



Analysis of the prognostic role and biological characteristics of circulating tumor cell-associated white blood cell clusters in non-small cell lung cancer

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Background: Recently, circulating tumor-cell-associated white blood cell (CTC-WBC) clusters have been reported to have prognostic value in some cancers. The prognostic role of CTC-WBC clusters in lung cancer has not yet been elucidated. Very little information is available about the biological characteristics of CTC-WBC clusters.

Methods: A total of 82 patients with non-small cell lung cancer (NSCLC) were included in this study, and 61 patients with advanced-stage disease were closely followed-up. All patients had blood drawn prior to treatment. Subtraction enrichment and immunostaining-fluorescence *in situ* hybridization (SE-iFISH) platform was used to isolate and identify CTCs and CTC-WBC clusters. Kaplan-Meier survival analysis and Cox regression analysis were applied to assess patient progression-free survival (PFS). Further, qualitative and quantitative analyses the size and ploidy characteristics of CTC-WBC clusters.

Results: Firstly, CTC-WBC clusters appeared more in the advanced (stage III and IV) stage (P=0.043) than in the early stage. Furthermore, the multivariable analysis (Cox proportional hazards model) revealed that the high-CTC ($\geq 7/6$ mL) group and CTC-WBC clusters ($\geq 1/6$ mL) positive group both had significantly worse PFS, with a hazard ratio (HR) of 2.89 [95% confidence interval (CI): 1.36–6.17, P=0.006] and 2.18 (95% CI: 1.07–4.43, P=0.031), respectively. In the conjoint analysis, compared to patients with < 7 CTCs/6 mL without CTC-WBC clusters, patients with ≥ 7 CTCs/6 mL with CTC-WBC clusters had the highest risk of progression (HR =7.13, 95% CI: 2.51–20.23, P<0.001). In addition, the presence of ≥ 3 -cell CTC-WBC clusters in patients may indicate a shorter PFS (P<0.05) and a higher risk of progression (HR =2.90, 95% CI: 1.06–7.89, P=0.037). Furthermore, compared with the characteristics of the total CTCs, almost all of the CTCs that could recruit WBCs were large cells (≥ 5 μ m) and exhibited polyploidy (\geq tetraploid) (both P<0.01).

Conclusions: The presence of CTC-WBC clusters was an independent prognostic factor for advanced NSCLC. The joint analysis of CTCs and CTC-WBC clusters could provide additional prognostic value to the enumeration of CTCs alone. Besides, most of the CTCs in CTC-WBC clusters were large polyploid cells.

Keywords: Circulating tumor cell (CTC); circulating tumor-cell-associated white blood cell cluster (CTC-WBC cluster); non-small cell lung cancer (NSCLC); progression-free survival (PFS)

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide. According to global cancer statistics 2020, lung cancer accounted for ~1.8 million deaths worldwide in 2020 (1). Roughly 90% percent of cancer deaths are caused by the metastatic spread of primary tumors (2). For patients with advanced lung cancer, it is important to identify real-time and effective biological markers to monitor disease progression, provide guidance for treatment measures, and improve the prognosis of patients. In addition to stage of the disease, mutated genes such as EGFR, KRAS have been validated as prognostic biomarkers in non-small cell lung cancer (NSCLC) (3,4). But obtaining such genetic information requires invasive procedures, and high sequencing costs. Currently, liquid biopsy provides a new method for predicting the prognosis of NSCLC, especially in advanced stage.

Circulating tumor cells (CTCs) are rare cancer cells that generally escape from primary or metastatic tumor lesions and enter the circulation (5), and are regarded as the “seeds” for tumor metastasis. In reality, the majority of CTCs perish within hours, primarily owing to the shear force exerted by the blood flow, immune attack, oxidative stress, and anoikis (6,7). Only very few CTCs survive, which eventually become involved in the development of relapses or metastases. Clinically, it is critical to focus on the subgroup of CTCs that are characterized by strong survival and metastatic potential. CTCs can not only exist as single cells, but also form heterogeneous clusters with white blood cells (WBCs) (8). Recent studies have revealed that WBCs play a crucial role in the promotion of tumor progression and metastasis, both in the bloodstream and tumor microenvironment (9,10). However, the role of circulating tumor-cell-associated white blood cell (CTC-WBC) clusters has been overlooked so far. The prognostic value of CTC-WBC cluster, a combination of “seed” and “soil”, has not been investigated in NSCLC. And information about the biological characteristics of CTC-WBC cluster is scarce.

The present study aimed to assess the prognostic clinical value of CTC-WBC clusters in patients with advanced NSCLC, and progression-free survival (PFS) was selected

as the study endpoint. We also assessed the biological characteristics of the subgroup of CTCs that can recruit WBCs. We present the following article in accordance with the REMARK reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-22-423/rc>).

Methods

Study subjects

This study was approved by the Ethics Committee of Huadong Hospital Affiliated to Fudan University (No. 2018K099). Patients with newly diagnosed NSCLC with no history of other malignant tumors were deemed eligible for inclusion in this study. A total of 82 patients with NSCLC were enrolled between October 2020 and June 2021. Among them, 21 patients were in early-stage (stages I and II), and 61 patients were in advanced stage (stages III and IV). All advanced patients included in the study were inoperable and were followed up. The endpoint analyzed in this study was the PFS based on the RECIST (Response Evaluation Criteria in Solid Tumors) criteria (11). PFS was calculated from the time of collection of the blood samples until the date of tumor progression or death. Individuals' age, gender, smoking history, pathology type stage and therapy elements were included as covariates to the regression analysis. Blood was drawn before the start of surgery or systemic therapies. Follow-up was conducted via clinic visits and telephone interviews. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and all patients provided informed consent.

Enrichment of CTCs from whole blood

CTCs were isolated via the subtraction enrichment according to the manufacturer's instructions (Cytelligen, San Diego, CA, USA) (12). For each patient, 6 mL of the antecubital blood was drawn into a tube containing acid citrate dextrose (ACD) anticoagulant before treatment. In most cases, the samples were processed within 24 h, with a maximum processing time of 48 h. At room temperature, the blood samples were centrifuged at 200 ×g for 15 min,

the supernatant was discarded, and 3 mL of CTC buffer was added to re-suspend the blood cells. Next, the sample was gently added to the non-hematopoietic cell separation matrix in a 50-mL centrifuge tube and centrifuged at room temperature for 6 min (350 \times g). The buffy coat was pipetted and transferred to a new 50-mL centrifuge tube and incubated with 300 μ L magnetic beads coated with the anti-Cluster Differentiation45 monoclonal antibody for 20 min at room temperature. The centrifuge tube was then placed on the magnetic frame (Promega, Madison, WI, USA) to remove the WBC-bound magnetic beads. The retained supernatant was subsequently centrifuged and cleaned to obtain approximately 100 μ L of CTCs and the WBC suspension for subsequent experimental procedures.

Immunostaining-fluorescence in situ hybridization (i-FISH)

The i-FISH was operated according to the method described in the instruction manual (Cytelligen) (13). First, immunofluorescence staining was performed on the CTCs, and the WBC suspension was obtained through subtraction enrichment. Next, 2 μ L of the citrate buffer solution was added into the cell suspension, mixed, and stood for 10 min. The staining solution was prepared by mixing 1 μ L of the CD45 staining solution and 1 μ L of the Vimentin staining solution into a 200- μ L blood cell analysis diluent. The staining solution was then thoroughly mixed with the cell suspension and incubated for 20 min at room temperature away from light. Subsequently, 5 mL of CRC was added to wash the cells and prepare 100 μ L of the cell suspension. Next, 100 μ L of the Cytelligen Fixative was added to the stained cell sample for cell fixation. The sample was then spread on Cytelligen format slides and dried overnight at 30 °C, followed by subjecting the samples to FISH. In the next step, 10 μ L of chromosome 8 (Chr8) centromeric probe (CEP8) was added to the glass slide, denatured for 10 min at 76 °C, and hybridized at 37 °C for 3 h. After washing, the slides were dried with a hairdryer, dropped into 10 μ L of the 4,6-diamino-2-phenyl indole dye solution on the specimen frame, and covered with a cover slide.

CTCs and CTC-WBC clusters image scanning and enumeration

Slides in the Cytelligen format were scanned to obtain and analyze images using the metafer-Ifish® Automated

Scanning and Image Analysis system (Carl Zeiss, MetaSystems, Cytelligen). Automatic X-Y plane scanning and the Z section were performed at 1-mm depth intervals to obtain complete fluorescence signals for each channel. The system automatically classifies and analyzes the CTCs and WBCs; CTC was defined as DAPI +/CD45-/Vim +/- with aneuploid Chr8 or DAPI +/CD45-/Vim+ with diploid Chr8; WBC was defined as DAPI+/CD45+ with diploid Chr8; and CTC-WBC cluster was defined as ≥ 1 CTC was adhered to by ≥ 1 WBC (*Figure 1*). The ploidy of Chr8 was precisely counted in all CTCs, and all samples were independently reviewed by two skilled researchers.

Statistical analyses

Categorical variables were expressed as counts (percentages) and compared using Pearson's Chi-squared tests or Yates' continuity correction analysis. The distribution of continuous variables was tested for normality via the Shapiro-Wilk test. Then, continuous variables were presented as median (first, third quartile) and compared using the Mann-Whitney *U*-test (two-sided). The survival curve was obtained by the Kaplan-Meier method and compared with the log-rank test. Associations between the presence of CTC-WBC cluster with PFS were presented as hazard ratios (HRs) and 95% confidence intervals (CI), calculated by univariate and multivariate Cox proportional hazards models. Multivariable analyses were adjusted by including clinical factors with $P < 0.2$ in univariate analysis. The α level for significance was set to 0.05. SPSS 22.0 (IBM Corporation, Chicago, USA) and GraphPad Prism 8.0.1 (GraphPad Corporation, San Diego, USA) were used for data analysis. $P < 0.05$ was considered statistically significant.

Results

The presence of CTC-WBC clusters tended to be late events in NSCLC

The present study included 82 patients with NSCLC. Among the 21 patients with early-stage cancer, 17 patients had adenocarcinomas and four patients had squamous cell carcinomas. Patients with advanced-stage cancer included 34 cases of adenocarcinomas and 27 cases of squamous cell carcinomas. Other clinical characteristics of the included patients are presented in *Table 1*. CTCs were detected in 78 of 82 (95.12%) patients, and CTC-WBC clusters were detected in 24 of 82 (29.27%) patients. The number of

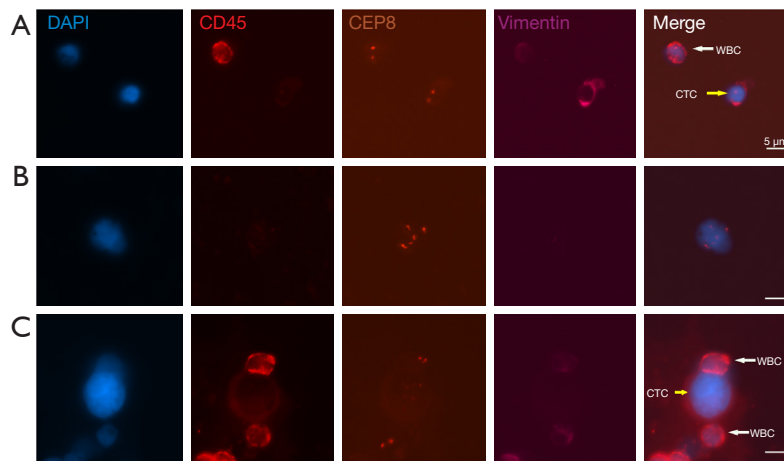


Figure 1 Examples of CTC (including diploid CTC and aneuploid CTC), WBC, and CTC-WBC cluster detected by the Subtraction Enrichment-iFISH platform. (A) Diploid CTCs are DAPI+/CD45-/Vimentin+ cells with diploid Chr8 (yellow arrow); WBCs are DAPI+/CD45+ cells with diploid Chr8 (white arrow). (B) Aneuploid CTCs are DAPI+/CD45- cells with aneuploid Chr8. (C) CTC-WBC cluster (the yellow arrow indicates CTC, and the white arrows indicate WBCs adhered to a CTC). The scale bars are 5 μ m. DAPI, 4,6-diamino-2-phenyl indole; WBC, white blood cell; CTC, circulating tumor cell; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; iFISH, immunostaining-fluorescence in situ hybridization.

Table 1 Correlation analysis between CTCs/CTC-WBC clusters and the patients' clinical characteristics

Variables	Total, n (%)	CTC count, median (IQR)	P	≥ 1 CTC-WBC cluster (%)	P
Age (years)					
≤ 60	29 (35.37)	5.00 (2.50–10.00)		8 (27.59)	
>60	53 (64.63)	7.00 (3.00–13.00)	0.321	16 (30.19)	0.804
Gender					
Male	45 (54.88)	6.00 (3.00–12.00)		11 (24.44)	
Female	37 (45.12)	6.00 (2.50–11.00)	0.914	13 (35.14)	0.290
Smoking history					
Yes	33 (39.51)	6.00 (3.00–14.00)		9 (27.27)	
No	49 (60.49)	6.00 (2.50–11.00)	0.729	15 (30.61)	0.744
Pathologic type					
Adenocarcinoma	51 (62.20)	5.00 (3.00–11.00)		16 (31.37)	
Squamous cell carcinoma	31 (37.80)	7.00 (2.00–11.00)	0.901	8 (25.81)	0.591
Stage					
I&II	21 (25.61)	3.00 (1.50–10.00)		2 (9.52)	
III&IV	61 (74.39)	7.00 (3.00–14.00)	0.022	22 (36.07)	0.043
Main therapy (III&IV)					
Targeted therapy	18 (29.51)	6.50 (4.75–22.25)		6 (33.33)	
Non-targeted therapy	43 (70.49)	7.00 (3.00–11.00)	0.330	16 (37.21)	0.774

CTC, circulating tumor cell; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; IQR, inter-quartile range.

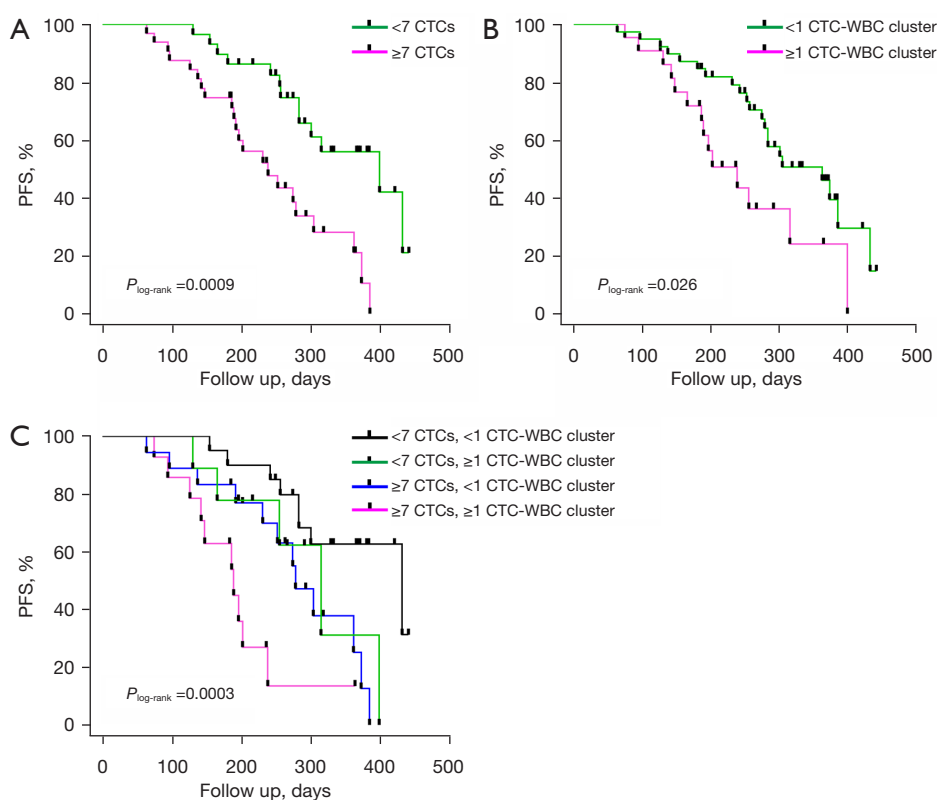


Figure 2 Analysis of CTC counts and CTC-WBC clusters with patient PFS. (A) Kaplan-Meier curves showing the difference in PFS between the high-CTC (≥ 7 CTCs/6 mL) and low-CTC (< 7 CTCs/6 mL) groups. (B) PFS analysis between CTC-WBC-cluster positive (≥ 1) and negative (< 1) groups. (C) Combined analysis of CTCs and CTC-WBC clusters with patient PFS. Kaplan-Meier curves displaying the PFS of patients with < 7 CTCs without CTC-WBC clusters, patients with < 7 CTCs with CTC-WBC clusters, patients with ≥ 7 CTCs without CTC-WBC clusters, and patients with ≥ 7 CTCs with CTC-WBC clusters. CTC, circulating tumor cell; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; PFS, progression-free survival.

CTCs and CTC-WBC clusters in each patient ranged from 0–78/6 mL and 0–17/6 mL, respectively. The results of the correlation analysis between the CTCs/CTC-WBC clusters and the clinical characteristics of the patients revealed that the total number of CTCs and CTC-WBC cluster events were only correlated with the patient's pathological stage ($P=0.022$ and 0.043 , respectively) (Table 1).

CTC counts and the presence of CTC clusters had prognostic predictive value

A total of 61 patients with advanced-stage cancer were followed up until December 24, 2021. The median follow-up time for the patients was 255 days (range, 63–441 days). Furthermore, the late-stage patients were divided into two groups based on the median number of CTCs: a high-CTC (≥ 7 CTCs/6 mL) group and a low-CTC (< 7 CTCs/6 mL)

group. There were 32 cases in the high-CTC group and 29 cases in the low-CTC group. CTC-WBC clusters were detected in 22 patients (CTC-WBC cluster positive group) and were not detected in 39 patients (CTC-WBC cluster negative group).

The PFS of patients with advanced cancer in the high-CTC group and CTC-WBC cluster positive group were significantly shorter compared to those in the low-CTC ($P=0.0009$) (Figure 2A) and CTC-WBC cluster negative ($P=0.026$) groups (Figure 2B). Univariate Cox regression analysis revealed an increased risk of progression in the high-CTC group (HR = 3.25, 95% CI: 1.56–6.78, $P=0.002$) and CTC-WBC cluster positive group (HR = 2.17, 95% CI: 1.08–4.36, $P=0.030$). Both these observations were markedly different in the multivariate Cox regression analysis, wherein other clinical variables were also included (described in Table 2).

Table 2 Univariate and multivariate analyses of NSCLC patients with PFS

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)*	P*
Gender				
Male vs. female	0.71 (0.36–1.38)	0.306		
Age				
≤60 vs. >60	0.83 (0.39–1.80)	0.643		
Smoking history				
Yes vs. no	1.17 (0.59–2.31)	0.661		
Stage				
III vs. IV	0.73 (0.34–1.57)	0.416		
Pathologic type				
Adenocarcinoma				
Squamous cell carcinoma	0.52 (0.26–1.06)	0.073	0.62 (0.27–1.43)	0.262
Main therapy (III&IV)				
Targeted therapy				
Non-targeted therapy	0.50 (0.21–1.17)	0.108	0.89 (0.33–2.39)	0.811
CTCs				
≥7 vs. <7	3.25 (1.56–6.78)	0.002	2.89 (1.36–6.17)	0.006
CTC-WBC clusters				
≥1 vs. <1	2.17 (1.08–4.36)	0.030	2.18 (1.07–4.43)	0.031

*, adjusted for pathologic type, main therapy, number of CTCs, and CTC-WBC clusters. HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer; CTC, circulating tumor cell; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster.

Combined analysis of CTCs and CTC-WBC clusters provided additional prognostic value

To further analyze the combined effect of CTCs and CTC-WBC clusters, patients with advanced cancer were divided into four risk groups: (A) patients with <7 CTCs/6 mL and without CTC-WBC clusters; (B) patients with <7 CTCs/6 mL and with CTC-WBC clusters; (C) patients with ≥7 CTCs/6 mL and without CTC-WBC clusters; and (D) patients with ≥7 CTCs/6 mL and with CTC-WBC clusters. The median PFS of patients in groups A, B, C, and D were 432, 315, 278, and 189 days, respectively. Compared to patients in group A, patients in groups C and D showed shorter PFS ($P_{\log\text{-rank}} < 0.05$, respectively) (Figure 2C), and had an increased risk of progression ($HR_{\text{group C}} = 3.09$, 95% CI: 1.15–8.36, $P = 0.026$; $HR_{\text{group D}} = 7.13$, 95% CI: 2.51–20.23, $P < 0.001$) (Table 3). Data from this cohort indicated that patients with ≥7 CTCs and CTC-WBC clusters had the highest risk of progression, and was identified as an

independent prognostic factor of PFS, which could provide additional prognostic value for CTC count alone.

Prognostic value of CTC-WBC cluster count and cluster size analysis

In order to further assess the relationship between the number of CTC-WBC clusters and patient prognosis, patients with CTC-WBC clusters were stratified according to the median number of the CTC-WBC clusters. Thirteen patients with ≤3 CTC-WBC clusters and nine patients with >3 CTC-WBC clusters were recorded. Patients without CTC-WBC clusters served as references. Patients with >3 CTC-WBC clusters showed shorter PFS ($P_{\log\text{-rank}} < 0.05$) (Figure 3A). However, this difference disappeared after adjusting for additional clinical risk factors. No significant difference in the risk of progression was observed between patients with ≤3 CTC-WBC clusters and those without CTC-WBC clusters (Table 4).

Table 3 Combined analysis of CTCs and CTC-WBC clusters with patient PFS

Variables	n (%)	CTCs		CTC-WBC	
		HR (95% CI)	P	HR (95% CI)*	P*
<7 CTCs, <1CTC-WBC cluster	20 (32.79)	1		1	
<7 CTCs, ≥1CTC-WBC cluster	9 (14.75)	2.43 (0.77–7.71)	0.130	2.77 (0.80–9.67)	0.110
≥7 CTCs, <1CTC-WBC cluster	18 (29.51)	3.25 (1.26–8.42)	0.015	3.09 (1.15–8.36)	0.026
≥7 CTCs, ≥1CTC-WBC cluster	14 (22.95)	8.03 (2.85–22.61)	<0.001	7.13 (2.51–20.23)	<0.001

*, adjusted for pathologic type, and main therapy. CTC, circulating tumor cell; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval.

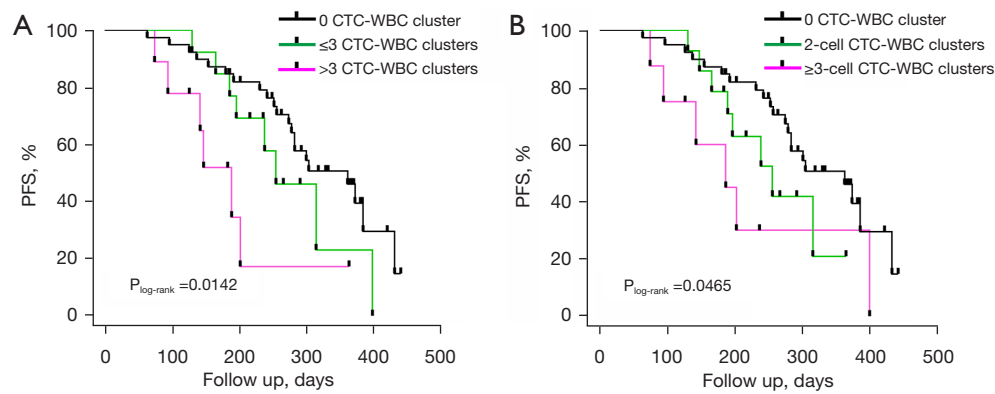


Figure 3 Prognostic value of CTC-WBC cluster count and cluster size analysis. PFS Kaplan-Meier curves for patients without CTC-WBC clusters, patients with ≤ 3 CTC-WBC clusters and patients with > 3 CTC-WBC clusters (A); and PFS Kaplan-Meier curves for patients without CTC-WBC clusters, patients with two-cell CTC-WBC clusters and patients with \geq three-cell CTC-WBC clusters (B). PFS, progression-free survival; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster.

We further evaluated the prognostic value of the CTC-WBC cluster size. In this study, the total number of CTCs plus WBCs in the CTC clusters was between two and five. Compared to patients without CTC-WBC clusters, patients with \geq three-cell CTC-WBC clusters had a shorter PFS ($P < 0.05$) (Figure 3B), and the multivariate Cox regression analysis showed an increased risk of progression (HR = 2.90, 95% CI: 1.06–7.89, $P = 0.037$) (Table 5). Patients with two-cell CTC-WBC clusters had a HR of 1.87 (95% CI: 0.81–4.34, $P = 0.142$).

Characteristics analysis of CTCs in CTC-WBC clusters

Given the clinical significance of ploidy of CTC chromosomes, CTCs were divided into three types (\leq triploid, tetraploid, and \geq pentaploid), according to the ploidy situation. The typical chromosome ploidy for CTCs is shown in Figure 4A. In our study, a total of 863 CTCs

were captured from 82 patients. Among these CTCs, 165 CTCs (19.12%) were found to be \leq triploid type, 134 (15.53%) were tetraploid type, and 564 (65.35%) were \geq pentaploid type. As for the CTC-WBC clusters, 85 CTCs were recorded in 82 CTC-WBC cluster events: two were triploid (2.35%) type; eight were tetraploid (9.41%) type; and 75 were \geq pentaploid (88.24%) type. A significant difference was recorded when the distribution of ploidy numbers was compared between the two groups ($P < 0.001$) (Figure 4B). This difference was mainly caused by more \geq pentaploid ($P < 0.05$) and less \leq triploid ($P < 0.05$) CTCs in the CTC-WBC clusters. In other words, more than 97% of the CTCs in the CTC-WBC clusters were recorded as polyploid in nature (tetraploid or higher ploidy).

CTCs are known to vary greatly in terms of size (14). Large CTCs are defined as having a maximum diameter of $\geq 5 \mu\text{m}$, while small CTCs are defined as have a maximum diameter of $< 5 \mu\text{m}$ (9). Representative images are shown

Table 4 Associations between the CTC-WBC cluster count and the PFS of NSCLC patients

Variables	n (%)	CTC-WBC		PFS	
		HR (95% CI)	P	HR (95% CI)*	P*
0 CTC-WBC cluster	39 (63.93)	1		1	
≤3 CTC-WBC clusters	13 (21.31)	1.67 (0.73–3.83)	0.226	2.18 (0.91–5.22)	0.079
>3 CTC-WBC clusters	9 (14.75)	3.72 (1.44–9.59)	0.007	2.18 (0.83–5.77)	0.116

*, adjusted for pathologic type, main therapy, and number of CTCs. CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; PFS, progression-free survival; NSCLC, non-small cell lung cancer; HR, hazard ratio; CI, confidence interval.

Table 5 Associations between the CTC-WBC cluster size and the PFS of NSCLC patients

Variables	n (%)	CTC-WBC		PFS	
		HR (95% CI)	P	HR (95% CI)*	P*
0 CTC-WBC cluster	39 (69.93)	1		1	
2-cell CTC-WBC clusters	14 (22.95)	1.82 (0.79–4.21)	0.163	1.87 (0.81–4.34)	0.142
≥3-cell CTC-WBC clusters	8 (13.11)	2.91 (1.14–7.43)	0.026	2.90 (1.06–7.89)	0.037

*, adjusted for pathologic type, main therapy, and number of CTCs. CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; PFS, progression-free survival; NSCLC, non-small cell lung cancer; HR, hazard ratio; CI, confidence interval.

in *Figure 4C*. In total CTCs, 158 (18.31%) small CTCs and 705 (81.69%) large CTCs were recorded in this study. In the CTC-WBC cluster populations, only two (2.35%) small CTCs and 83 (97.65%) large CTCs were observed ($P < 0.001$) (*Figure 4D*).

Generally, it is expected that the vast majority of CTCs would undergo rapid apoptosis or are cleared by immune cells. However, in the present study, we speculated that some CTCs in the CTC-WBC clusters would be non-senescent or apoptotic cells, whereas a group of cells would exhibit active proliferation. As shown in *Figure 4E*, a CTC in a CTC-WBC cluster was observed to be in the state of cell division.

Discussion

CTCs are rare cancer cells that are known to escape from primary lesions to enter the circulatory system. Generally, CTCs are considered to be seeds of metastasis in cancers (6,15). Several previous studies reported that the number of CTCs was related to the prognosis of multiple cancer types, including lung (16), breast (17), colon (18), and prostate (19) cancers. In the present study, it was also demonstrated that NSCLC patients with a high number of CTCs exhibited a shorter PFS.

Nevertheless, CTCs encounter a harsh environment

once within the circulation and they adopt multiple strategies to ensure their survival (20). Previous studies have demonstrated that CTCs can combine with stromal cells, such as immune cells (21), fibroblasts (22), and platelets (23). The present study focused on the prognostic value of CTC-WBC clusters and their biological characteristics in patients with advanced NSCLC. These heterogeneous CTC clusters could promote the survival of CTCs, and enhance the metastatic potential via different mechanisms. In particular, neutrophils, which account for the majority of WBCs, play a significant part in tumor growth and metastasis (24,25). Recent advances in research have indicated that neutrophils exhibit a longer life cycle and are characterized by the ability to secrete active biomolecules that promote tumor progression. Neutrophils are primarily anchored by the endothelial glycocalyx via autocrine Interleukin 8 and tumor-derived C-X-C motif chemokine ligand-1, which are known to disrupt the endothelial barrier and promote the extravasation of attached tumor cells (26).

Currently, only a few clinical studies are available on CTC-WBC clusters. To the best of our knowledge, no relevant clinical research has been previously conducted in the field of lung cancer. In our study, it was observed that CTC-WBC cluster events were more likely to occur in advanced stages of NSCLC. Moreover, the presence of CTC-WBC clusters was identified as an independent

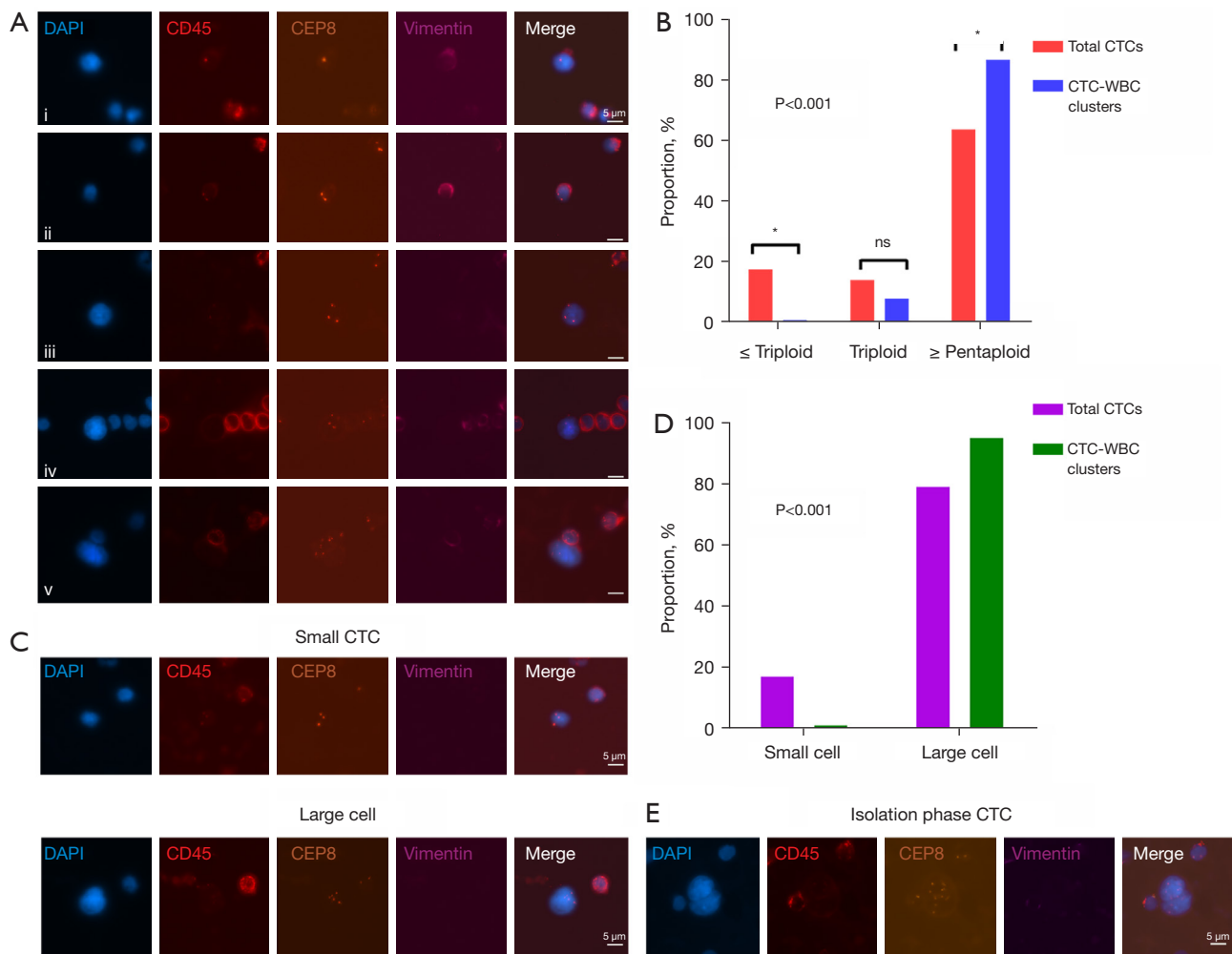


Figure 4 Characteristics analysis of CTCs in CTC-WBC clusters. (A) Schematic diagram of CTC chromosome ploidy detected by Subtraction Enrichment-iFISH platform. i-v represent CTCs with haploid, diploid, triploid, tetraploid, and \geq pentaploid Chr8, respectively. (B) Histogram of CTC ploidy distribution difference in the total CTCs and CTC-WBC clusters (*, denotes $P < 0.05$; ns, non-significant). (C) A typical image of small ($< 5 \mu\text{m}$) and large ($\geq 5 \mu\text{m}$) CTCs detected by Subtraction Enrichment-iFISH platform. (D) Histogram of the ratio of large and small CTCs to the total CTCs and CTC-WBC clusters. (E) A CTC in a CTC-WBC cluster displays a dividing nucleus with an intact cell membrane. The scale bars are $5 \mu\text{m}$. DAPI, 4,6-diamino-2-phenyl indole; CTC, circulating tumor cell; CTC-WBC cluster, circulating tumor-cell-associated white blood cell cluster; iFISH, immunostaining-fluorescence in situ hybridization.

prognostic factor for PFS. These results could help clinicians assess patient outcomes and develop treatment strategies. Similar findings have been demonstrated for advanced renal cell carcinoma (27,28), liver cancer (29), breast cancer, and gastric cancer (30,31). In addition, according to the combined analysis, patients with ≥ 7 CTCs and with CTC-WBC clusters had the highest risk of progression. Considering that the number of CTC does not always act as a reliable predictor of prognosis, the additional predictive value provided by the CTC-WBC

clusters is needed as it reinforces the clinical value of CTC enumeration without any additional testing.

In the analysis of the relationship between the number of CTC clusters and prognosis, > 3 CTC clusters exhibited a shorter PFS ($P_{\log\text{-rank}} < 0.05$), but this statistically significant difference disappeared after adjustment for other clinical factors, which may be related to the number of CTCs included in the multiple factors. In addition, another new finding of this study is the prognostic value of the size of CTC-WBC clusters. Specifically, patients with \geq three-cell

CTC-WBC clusters had a higher risk of progression (HR =2.90, P=0.037). Such heterogeneous clusters containing multiple cells may confer proliferative and metastatic advantages to CTCs (32). However, the number of patients with \geq three-cell CTC-WBC clusters was small, and thus, further evaluation of this conclusion is needed.

At present, no general understanding exists regarding the frequency of CTC-WBC clusters in various cancer types. Previous studies have reported the frequency of patients positive for CTC-WBC clusters to be in the range of 3.4–41.6% (27,29,30). Meanwhile, in the present study, the frequency of these patients was 29.27%, which might be related to the tumor types and enrichment methods. Thus, more data is required from clinical studies in order to obtain a more comprehensive understanding of CTC-WBC clusters.

The present study analyzed the characteristics of CTCs adhered to by WBCs, and we observed that most of these CTCs were large cells that exhibited polyploidy (tetraploid or higher ploidy). Polyploid tumor cells often represent an increase in genetic material and produce more mutations, which could contribute to tumor progression and drug resistance, further promoting malignant behavior (33–36). Furthermore, the recruitment of WBCs by CTCs might be related to the induction of specific mutations. In a previous study, Szczerba *et al.* reported that a group of CTCs exhibited high-impact mutations in genes, such as TLE1, which further facilitated neutrophil recruitment to CTCs (37). Moreover, WBCs might also be involved in promoting the proliferation of CTCs (37). This study provided direct evidence that CTCs in CTC clusters possess splitting ability.

The present study had some limitations that should be noted. Firstly, this study involved a small sample size from a single-center, which may limit the ability of statistical analysis. Our conclusions will be more reliable if they are subsequently verified by large multi-center clinical studies. In addition, the follow-up period in this study was inadequate, and overall survival data was not included as an endpoint of follow-up. More patients will be included and follow-up will be extended in future research.

Conclusions

In conclusion, the presence of CTC-WBC clusters could be used as an independent prognostic factor for advanced NSCLC. The combined analysis results revealed that the presence of CTC-WBC clusters could provide additional

prognostic value to the enumeration of CTCs alone. Furthermore, a larger-size CTC-WBC cluster represented a higher risk of shorter PFS. CTCs that can recruit WBCs are likely to be a specific subtype of tumor cells with proliferative ability. Therefore, attention should be paid to the phenomenon of CTC-WBC clusters both in clinical practice and basic research.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki

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