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ORIGINAL ARTICLE

The Clinical Values of Circulating Tumor Cells and T Lymphocyte Subsets in Predicting a Prognosis of Lung Cancer

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SUMMARY

Background: **Lung cancer is the most lethal cancer in men and women. Recently, it has been reported that circulating tumor cells (CTCs) are sensitive and reliable biomarkers for tracing relapse and metastasis of cancer patients. Many studies also showed that immune cellular dysfunctions in lung cancer patients are major reasons for cancer development. In this study, we explored the clinical significance of CTCs and T lymphocyte subtypes in lung cancer patients.**

Methods: **A total of 92 patients with diagnosed lung cancer, including 23 squamous-cell carcinoma and 69 adenocarcinoma, were enrolled in this study. Another 10 patients with non-carcinoma nodules in their lungs were also recruited as a control group. Peripheral blood samples were drawn from the patients with lung cancer and from the control cases before the treatment. The identification of CTCs was carried out by a PatrolCTC detection method. The T lymphocyte subtypes were characterized by flow cytometry (FACS). Cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-17A (IL-17A), interleukin-10 (IL-10), and interferon γ (IFN-γ) were detected by meso scale discovery (MSD) assay.**

Results: **Out of the enrolled patients, 69 (75%) patients with non-small cell lung cancer (NSCLC) were male and 23 (25 %) were female. Smoking and non-smoking history was 50% (46 cases) each. The case numbers for I - IV tumor-node-metastasis (TNM) stages were 23 (25.0%), 28 (30.4%), 16 (17.4%), and 25 (27.2%), respectively. The positive rates of the CTCs before treatment were 8.7% (2/23), 17.6% (5/28), 81.3% (13/16), and 100% (25/25) in stage I, II, III, and IV patients, respectively. Total CTCs, mixed CTCs, and mesenchymal CTCs (MCTCs) were strongly related to the progression-free survival (PFS) of the patients. In addition, total CTCs (≥ 6) and positive MCTCs also significantly correlated with recurrence and metastasis. The patients with high CTCs also had low levels of CD4, CD4/CD8 ratio, IL-2, and IFNγ. In contrast, IL-10 in high CTCs patients was significant elevated. These results indicate that the CTC numbers in lung cancer patients are an independent indicator for a worse PFS.**

Conclusions: **Higher total CTCs, mixed CTCs, and MCTCs in peripheral blood were significant biomarkers for predicting the prognosis of lung cancer patients. High CTCs also had a strong correlation with weak cellular immunity functions.**

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KEYWORDS

circulating tumor cells, non-small cell lung cancer, metastasis, recurrence, progression-free survival, cellular immunity

INTRODUCTION

Worldwide, non-small cell lung cancer is the most lethal cancer in men and women and accounts for 85% of the total lung cancer cases. The five-year survival rate of lung cancer depends on its tumor-node-metastasis (TNM) staging, ranging from 47% - 1% in stage I - IV, respectively. The major types of NSCLC include squamous-cell carcinoma, large-cell carcinoma, and adenocarcinoma. Among those three common types, lung adenocarcinoma (LUAC) accounts for 40% of lung cancers and occurs in non-smokers [1]. In contrast, squamous-cell carcinoma exists in smokers [2]. The therapy benefits of lung cancer also depend on its TNM staging. Generally, NSCLCs are not sensitive to radiation and chemotherapy [3]. Surgery resection is without a doubt the most effective therapeutic tool at the early stage of the cancer [4]. However, the most NSCLC patients were already in their advanced stages at the moment of their initial diagnosis, because patients frequently ignore minor symptoms and do not see doctors on time [5]. Recently, with the discovery of many specific gene mutations, including epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase [6,7], many targeted therapy drugs have been developed [6,8]. In addition, a few immune-checkpoint inhibitors, like nivolumab and pembrolizumab, also were used to treat metastatic lung cancer patient [9-11]. However, although these great progresses have happened, NSCLC still has a poorer prognosis, because it is lacking specific biomarkers for a diagnosis in its early stage. Therefore, it is urgent to identify more sensitive and reliable biomarkers for precision therapy of patients at its early stage to predict the prognosis of patients.

So far, the diagnosis of lung cancer still depends on tumor biopsies. Recently, the monitoring of circulating tumor cells (CTCs) and/or circulating tumor DNA (ctDNA) opened a new door for the management of cancer patients. There is much evidence revealing that epithelial to mesenchymal transition (EMT) mechanism contributed this process, because EMT promotes phenotypic conversion from epithelial to mesenchymal features of tumor cells [12,13]. This process usually promotes tumor cell migration, invasion, and resistance to apoptosis. When CTCs travel from primitive tumor sites to distant organs, they can perform the mesenchymal - epithelial transition [14] process to induce a new metastasis [15]. CTCs are divided into three types: epithelial CTCs (eCTCs), mesenchymal CTCs (MCTCs), and mixed CTCs, based on their surface markers [16]. CTC detection is a noninvasive method and can obtain information of metastatic tumorigenic clones [17]. In addition, peripheral blood samples from the patients are easily obtained at one or multiple time points during the diagnosis and treatment of the disease. These advantages have driven the CTC detection in lung cancer patients and achieved great benefits for the prognosis of patients.

So far, reports about the prognosis values of CTCs in lung cancer patients are limited. A study showed that monocarboxylate transporter 4 (MCT4) of CTCs in lung cancer patients indicated its metastatic status [18]. Evangelia et al. [19] reported that the detection of CTCs in lung cancer patients can guide the treatment of patients. In addition, recent studies indicate that cellular immunity dysfunctions were critical factors in the tumor genesis [20,21]. T lymphocyte is divided into CD4 and CD8 subtypes, according to its functions and surface markers [22]. CD4 T lymphocyte is further characterized into Th1, Th2, Th17, and T reg, with specific transcription factor and secreting cytokines [23,24]. Here, we investigated profiles of CTCs and T lymphocyte subsets in the peripheral blood, obtained from NSCLC patients. Our goal was to explore the relationships between CTCs or T lymphocyte subsets and the prognosis for patients with lung cancer. These results will guide the prediction of prognosis in lung cancer patients.

MATERIALS AND METHODS

Patient samples

We, retrospectively, reviewed 92 NSCLC patients with well-established tumor, node, and metastasis (TNM) stages, including 23 squamous-cell carcinoma and 69 adenocarcinoma lung cancer patients, that underwent diagnosis between January 2017 and May 2020 at the Jiangxi Cancer Hospital. Their diagnoses were confirmed by clinical pathologists using biopsy samples combined with CT or MRI image data. This study protocol was reviewed and approved by the review board and ethics committee of the Jiangxi Cancer Hospital. Informed consent was obtained from all participants before our study was conducted. For CTCs identification and T lymphocyte subsets profiles, 10 mL peripheral blood samples were obtained from all patients as well as from the control group before treatment. The follow-up of patients started 3 months after treatments and was then repeated every three months in the first year. The final cutoff time was one year after CTCs detection.

Identification of CTCs via the CanPatrol system combined with the tricolor RNA-ISH method

The isolation and characterization of the CTCs in the NSCLC patients and the control group was done according to the abovementioned method [25]. Briefly after the nodules from a patient's chest were found and the lung cancer diagnosis was confirmed by tumor biopsy, 5 mL peripheral blood were drawn and transferred into ethylenediaminetetraacetic acid (EDTA)-coated tubes, which were then spun for 30 minutes at 1,500 rpm with Ficoll 400 density gradient liquid (GE healthy, USA). The plasma was collected and stored at -20°C for cytokines assay. The remaining cells were further separated by CanPatrol CTC enrichment technique (SurExam, Guangzhou, China).

For identification of the different CTCs subtypes, the cells were incubated with Alexa Fluor 594 conjugated epithelial markers EpCAM, CK8/18/19, Alexa Fluor 488 conjugated mesenchymal markers vimentin and Twist, and 4′,6-diamidino-2-phenylindole (DAPI) stained nucleus, respectively. After staining 30 minutes at 4°C, the cells were washed with 2% serum PBS solution, and pictures were taken under an immunofluorescence microscope. The representative pictures are shown in Figure 1.

T lymphocyte subset measurement by flow cytometry

A total of 5 mL peripheral blood mononuclear cells (PBMCs) were isolated with the abovementioned procedure. PBMCs were blocked with FcR and live dye-incubated for 15 minutes. Cells were washed twice with FACS buffer. Firstly, anti-Foxp3 antibody was incubated for 30 minutes at 4°C in intracellular staining reagents, then it was washed twice with FACS buffer. For surface markers staining, anti-human CD4, CD8, CD14, CD16, CD56, and CD25 antibodies were added into cells and incubated for 30 minutes at 4°C. Cell analyses were performed with the Fortassa FACS machine (BD pharmingen, USA). Cell populations were analyzed by flow jo software (version 10.7.1, Tree Star, Ashland, OR, USA).

Cytokine levels were detected by MSD assay

Previous frozen serum was thawed at room temperature. Each sample was diluted at a 1:20 ration of dilute2 solution. The operation steps follow the manufacturer 's instructions. The results were analyzed with the MSD bench work software.

Statistical analysis

The association of the CTC levels and the clinicopathological characteristics were analyzed with the chi-squared test. A comparison of the cytokine levels was performed with two-tailed Student´s *t*-test. PFS was calculated with the Kaplan–Meier method and compared with the log-rank test. Analyses were performed in Graphpad prism 9.0. software. All two-sided p-values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 92 NSCLC patients with TNM (T_{I-IV} N_{1-3} M_{0-1c}) and 10 cases of benign nodules were enrolled in this study. The clinicopathological features of the patients are shown in Table 1, including age, gender, TNM stage, and CTCs count. The results indicate that the below 60 years-old patients accounted for 47.8% (45/92) of all cases, and the above 60 years-old patients accounted for 52.2% (48/92). Out of all patients, 23.9% (22/92) were female and 76.1% (70/92) were male. I, II, III, and IV TNM stage cases amounted to 23 (25.0%), 28 (30.4%), 16 (17.4%), and 25 (27.2%), respectively.

Profiles of CTC subtypes in the NSCLC patients

To assess the profiles of CTC numbers in different staging, we performed CTC subtypes from peripheral blood of 92 patients and 10 controls by using Canpatrol combined with the tricolor RNA-ISH technique. CTCs were identified based on their surface markers. The epithelial CTCs have positive EpCAM and CK8/18/19 expression. MCTCs have positive vimentin and Twist. In addition, all cellular nucleuses were labeled with DAPI staining. The epithelial CTCs, MCTCs, and mixed CTCs can be distinguished by different immunofluorescence dye staining (Figure 1). In the present study, 47 patients as well as the 10 controls had no CTCs detected, including 21 stage I, 23 stage II, and 3 stage III patients. Interestingly, all 25 stage IV patients had positive CTCs. There were no significant differences found in the CTC positive and negative cases of age and gender (Table 1). In contrast, all 25 stage IV patients had positive CTCs compared with stage I and II patients ($p = 0.0001$).

The association between CTCs and patients' prognoses

To validate the clinical significance of CTCs in predicting the prognosis of the NSCLC patients, we compared the PFS of the patients with a median number of total CTC counts (CTC < 6 or CTC ≥ 6), mixed CTCs, and MCTCs. Out of the 45 patients with positive CTCs cells, 20 (44.4%) had CTCs > 6 and 25 (55.6%) had CTC ≤ 6, at baseline. The follow-up duration of all patients was 12 months after treatment. In total, only 7 patients (15.5%) had not experienced a clinical relapse or metastasis by the end of the follow-up. The Kaplan–Meier's survival curves revealed that patients with CTCs > 6 had significantly poorer PFS ($p < 0.001$) than those with CTC ≤ 6 (Figure 2). Interestingly, more than 6 CTCs/5mL peripheral blood in total CTCs (Figure 2A, $p < 0.0001$), mixed CTCs (Figure 2C, $p < 0.0001$), and MCTCs (Figure2 D, $p < 0{,}0001$) had poorer PFS than epithelial CTCs (Figure 2B, $p = 0.54$). In a multivariate Cox regression analysis, we found more than 6 total CTCs (hazards ration [HR], 2.556, 95% confidence interval (CI) 1.157 - 5.645, $p = 0.0001$), mixed CTCs (HR, 3.122, 95% CI 1.391 - 7, $p = 0.0001$), and MCTCs

Table 1. Relationship between the presence of circulating tumor cells (CTCs) and the clinical features of NSCLC patients.

	Number of cases	CTC-positive (%)	CTC-negative (%)	χ^2	p-values
Age					
≤ 60-year-old	44 (47.8%)	24 (54.5%)	20 (45.5%)	1.86	0.32
> 60-year-old	48 (52.2%)	21 (43.8%)	27 (56.2%)		
Gender					
Female	22 (23.9%)	10 (45.5%)	12 (54.5%)	1.02	0.74
Male	70 (76.1%)	35 (50.0%)	35 (50.0%)		
Pathological stage					
I	23 (25.0%)	2 (8.7%)	21 (91.3%)	20.63	0.0001
II	28 (30.4%)	5 (17.8%)	23 (82.2%)		
III	16 (17.4%)	13 (81.3%)	3 (18.7%)		
IV	25 (27.2%)	25 (100%)	0 (0.0%)		

Table 2. Comparison of PFS in different CTC cell numbers.

Variables	PFS in > 6 CTC (months)	PFS in ≤ 6 CTC (months)	HR (Logrank)	95% CI	p-value
Total CTC	6	7	2.556	1.157 to 5.645	0.0001
eCTC	6	7	0.726	0.2633 to 2.002	0.54
Mixed CTC	5	9	3.122	1.391 to 7.00	0.0001
MCTC	6	8	3.018	1.319 to 6.90	0.0001

Table 3. Association between NSCLC and different subtypes of T lymphocyte (mean ± SD).

Variables (%)	Control (n = 10)	Stage I (n = 23)	Stage II (n = 28)	Stage III (n = 16)	Stage IV (n = 25)	F-value	p-value
CD3	72.67 ± 3.16	65.47 ± 5.13	60.37 ± 6.26	54.06 ± 9.15	51.2 ± 7.32	9.73	0.0001
CD4	45.47 ± 3.19	40.37 ± 2.74	31.18 ± 6.36	20.68 ± 7.13	19.05 ± 3.88	12.26	0.0001
CD8	23.37 ± 3.02	20.19 ± 2.89	22.59 ± 4.88	22.76 ± 7.08	16.75 ± 2.51	5.34	0.23
CD4/8	1.56 ± 0.14	1.42 ± 0.37	1.21 ± 0.39	1.27 ± 0.43	0.98 ± 0.20	5.17	0.002
NK cells	21.05 ± 2.27	15.36 ± 2.07	14.47 ± 3.25	13.76 ± 2.27	11.31 ± 2.04	6.75	0.001
Treg cells	5.6 ± 1.47	8.01 ± 2.42	8.4 ± 2.26	8.72 ± 1.72	9.8 ± 2.02	7.39	0.001

NSCLC - non-small cell lung cancer, NK - natural killer, T reg - T lymphocyte regulatory.

(HR 3.018, 95% CI 1.319 - 6.90, p = 0.0001) (Table 2). These data indicate that high CTC numbers in total CTCs, mixed CTCs, and MCTCs had a significant higher risk of progression compared with those in epithelial CTCs.

Characteristics of T lymphocyte subsets in NSCLC patients

To investigate the cellular immunity function in lung cancer patients, we performed a T lymphocyte subtype analysis in the NSCLC patients. The results are shown in Table 3. Compared to benign chest nodule patients, CD3, CD4, and NK cell percentages dramatically de-

Table 4. Association between NSCLC and cytokines (mean ± SD).

Variables (%)	Control (n = 10)	Stage I (n = 23)	Stage II (n = 28)	Stage III (n = 16)	Stage IV (n = 25)	F-value	p-value
IL-2	16.15 ± 3.15	12.27±2.78	9.79±1.57	8.71±3.66	7.19±1.63	7.46	0.0001
IL-4	9.69±4.72	8.34±2.78	8.77±3.41	8.66±3.01	8.68±1.59	2.073	0.42
IL-17A	7.26±2.35	8.27±1.27	7.27±2.59	7.07±1.77	7.4±2.30	1.96	0.67
IL-10	21.08±3.54	28.67±2.89	32.41±2.84	32.24±2.06	39.55±2.39	7.56	0.0001
INFγ	7.73±2.75	5.45±1.47	4.39±1.84	3.74±1.08	1.97±1.35	10.30	0.0001

IL-2 - interleukin-2, IL-4 - interleukin-4, IL-17A - interleukin-17A, IL-10 - interleukin-10, INFγ - interferon γ.

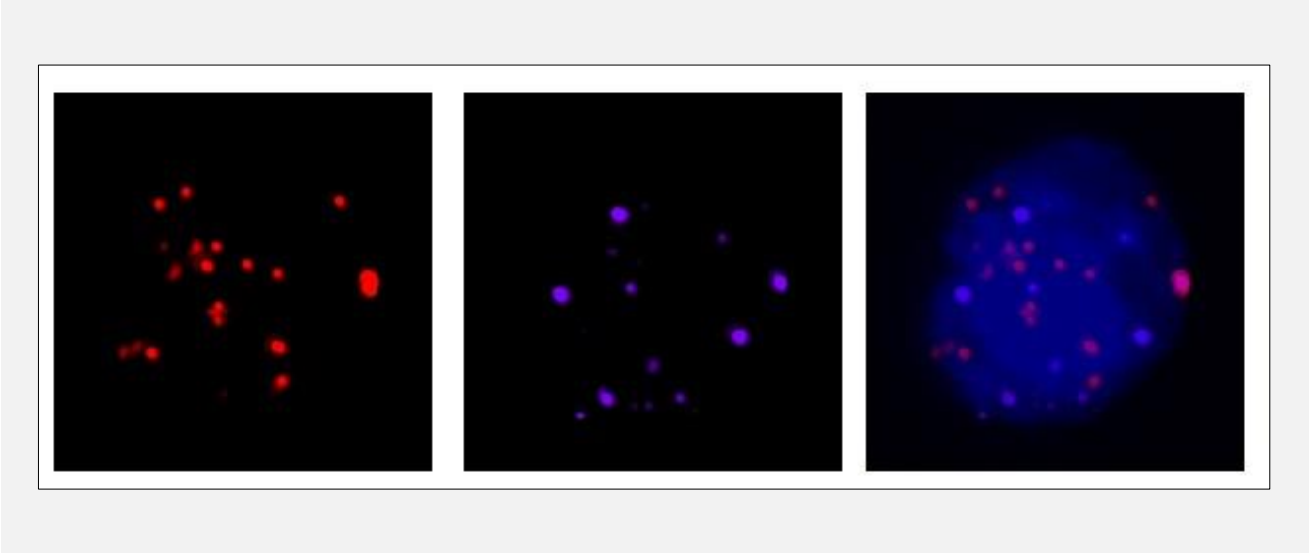

Figure 1. EMT phenotypes detected by the RNA in situ hybridization in lung cancer patients.

clined in the NSCLC patients (p = 0.0001). In contrast, CD8 had no big difference in control and NSCLC patients (p = 0.23). In addition, the Treg cells in the NSCLC patients were significantly increased compared to the control group (p = 0.0001). These results reveal that T lymphocyte is dysfunctional in NSCLC patients.

Cytokine levels of NSCLC patients

To further evaluate the T cellular immunity function in NSCLC patients, we measured IL-2, IL-4, IL-17A, IL-10, and IFN-γ with MSD assay. The results show that the IL-2 and IFN-γ levels in the lung cancer patients significantly declined in comparison to the control patients (p = 0.0001 and 0.0001, respectively, Table 4). In contrast, IL-4 and IL-17A had no dramatic changes to the control patients (p = 0.42 and 0.67, respectively). Moreover, IL-10 in the NSCLC patients was markedly higher than in the control patients. These data further confirm that the T cell immunity functions were disordered.

DISCUSSION

NSCLC currently has a high incidence and lethal rate in either men or women all over the world [26]. Its clinical characteristics show a very aggressive course and are strongly associated with smoking history [27]. So far, no specific biomarkers are available for the diagnosis of NSCLC patients at its early stage. Recently, many studies have shown that CTCs are a critical biomarker for tracing metastatic cascades and predicting the prognosis of cancer patients [13,28,29]. As for lung cancer, several published studies have also focused on CTCs in advanced stages with various techniques [30,31]. In the present study, we mainly detected total CTCs, mixed CTCs, and MCTCs in NSCLC cancer patients and found that these CTC subtypes have a significant clinical association with lung cancer progress predication. Also, we found that the T lymphocyte subtype in NSCLC patients is dysfunctional.

CTCs in the bloodstream can be divided into three types such as epithelial CTCs, MCTCs, and mixed CTCs.

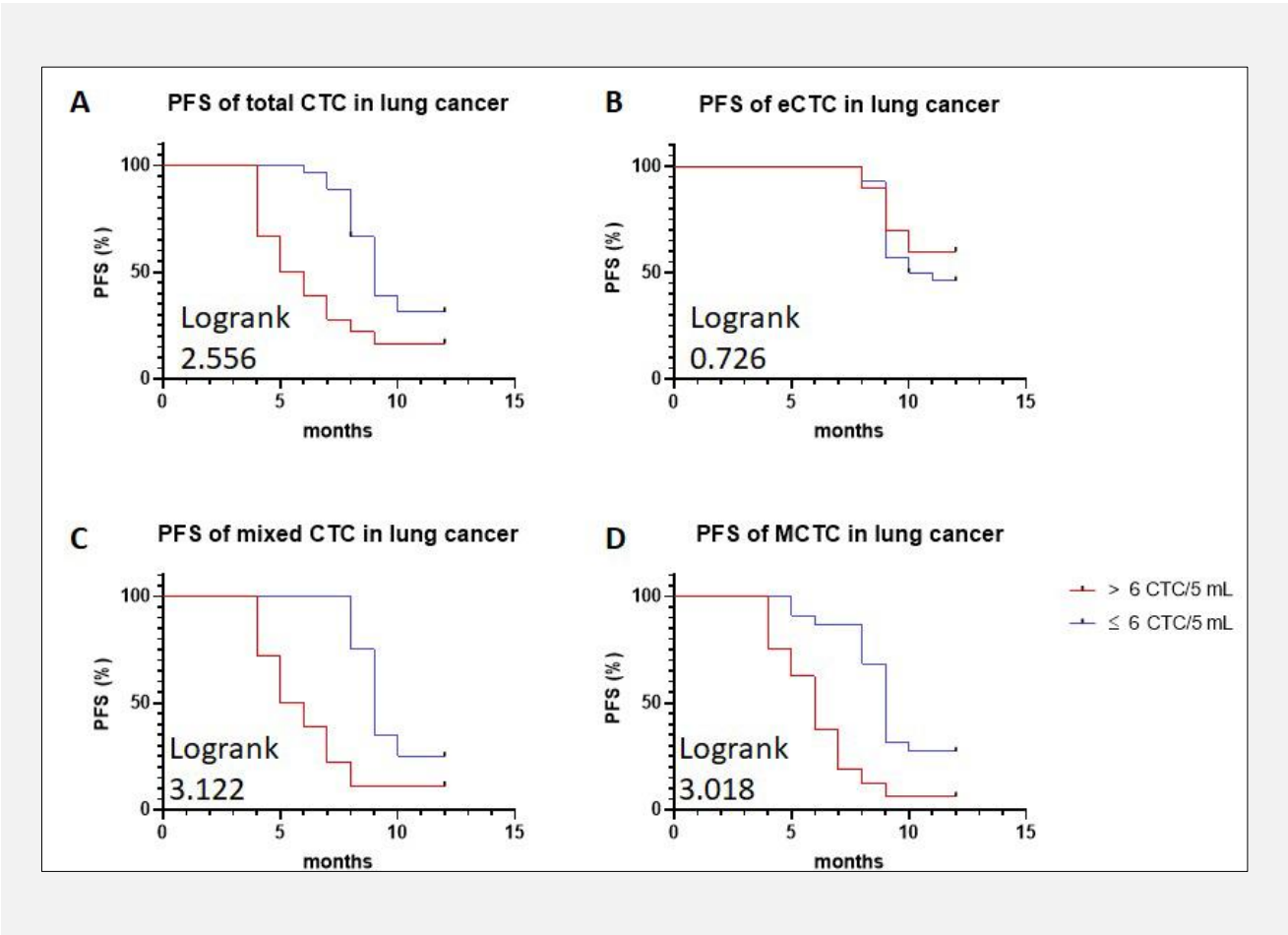

Figure 2. Kaplan-Meier curves for progression-free survival (PFS) of patients according to CTC, epithelial CTCs, mixed CTCs, and mesenchymal CTC (MCTC).

A - Total CTC, B - epithelial CTCs, C, D - mixed MCTC and MCTC with PFS. n = 45.
CTC - circulating tumor cells, WBC - white blood cell, PFS - progression-free survival, MCTC - mesenchymal CTC.
A - Fluorescence microscopy images show three types of CTCs with a positive expression of the epithelial markers (EpCAM and CK8/18/19, red dots), B - mesenchymal markers (Vimentin and Twist, green dots), C - DAPI stained nuclear, and D - biphenotypic markers, pictures were taken under an immunofluroscence microscope by 40 x magnification.

Their detection mainly relies on a combination of membrane filtration and cellular surface markers. Recent reports indicated that the expression of EMT markers in CTCs is a relevant process for invasion and metastasis in several cancers, such as breast, colorectal, non-small cell lung, gastric, and prostate cancers [32-35]. The CTC detection method in the present study allowed the isolation of pivotal EMT CTCs in NSCLC. Total CTCs and MCTCs cluster detection could contribute to improving the accuracy and clinical implications of the CTCs. We initially found that age and gender were not relevant to positive CTCs. However, positive CTCs were strongly associated with T stages. Similar results were found in non-metastatic breast cancer, where preoperative CTCs were poorly associated with tumor size, grade, or lymph node status [36]. However, CTCs appear to provide important reference information regarding an individual patient's risk for relapse or progression. In the present study, patients with higher MCTCs were more likely to have a bad clinical outcome during follow up. More than six total CTCs, mixed CTCs, and higher positive MCTCs were independent prognostic indicators for a poorer PFS.

Recent clinical studies showed that T cell dysfunction in cancer patients are a critical factor in the cancer progress [21]. T lymphocyte is divided into different subsets, like Th1, Th2, Th17, and Treg, according to their specific transcription factor and secreting cytokines. These subtypes play an extremely important function in regulating the whole-body functions [37,38]. Indeed, our current data indicate that CD3, CD4, and NK cells significantly declined in lung cancer patients. In contrast, Treg cells were dramatic increased. IL-2 and IFN-γ were greatly decreased. These results confirmed that

the T cell function is important in the lung cancer progress.

Previous studies have typically focused on the respective roles of CTCs and T-lymphocyte subtypes, e.g. the number of CTCs has been used to assess tumor load and prognosis [39], whereas changes in T-lymphocyte subtypes have been correlated with immune escape from the tumor and patient response to immunotherapy [40]. However, the complexity of NSCLC means that a single biomarker may not be sufficient to fully predict the disease progression and response to therapy. In this study, the detection of CTCs and T-lymphocyte subtypes by blood samples improved the accuracy of blood biopsies for NSCLC and avoided the risks and discomfort of invasive biopsies, making it easier for patients to undergo continuous monitoring. Changes in CTCs and T-lymphocytes reflected the growth and spread of the tumor. This study found that the number of CTCs and the composition of T-lymphocyte subtypes are closely associated with the prognosis of NSCLC patients, which could help to predict the survival and recurrence risk of patients. Overall, the clinical significance of CTCs and T-lymphocyte subtypes explored in this study can provide a more comprehensive and precise treatment and prognosis assessment for NSCLC patients, which is an important direction for modern oncology research.

CONCLUSION

In conclusion, the current study investigated the relationship between the CTC subtypes and the prognosis in NSCLC patient. Our results demonstrate that total CTCs (CTC > 6), mixed CTCs, and positive MCTCs are associated with shorter PFS, and the T lymphocyte subsets in the NSCLC patients were dysfunctional.

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Availability of Data and Materials:
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethical Approval:
All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration, and its later amendments or comparable ethical standards. All subjects were approved by the Jiangxi Cancer Hospital.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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