PD-L1-positive and (B) PD-L1-negative IHC staining in tumor tissues of patients with NSCLC. (C) PD-L1-positive and (D) PD-L1-negative HE staining in tumor tissues from patients with NSCLC (Scale bar, 100 μm). (E) Kaplan-Meier overall survival probability analysis of PD-L1-positive and PD-L1-negative patients. HE, hematoxylin and eosin; IHC, immunohistochemistry; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1.

of M-CTCs in the PD-L1-negative group was 3.90±0.25 and the quantity of M-CTCs in the PD-L1-positive group was 6.79±0.54. The quantity of M-CTCs in the PD-L1-positive group was significantly higher compared with that in the PD-L1-negative group (P=0.001) (Fig. 2C).

The quantity of CTCs in the stage I-II NSCLC group was 10.07±0.55 and the quantity of CTCs in the stage III-IV NSCLC group was 11.91±0.46. The quantity of CTCs in the stage III-IV NSCLC group was significantly higher compared with that in the stage I-II NSCLC group (P=0.02). Furthermore, the quantity of E-CTCs in the stage I-II NSCLC group was 5.77±0.40 and the quantity of E-CTCs in the stage III-IV NSCLC group was 6.30±0.28. There was no statistically significant difference between the two groups (P=0.29). Furthermore, the quantity of

Table I. Baseline demographic and clinical characteristics of the patients with non-small cell lung cancer.

Characteristic	PD-L1-positive, n (n=60)	PD-L1-negative, n (n=40)	χ^2	P-value
Sex			0.109	0.741[a]
Male	25	18		
Female	35	22		
Age, years			1.127	0.288[a]
≤60	32	17		
>60	28	23		
Smoking history			1.255	0.262[a]
Yes	23	11		
No	37	29		
Pathological type				0.678[b]
Adenocarcinoma	48	30		
Squamous cell carcinoma	11	10		
Mixed carcinoma	1	0		
TNM stage			4.342	0.037[a,c]
Stage I-II	15	18		
Stage III-IV	45	22		
Tumor differentiation			5.402	0.020[a,c]
High-moderate	19	22		
Poor	41	18		
Tumor location			0.432	0.511[a]
Left	28	16		
Right	32	24		
Tumor diameter, cm			3.991	0.045[a,c]
≥3	38	17		
<3	22	23		
Lymphatic metastasis			6.750	0.009[a,c]
Yes	41	18		
No	19	22		

[a]χ^2 test. [b]Fisher's exact test. [c]P<0.05. PD-L1, programmed death ligand 1.

M-CTCs in the stage I-II NSCLC group was 4.30±0.41 and the quantity of M-CTCs in the stage III-IV NSCLC group was 5.61±0.71. There was no statistically significant difference between the two groups (P=0.06) (Fig. 2D).

A previous study has reported an association between a baseline level of ≥5 CTCs/7.5 ml peripheral blood and poor OS in patients with prostate cancer (24). By grouping patients based on the number of CTCs (>5 or ≤5), it was demonstrated that CTCs >5, patients in the PD-L1-positive group had a worse overall survival compared to PD-L1-negative group, while CTCs ≤5, patients in the PD-L1-negative group had an improved overall survival compared with PD-L1-positive group (Fig. 2B).

Furthermore, patients with PD L1$^+$ M-CTCs had a worse prognosis compared with those with PD L1-M-CTCs (P=0.03; Fig. 2E); however, there was no significant difference in prognosis between the two groups based on E-CTCs (P=0.31; Fig. 2F).

PD-L1 expression in patients with different CTC numbers and subtypes and its relationship with prognosis. Immunofluorescence staining was performed to detect the protein expression levels of PD-L1 in CTCs from the peripheral blood samples of 100 patients with NSCLC (Fig. 3A). The results demonstrated that the quantity of PD-L1$^+$ CTCs in the PD-L1-negative group was 5.79±0.32 and the quantity of PD-L1$^+$ CTCs in the PD-L1-positive group was 7.89±0.51. The quantity of PD-L1$^+$ CTCs in the PD-L1-positive group was significantly higher compared with that in the PD-L1-negative group (P<0.001). The quantity of PD-L1$^+$ M-CTCs in the PD-L1-negative group was 2.65±0.25 and the quantity of PD-L1$^+$ M-CTCs in the PD-L1-positive group was 4.50±0.44. The quantity of PD-L1$^+$ M-CTCs in the PD-L1-positive group was significantly higher compared with that in the PD-L1-negative group (P<0.001). The quantity of PD-L1$^+$ E-CTCs in the PD-L1-negative group was 3.15±0.19 and the quantity of PD-L1$^+$ E-CTCs in the PD-L1-positive group was 3.39±0.23. There was no statistically significant difference between the two groups (P=0.43) (Fig. 3C).

Furthermore, the quantity of PD-L1$^+$ CTCs in the stage I-II NSCLC group was 5.80±0.41 and the quantity of PD-L1$^+$ CTCs in the stage III-IV NSCLC group was 7.34±3.26. The

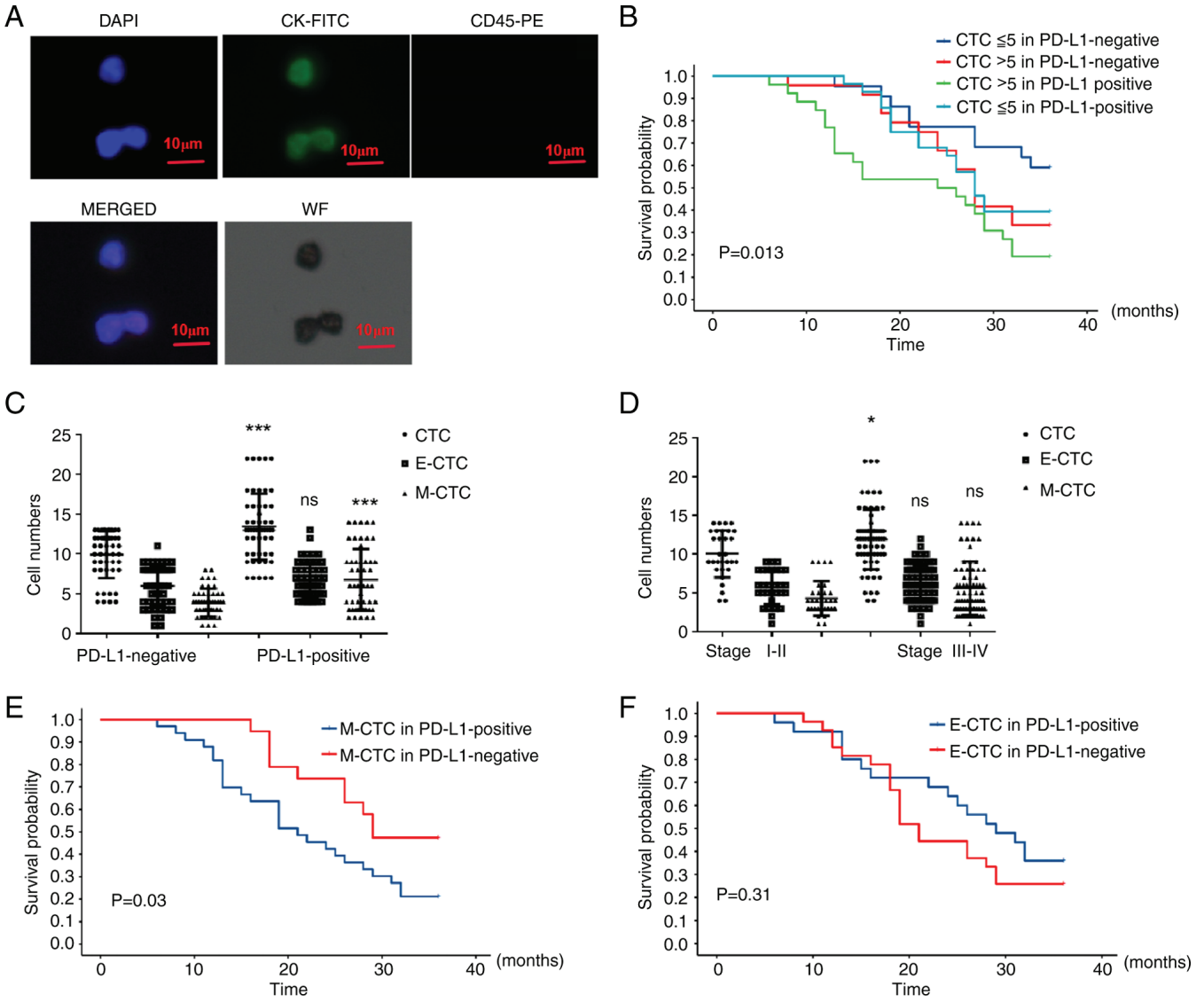

Figure 2. Quantification of CTCs in patients with NSCLC and association with prognosis. (A) Representative images of cells stained with DAPI, CK-FITC and CD45-PE to identify CTCs in peripheral circulation of patients with NSCLC by immunofluorescence staining. Scale bar, 10 μm. (B) Kaplan-Meier analysis of OS in ≤5 CTC and >5 CTC groups of PD-L1-positive and PD-L1-negative patients. Quantification of CTCs, E-CTCs and M-CTCs in peripheral circulation of (C) PD-L1-positive and PD-L1-negative groups and (D) stage I-II and stage III-IV groups. Data are presented as the mean ± SD. Kaplan-Meier analysis of survival probability in (E) The OS of M-CTC on the PD-L1$^+$-group and PD-L1$^-$-group; (F) The OS of E-CTC on the PD-L1$^+$-group and PD-L1$^-$-group. *P<0.05, ***P<0.001. CK, cytokeratin; CTC, circulating tumor cell; E-CTC, epithelial-type CTC; M-CTC, mesenchymal-type CTC; ns, not significant; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; PE, phycoerythrin; WF, white field.

quantity of PD-L1$^+$ CTCs in the stage III-IV NSCLC group was significantly higher compared with that in the stage I-II NSCLC group (P=0.02). The quantity of PD-L1$^+$ M-CTCs in the stage I-II NSCLC group was 2.70±0.39 and the quantity of PD-L1$^+$ M-CTCs in the stage III-IV NSCLC group was 3.98±0.35. The quantity of PD-L1$^+$ M-CTCs in the stage III-IV NSCLC group was significantly higher compared with that in the stage I-II NSCLC group (P=0.04). The quantity of PD-L1$^+$ E-CTCs in the stage I-II NSCLC group was 2.90±0.24 and the quantity of PD-L1$^+$ E-CTCs in the stage III-IV NSCLC group was 3.43±0.19. There was no significant difference between the two groups (P=0.11) (Fig. 3D).

By grouping patients based on the number of PD-L1$^+$ CTCs (>5 and ≤5), it was demonstrated that patients in the PD-L1-positive group with >5 PD-L1$^+$ CTCs had a worse overall survival compared to PD-L1-negative group, while patients in the PD-L1-negative group with ≤5 PD-L1$^+$ CTCs had the better overall survival compared to PD-L1-positive group (P<0.0001, Fig. 3B). Patients with PD-L1$^+$ M-CTCs >5 in PD-L1-positive group had a worse prognosis compared with those in PD-L1-negative group (P<0.0001, Fig. 3E); however, there was no significant difference in prognosis between the two groups based on PD-L1$^+$ E-CTCs >5 (P=0.14, Fig. 3F).

Univariate and multivariate analysis of clinicopathological parameters associated with OS. To further determine independent prognostic factors, univariate and multivariate analysis was performed. Univariate Cox regression analysis demonstrated an association between OS and TNM stage [hazard ratio (HR), 3.943; 95% CI, 0.982-14.231; P=0.049], the quantity of M-CTCs (HR, 3.063; 95% CI, 0.560-10.353;

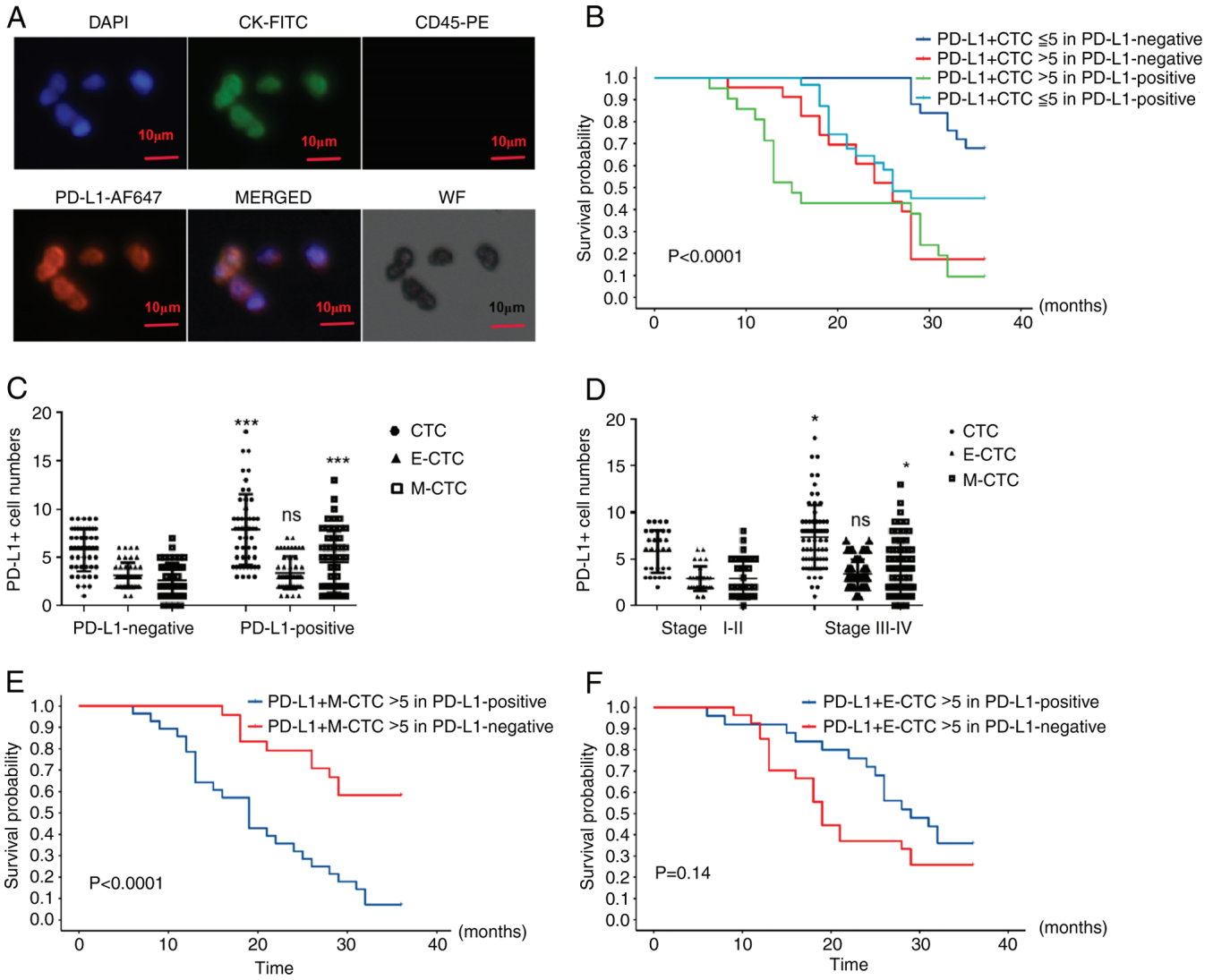

Figure 3. Quantification of PD-L1+ CTCs and CTC subtypes in patients with NSCLC. (A) Representative images of cells stained with DAPI, pcytokeratins (CK-FITC), CD45-PE and PD-L1 (AF647) to identify PD-L1$^+$ CTCs in peripheral circulation of patients with NSCLC by immunofluorescence staining. Scale bar, 10 μm. (B) Kaplan-Meier analysis of survival probability in ≤5 PD-L1$^+$ CTCs and >5 PD-L1$^+$ CTCs groups of PD-L1-positive patients, and ≤5 CTCs and >5 CTCs groups of PD-L1-negative patients. Quantification of PD-L1$^+$ CTCs, PD-L1$^+$ E-CTCs and PD-L1$^+$ M-CTCs in peripheral circulation of (C) PD-L1-positive and PD-L1-negative groups and (D) stage I-II and stage III-IV groups. Data are presented as the mean ± SD. Kaplan-Meier analysis of survival probability in (E) PD-L1$^+$ M-CTC on the PD-L1$^+$-group and PD-L1$^-$-group; (F) OS of PD-L1$^+$ E-CTC on the PD-L1$^+$-group and PD-L1$^-$-group. *P<0.05, ***P<0.001. CK, cytokeratin; CTC, circulating tumor cell; E-CTC, epithelial-type CTC; M-CTC, mesenchymal-type CTC; ns, not significant; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; PE, phycoerythrin; WF, white field.

P=0.036) and the quantity of PD-L1$^+$ M-CTCs (HR, 3.677; 95% CI, 0.108-6.579; P=0.027). Multivariate Cox regression analysis revealed an association between OS and the quantity of PD-L1$^+$ M-CTCs (HR, 4.112; 95% CI, 0.288-9.417; P=0.039) and E-CTCs (HR, 4.057; 95% CI, 1.305-20.237; P=0.013). The results demonstrated that the quantity of PD-L1$^+$ M-CTCs in the peripheral blood of patients with NSCLC was an independent prognostic factor associated with OS (Table II).

Discussion

The present study assessed the association between the number of CTCs, CTC subtypes and PD-L1 expression in CTCs and the clinical characteristics or prognosis of patients with NSCLC. The aim of the present study was to evaluate the association of PD-L1 expression on CTCs with advanced staging and poor prognosis, and to assess PD-L1$^+$ M-CTCs as potential markers for prognostic assessment and clinical staging evaluation.

CTCs are tumor cells that enter the circulatory system, which have been confirmed as a basis for tumor metastasis (7,25). With ongoing research, studies have reported the use of CTCs in early diagnosis, early chemotherapy response assessment and prognostic evaluation in various solid tumors, such as colorectal cancer, breast cancer and genitourinary tumors (26-29). The present study also demonstrated that a higher number of CTCs in the peripheral circulation of patients with NSCLC was associated with worse clinical staging and prognosis, which is consistent with the results of national and international research (15,30). Previous studies (14,15) have reported the association of total CTCs and M-CTCs with clinical characteristics, tumor genotypes and survival rates. A baseline CTC count of >5 is a poor prognostic factor for

Table II. Univariate and multivariate Cox hazard regression analysis in patients with non-small cell lung cancer.

	Univariate analysis		Multivariate analysis	
Parameter	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Sex (female vs. male)	1.527 (0.538-3.975)	0.421	2.282 (0.810-7.592)	0.649
Age (<60 vs. ≥60 years)	2.329 (0.271-17.354)	0.612	4.325 (1.339-16.354)	0.238
Smoking history, yes vs. No	0.413 (0.065-3.543)	0.214	1.267 (0.482-6.834)	0.532
Tumor differentiation (high-moderate vs. poor)	1.365 (0.914-5.951)	0.679	4.817 (1.254-10.691)	0.317
Tumor size (<3 vs. ≥3 cm)	1.987 (1.443-7.541)	0.871	2.541 (1.637-9.572)	0.657
Lymphatic metastasis (yes vs. no)	1.085 (0.833-5.955)	0.163	1.975 (0.612-7.833)	0.320
TNM stage (III- vs. I-II)	3.943 (1.232-14.231)	0.049[a]	4.057 (1.254-8.941)	0.175
CTC (≤5 vs. >5)	2.561 (0.829-5.124)	0.294	3.079 (0.927-6.938)	0.598
E-CTC (≤5 vs. >5)	3.473 (1.220-14.274)	0.096	4.057 (1.305-20.237)	0.013[a]
M-CTC (≤5 vs. >5)	3.063 (0.560-10.353)	0.036[a]	5.433 (0.341-12.174)	0.066
PD-L1$^+$ CTC (≤5 vs. >5)	3.641 (0.894-6.117)	0.053	3.221 (0.904-7.892)	0.779
PD-L1$^+$ M-CTC (≤5 vs. >5)	3.677 (0.108-6.579)	0.027[a]	4.112 (0.288-9.417)	0.039[a]
PD-L1$^+$ E-CTC (≤5 vs. >5)	1.527 (0.538-3.975)	0.421	2.282 (0.810-7.592)	0.649

[a]P<0.05. CTC, circulating tumor cell; E-CTC, epithelial-type CTC; M-CTC, mesenchymal-type CTC; PD-L1, programmed death ligand 1.

advanced NSCLC (27). Chen *et al* (31) reported that EMT was associated with tumor resistance while M-CTCs may suggest the progression of NSCLC. The results of the present study suggested an negative association between peripheral circulation of M-CTCs and the clinical staging and prognosis of patients with NSCLC. As previously reported (31), M-CTCs may potentially promote tumor cell dissemination and metastasis via EMT. However, there are limitations of CTC detection. Although liquid biopsy is a powerful method in oncology, it has disadvantages. Notably, technical inconsistencies and a lack of standardization have hindered its widespread and routine use in clinical practice. In patients with NSCLC, numerous CTCs do not have sufficient epithelial characteristics and may therefore evade detection. Therefore, the limitation of CTC detection is the lack of standardized and unified procedures. This highlights the need for multifaceted efforts to optimize and standardize accessible and efficient methods of CTC (11,13).

Due to the lack of specific clinical manifestations of NSCLC, numerous patients are diagnosed at an advanced stage and their survival time can only be extended through non-surgical treatment methods. Currently, the efficacy and safety of PD-1 inhibitors in the second-line therapy of NSCLC have been demonstrated. However, research data show that few patients with advanced NSCLC benefit from PD-1 inhibitor treatment (20). Therefore, it is necessary to identify suitable indicators to accurately assess and predict the effectiveness of PD-1 inhibitor treatment in patients with NSCLC. Currently, research on PD-L1 mainly focuses on tissue samples, with little research conducted on the evasion of immune surveillance by CTCs in the blood. During circulation in the bloodstream, most CTCs undergo EMT (15,31). Some CTCs, such as those expressing PD-L1, possess a higher degree of metastatic characteristics, possibly because this subset of CTCs is more likely to evade immune surveillance and promote tumor metastasis (32). The results of the present study also demonstrated that higher counts of PD-L1$^+$ CTCs were associated with a worse prognosis and that PD-L1$^+$ M-CTCs served as an independent risk factor for OS in NSCLC. International research on lung cancer has suggested that the proportion of PD-L1$^+$ CTCs increases after the start of radiotherapy and chemotherapy in patients with lung cancer, and the increased PD-L1 expression in CTCs is associated with poor prognosis (33). The detection of PD-L1 expression in CTCs has potential clinical applications in evaluating tumor prognosis and guiding personalized immunotherapies (34). We hypothesized that CTCs may trigger the EMT process in tumor cells via PD-L1, thereby promoting tumor progression and affecting the prognosis of patients with NSCLC. This should be verified in further study. The detection of PD-L1 expression and EMT status in CTCs may assist in assessing disease progression and prognosis in patients with NSCLC, assisting clinicians in making decisions regarding drug treatments.

The present study had numerous limitations, including the small number of samples and the fact that it is not clear whether CTCs had undergone the EMT process during immunotherapy for lung cancer. Therefore, future studies with an increased number of samples and long-term CTC detection will strengthen the current results on the efficacy of these markers. Another limitation is that the number of total CTCs in peripheral blood samples may be underestimated, since CTCs are difficult to identify and separate. There may be some non-specific fluorescence identified in the fluorescence immunoassay, and the method of detecting CTCs needs to be further optimized in the future.

The present study aimed to evaluate PD-L1 expression in the tumor tissues of patients with NSCLC, and assessed the number of CTCs, CTC subtypes and PD-L1 expression in different types of peripheral blood CTCs. The association between these factors and clinical staging and prognosis of

patients was also assessed. The results of the present study suggested that M-CTCs and PD-L1$^+$ M-CTCs on the peripheral blood CTCs may be an independent risk factor for poor prognosis in patients with NSCLC, enabling an improved evaluation of tumor occurrence and development, and potentially improving the prognosis of patients with NSCLC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HW and HC conceived and designed the study. JJ, WM and XL designed the methodology and contributed to data acquisition. JJ and DC performed the statistical analyses. JJ and HW drafted the manuscript. JJ, HC and HW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study complied with the tenets of The Declaration of Helsinki and was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Jiaxing University (approval no. JXEY-2021SW007; Jiaxing, China). Written informed consent for participation was obtained from all patients in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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