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The Presence of Small-Size Circulating Tumor Cells Predicts Worse Prognosis in Non–Small Cell Lung Cancer Patients

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• **Context.**—Most patients with non–small cell lung cancers (NSCLC) are diagnosed at advanced stages. The 5-year survival rate of patients with advanced lung cancer is less than 20%, which makes lung cancer the leading cause of cancer-related deaths worldwide.

Objective.—To identify indicators that can predict the prognosis of lung cancer patients.

Design.—To determine the correlation between circulating tumor cells (CTCs), circulating tumor-derived endothelial cells (CTECs), and their subtypes and the prognosis of patients with NSCLC, 80 patients with lung cancer were recruited and 48 patients who met the enrollment criteria were selected in this study. Peripheral blood was collected from the enrolled patients before any treatment and analyzed by the subtraction enrichment and immunostaining–fluorescence in situ hybridization technique to determine the correlation between CTCs and

CTECs and lung cancer disease progression and to identify prognostic indicators.

Results.—In all patients, the positive rate of CTCs was 100% and the positive rate of CTECs was 81.3%. The CTEC positivity rate was higher in late-stage patients than in early-stage patients ($P = .03$). Patients with advanced or lymph node metastases had a higher rate of small-size CTC positivity than those with early or no lymph node metastases. Large-size CTEC positivity was higher in patients with advanced NSCLC than in early-stage patients. Patients with ≥ 1 small-size CTC had shorter progression-free survival, and it was an independent prognostic factor.

Conclusions.—Small-size CTCs are a reliable prognostic indicator and a probable predictor of the severity of disease in NSCLC patients.

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Lung cancer includes non–small cell lung cancers (NSCLCs), and small cell lung cancer (SCLC), of which NSCLC accounts for about 85%.¹ Today, lung cancer has become the most commonly diagnosed tumor in the world. Although lung cancer mortality has declined in recent years due to environmental improvements, active smoking cessation, and early screening, the overall mortality rate is still high among all cancer types. Worldwide, lung cancer remains the leading contributor to cancer deaths among men and the

second leading contributor among women.² For all lung cancer types, the 5-year survival rate is 19%, with NSCLC at 23% and SCLC at 6%.³ Since many lung cancer patients are asymptomatic in the early stages and the cancer is late-stage when detected, it is especially important to find indicators that can screen for early lung cancer and predict its progression. In recent years, many studies have demonstrated that low-dose computed tomography can effectively improve the screening of early-stage lung cancer,⁴ thus allowing a large proportion of patients to achieve early diagnosis and treatment, consequently greatly improving progression-free survival (PFS), overall survival, and survival rates. Despite this encouraging result, many patients with early-stage lung cancer still experience a recurrence years or even months later, and the cancer further develop into advanced lung cancer. Therefore, predicting lung cancer progression as early and efficiently as possible may improve patients' survival.

Liquid biopsy is a recently emerging technique.⁵ This technique allows tissue samples to be obtained from the patient's body through a noninvasive method. In brief, liquid biopsy detects tumor cells mainly through blood or secretions, hence allowing for timely, multiple, dynamic observation of changes in the patient's condition so that appropriate medical treatment can be taken for the patient. Liquid biopsies help further elucidate the characteristics of lung cancer by identifying tumor cells or tumor DNA released into the bloodstream by cancer cell growth and/or apoptosis. Thus, blood testing is a highly sensitive technique that not

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Table 1. Patient Characteristics and Circulating Tumor Cell (CTC) and Circulating Tumor-Derived Endothelial Cell (CTEC) Status of Non–Small Cell Lung Cancer Before Any Therapy

Characteristics	Patients	CTCs ≥ 1, n (%)	CTCs < 1, n (%)	CTECs ≥ 1, n (%)	CTECs < 1, n (%)
Total	48	48 (100%)	0	39 (81.3%)	9 (18.7%)
Median age, y (range)	60.5 (31–74)				
≤60	24 (50%)	24 (50)	0	22 (56.4)	2 (22.2)
>60	24 (50%)	24 (50)	0	17 (43.6)	7 (77.8)
Sex					
Female	25 (52.1%)	25 (52.1)	0	19 (48.7)	6 (66.7)
Male	23 (47.9%)	23 (47.9)	0	20 (51.3)	3 (33.3)
Smoking					
No	33(68.8%)	33 (68.8)	0	25 (64.1)	8 (88.9)
Yes	15 (31.2%)	15 (31.2)	0	14 (35.9)	1 (11.1)
Family cancer history					
No	31(64.6%)	31(64.6)	0	26 (66.7)	5 (55.6)
Yes	17(35.4%)	17(35.4)	0	13 (33.3)	4 (44.4)
Histology					
Adenocarcinoma	41 (85.4%)	41(85.4)	0	34 (87.2)	7 (77.8)
Squamous carcinomas	5 (10.4%)	5 (10.4)	0	4 (10.3)	1 (11.1)
Others	2 (4.2%)	2 (4.2)	0	1 (2.5)	1 (11.1)
Size					
≤2 cm	24 (50.0%)	24 (50.0)	0	18 (46.2)	6 (66.7)
>2 cm	24 (50.0%)	24 (50.0)	0	21 (53.8)	3 (33.3)
Stage					
Early	27 (56.3%)	27 (56.3)	0	19 (48.7)	8 (88.9)
Late	21 (43.7%)	21 (43.7)	0	20 (51.3)	1 (11.1)
Lymph node metastasis					
No	29 (60.4%)	29 (60.4)	0	21 (53.8)	8 (88.9)
Yes	19 (39.6%)	19 (39.6)	0	18 (46.2)	1 (11.1)
Distant metastasis					
No	30 (62.5%)	30 (62.5)	0	22 (56.4)	8 (88.9)
Yes	18 (37.5%)	18 (37.5)	0	17 (43.6)	1 (11.1)

only diagnoses cancer, but also reveals key features of the tumor, such as the type of tumor cells, tumor markers on the surface of the tumor cells, and whether the cancer cells have significant genetic mutations, among others. Currently, liquid biopsies usually detect circulating tumor cells (CTCs), circulating tumor-derived endothelial cells (CTECs), circulating tumor DNA, exosomes, micro-RNAs, circulating RNA in peripheral blood, and tumor-educated blood platelets,⁶ with CTCs, circulating tumor DNA, and exosomes being the most common biomarkers.⁷

CTCs are formed when tumor cells from the primary or metastatic lesions are released into the bloodstream. When encountering the right tumor microenvironment, they can come to form new metastases. CTECs are tumor-derived endothelial cells shed into the peripheral circulation.⁸ CTCs and CTECs together constitute a pair of “cell-based circulating tumor biomarkers,” both of which have the typical hallmark of tumors: chromosomal allopolyploidy, suggesting that they are associated with tumor malignancy. Peter Ping Lin et al⁹ developed an improved subtraction enrichment and

immunostaining–fluorescence in situ hybridization (SE-iFISH) method, which could codetect CTCs and CTECs. The SE-iFISH technique has been employed in various cancers. CTCs were prognostic for survival in SCLC patients and decreasing CTCs during treatment corresponds well to tumor responses.¹⁰ Elevated levels of baseline CTECs were found to be a high-risk factor for poor outcomes in NSCLC patients.¹¹ CTC counts at the beginning of treatment could predict patient survival in metastatic castration-resistant prostate cancer patients.¹² CTECs could be used to detect treatment effects in breast cancer patients.¹³ However, there are few studies comprehensively expounding the characteristics of CTECs and CTCs and their clinical prognostic significance in NSCLC.

In this paper, we recruited 48 NSCLC patients who did not undergo any treatment. After drawing 6 mL peripheral blood, CTCs, CTECs, and their subtypes of cells were detected by the SE-iFISH technique in an attempt to identify predictive indicators associated with the prognosis and extent of disease in lung cancer patients.

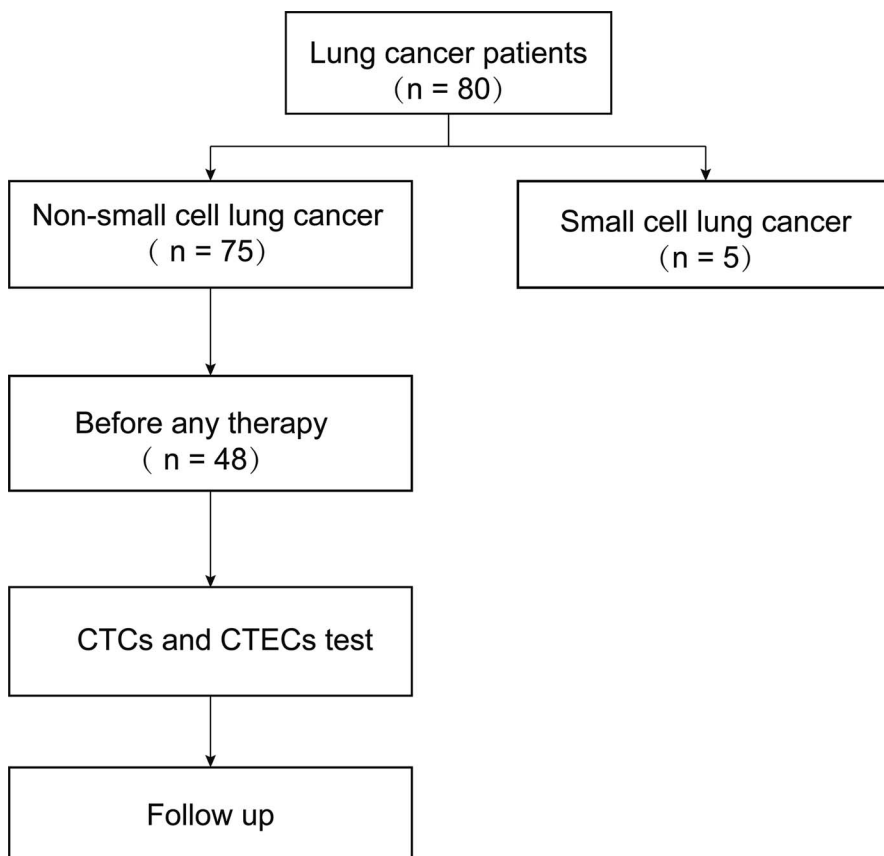


Figure 1. Flowchart of patient inclusion: 48 patients with non-small cell lung cancer were eventually included. Abbreviations: CTC, circulating tumor cell; CTEC, circulating tumor-derived endothelial cell.

MATERIALS AND METHODS

Participants and Sample Collection

A total of 80 lung cancer patients were recruited from Beijing Shijitan Hospital of Capital Medical University (Beijing, China) between February 2021 and July 2023. The inclusion criteria were age 18–80 years, newly diagnosed, never previously treated with any treatment, and NSCLC. Forty-eight patients were eligible and information on their clinical characteristics is presented in Table 1. There were 27 patients with early-stage lung cancer, and 21 with late-stage lung cancer, and the staging was based on tumor node metastasis (TNM) version 8 with I–IIIA as early stage and IIIB–IV as late stage. Pathologic types included 42 of 48 adenocarcinomas (87.5%, with 1 mucinous adenocarcinoma), 5 of 48 squamous carcinomas (10.4%) and 1 of 48 large cell carcinoma (2.1%). To detect CTCs and CTECs, 6 mL of peripheral blood was drawn from each of the 48 NSCLC patients. These 48 NSCLC patients had not undergone any treatment at the time of blood sampling and they were followed up afterward. The design of the study is shown in Figure 1.

This study involved human participants and was approved by the Ethical Committee of Beijing Shijitan Hospital, affiliated with Capital Medical University. The experiments were conducted after collecting the informed consent of each subject, and the study conformed with the Code of Ethics of the World Medical Association¹⁴ (Declaration of Helsinki) in the British Medical Journal (18 July 1964).

Subtraction Enrichment

Subtraction enrichment (SE) was performed according to the manufacturer’s updated instruction with minor modifications (Cytelligen, San Diego, California). In its simplest terms, 6 mL of blood were collected into a tube containing an anticoagulant solution and centrifuged to separate plasma. Sedimented blood cells

were resuspended with 3 mL human CTCs buffer and loaded on the nonhematologic cell separation matrix in a 50-mL tube. Samples were centrifuged, followed by the collection of the solution containing white blood cells (WBCs) and tumor cells above red blood cells. The solution containing WBCs was incubated with magnetic beads conjugated to a cocktail of anti-leukocyte monoclonal antibodies for 30 minutes. WBCs bound to immuno-beads were subsequently removed using a 50-mL magnetic separator (Cytelligen). The remaining nonhematologic cells were mixed with cell fixative, then smeared on formatted and coated CTC slides and dried for subsequent immunostaining–fluorescence in situ hybridization (iFISH) processing.

Immunostaining–Fluorescence In Situ Hybridization

Regarding iFISH, dried monolayer cells on the coated CTC slides were hybridized with centromere probe 8 (CEP8) SpectrumOrange (Vysis, Abbott Laboratories, Chicago, Illinois), which has been approved by the US Food and Drug Administration to identify aneuploid solid tumor cells. Samples were subsequently incubated with the indicated monoclonal antibodies, including Alexa Fluor (AF)594–anti-CD45 (clone 9.4) and Cy5–anti-CD31(clone WM59). Conjugation of diverse antibodies to each specific fluorescent dye was performed at Cytelligen. After washing, samples were mounted with mounting media containing DAPI (Vector Laboratories, Burlingame, California) and subjected to the automated iFISH CTC 6-channel 3D scanning and image analyzing system codeveloped by Carl Zeiss (Oberkochen, Germany), MetaSystems (Altussheim, Germany), and Cytelligen. CTC slides were automatically loaded on a Zeiss fluorescence microscope (AXIO Imager Z2) and afterward subjected to automated X–Y scanning with cross Z-sectioning of all cells performed at 1- μ m steps of depth. X–Y–Z 3D scanning was performed in each of the 6 fluorescence color channels. Positive target cells were defined as

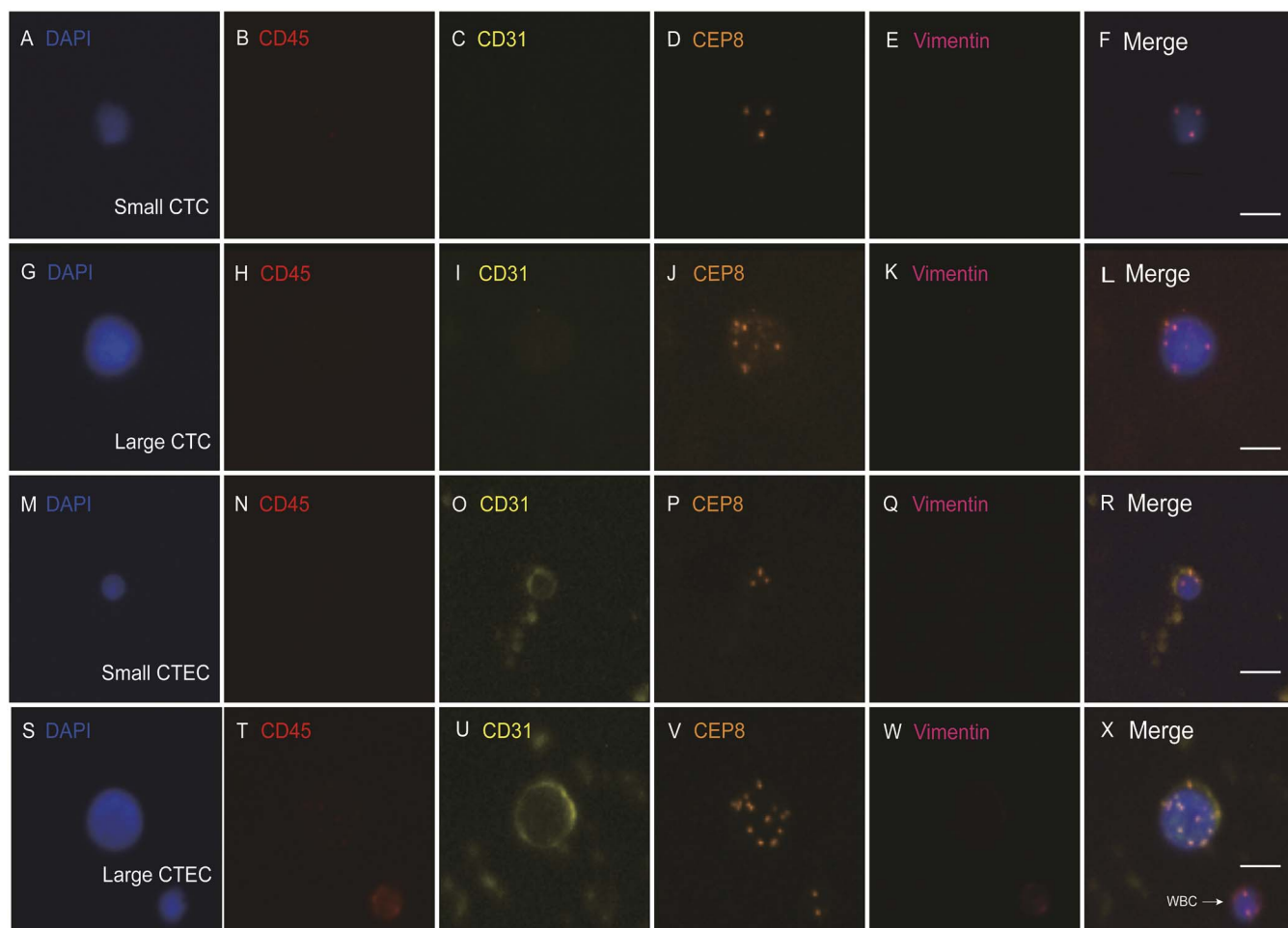


Figure 2. Representative images of CTC and CTEC subtypes identified by SE-iFISH. $DAPI^+/CD45^-/CD31^-/CEP8^+$ represent CTCs; $DAPI^+/CD45^-/CD31^+/CEP8^+$ represent CTECs. (A through F) A small ($\leq 5 \mu m$ WBC) $vim^-/CD31^-$ triploid CTC (small CTC). (G through L) A large $vim^-/CD31^-$ multiploid CTC (large CTC). (M through R) A small $vim^-/CD31^+$ triploid CTEC (small CTEC). (S through X) A large ($> 5 \mu m$) $vim^-/CD31^+$ multiploid (\geq pentasomy 8) CTEC (large CTEC) (original magnification $\times 400$ [A through X]). Arrow: negative control cell – a diploid WBC: $DAPI^+/CD45^+/CD31^-/CEP8^+/Vim^-$. Abbreviations: CTC, circulating tumor cell; CTEC, circulating tumor-derived endothelial cell; WBC, white blood cell; SE-iFISH, subtraction enrichment and immunostaining–fluorescence in situ hybridization; CEP8, centromere probe 8. Bars = $5 \mu m$.

$DAPI^+/CD45^-/CD31^+$ or $DAPI^+/CD45^-/CD31^-$ with diploid or aneuploid chromosome 8.

Following high-throughput scanning and acquiring and processing cell images, subsequent comprehensive characterization and classification of $CD31^-$ CTCs and $CD31^+$ CTECs as well as statistical analyses were performed upon phenotypic, karyotypic, and cell morphological characterization of the trielement in the intracellular bio-chain, with particular focus on cell size and cell cluster. In this technique, leukocytes were used as negative control cells while the definition of large- and small-size cells¹⁵ was generally based on the size of leukocytes ($5 \mu m$) as a threshold, with cells larger than $5 \mu m$ being called large-size cells and those smaller than $5 \mu m$ designated as small size cells (Figure 2, A through X).

Statistical Analysis

Statistical analysis and graphical plots were performed using SPSS 26 (IBM) and GraphPad Prism 9 (La Jolla, California). The differences of categorical variables in distribution among groups were analyzed using the χ^2 test or the Fisher exact test. Differences of continuous variables with normal distribution among 2 groups were compared by *t* test. Receiver operating characteristic (ROC) curve analysis was used to evaluate diagnostic accuracy and to determine cutoff points. Kaplan-Meier survival curves and log-rank tests were used to compare the differences in PFS rates between the

2 groups. The Cox proportional hazards regression model was used to determine hazard ratios for PFS. $P < .05$ was considered to indicate a statistically significant difference.

RESULTS

Patient Characteristics and Analysis of CTCs and CTECs in NSCLC Patients

First, we analyzed the proportional distribution of the number of CTCs and CTECs in patients (Table 1). We defined CTCs or CTECs ≥ 1 as positive; otherwise as negative. CTCs were present in all patients. The CTEC positivity rate was 81.3% (39 of 48) and it was higher in late-stage patients (95.2%, 20 of 21) than in early-stage patients (70.4%, 19 of 27), and statistically significant ($P = .03$). Meanwhile, we compared the differences in CTC and CTEC counts between lung cancer stages. However, there was no significant difference between the early and late stages for either the CTC number (Figure 3, A; $P = .67$) or the CTEC number (Figure 3, C; $P = .50$). We further stratified CTCs and CTECs (Figure 3, B and D), then finding that the number of early-stage patients with either CTCs or CTECs was not less than that of late-stage patients.

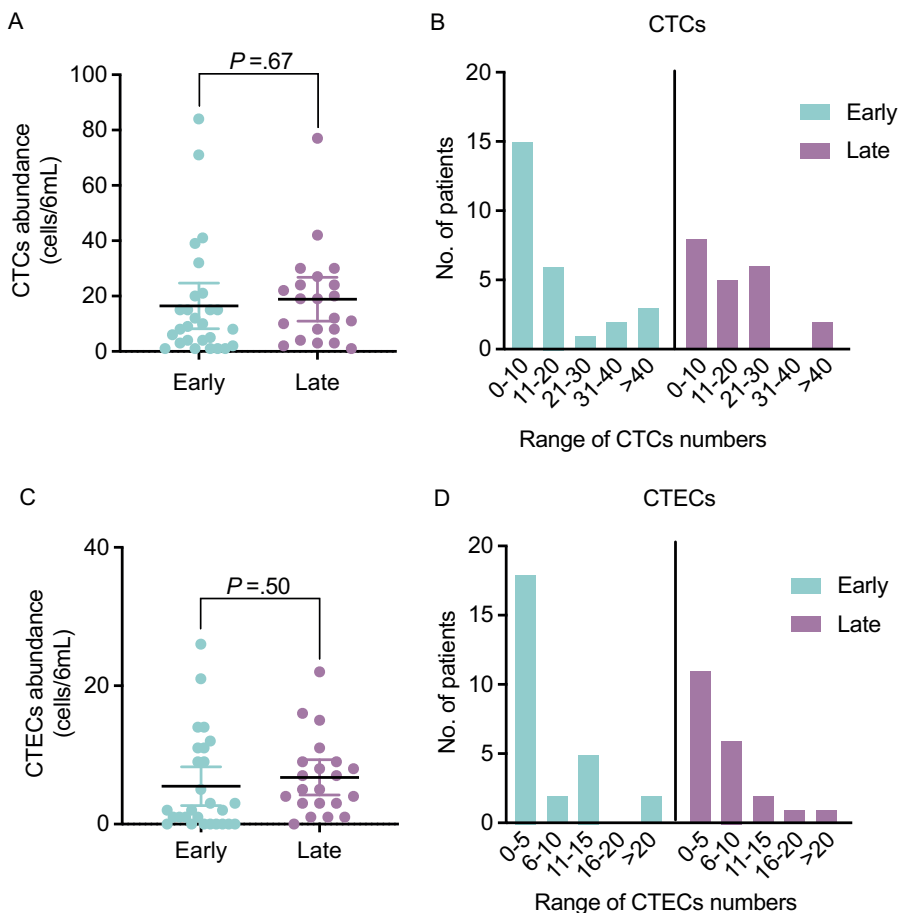


Figure 3. Distribution of the number of CTCs and CTECs in different stages. (A and C) There was no significant difference between the early and late stages for either the CTC number ($P = .67$) or the CTEC number ($P = .50$). (B and D) More early-stage patients were found with a higher number of CTCs or CTECs compared to late-stage patients. Abbreviations: CTC, circulating tumor cell; CTEC, circulating tumor-derived endothelial cell.

CTCs and CTECs had diverse cell subtypes and differed in their distribution in the blood (Figure 4, A). The size of CTCs and CTECs in peripheral blood differed. Therefore, we categorized CTCs and CTECs. Classification was based on the size of leukocytes ($5\ \mu\text{m}$): when a cell was larger than that it was called large size and it was small size when less than $5\ \mu\text{m}$. Among the 48 newly diagnosed NSCLC patients, 840 CTCs (74.3%, 840 of 1130) and 290 CTECs (25.7%, 290 of 1130) were detected (Figure 4, A). CTCs comprised the majority of the blood in NSCLC patients, and large-size CTCs dominated among CTCs. Small-size CTCs accounted for 19.0% (160 of 840) of CTCs, while large-size CTCs were in the majority (81.0%, 680 of 840); the situation was similar for CTECs, with small-size CTECs being 13.1% (38 of 290) and large-size CTECs being 86.9% (252 of 290; Figure 4, B and C).

Analysis of Various Sizes of CTCs and CTECs in NSCLC Patients

Using small- and large-size cells as categories, we grouped all cells as negative or positive (Table 2). Among CTCs, the positivity rates of small- and large-size CTCs were 66.7% (32 of 48) and 93.8% (45 of 48), respectively; within CTECs, the positivity rates of small- and large-size CTECs were 27.1% (13 of 48) and 81.2% (39 of 48), correspondingly. By analyzing the relationship between different clinical features (Figure 4, D through F), we discovered that the positivity rate of small-size CTCs was higher in patients with lymph node metastasis than in those without lymph node metastasis ($P = .04$); although small-size CTCs were

more often positive in patients with late-stage compared to early-stage lung cancer, it was not statistically significant ($P = .06$); nevertheless, this was not the case for large-size CTCs. For various CTECs, large-size CTECs were more often positive in patients with late-stage compared to early-stage lung cancer ($P = .03$).

Clinical Prognostic Significance of CTCs and CTECs in NSCLC Patients

To investigate whether CTCs and CTECs of different cell sizes had an impact on the prognosis of patients with NSCLC, we followed all patients until July 2023 and used death or relapse as a marker for termination of follow-up. The median follow-up time was 11.3 months (0.5–29.3 months). During the follow-up period, 22 patients had a recurrence, 23 patients were progression-free, and death occurred in 3 patients. First of all, we figured out that small-size CTCs and large-size CTECs had the highest diagnostic value by plotting ROC curves, and then calculated the optimal cutoff values for each subtype of cells (Figure 4, G). In view of the above results, we separately plotted the PFS curves for all patients as well as only late-stage patients (Figure 5, A through H). Presence of ≥ 1 small-size CTC was a significant risk factor for reducing the PFS of NSCLC patients ($P = .007$; Figure 5, A). In other sizes of CTCs, CTECs had no significant influence on PFS. We continued to analyze the survival of patients with late-stage lung cancer and realized that presence of ≥ 1 small-size CTC was equally clinically significant for PFS ($P = .04$; Figure 5, B).

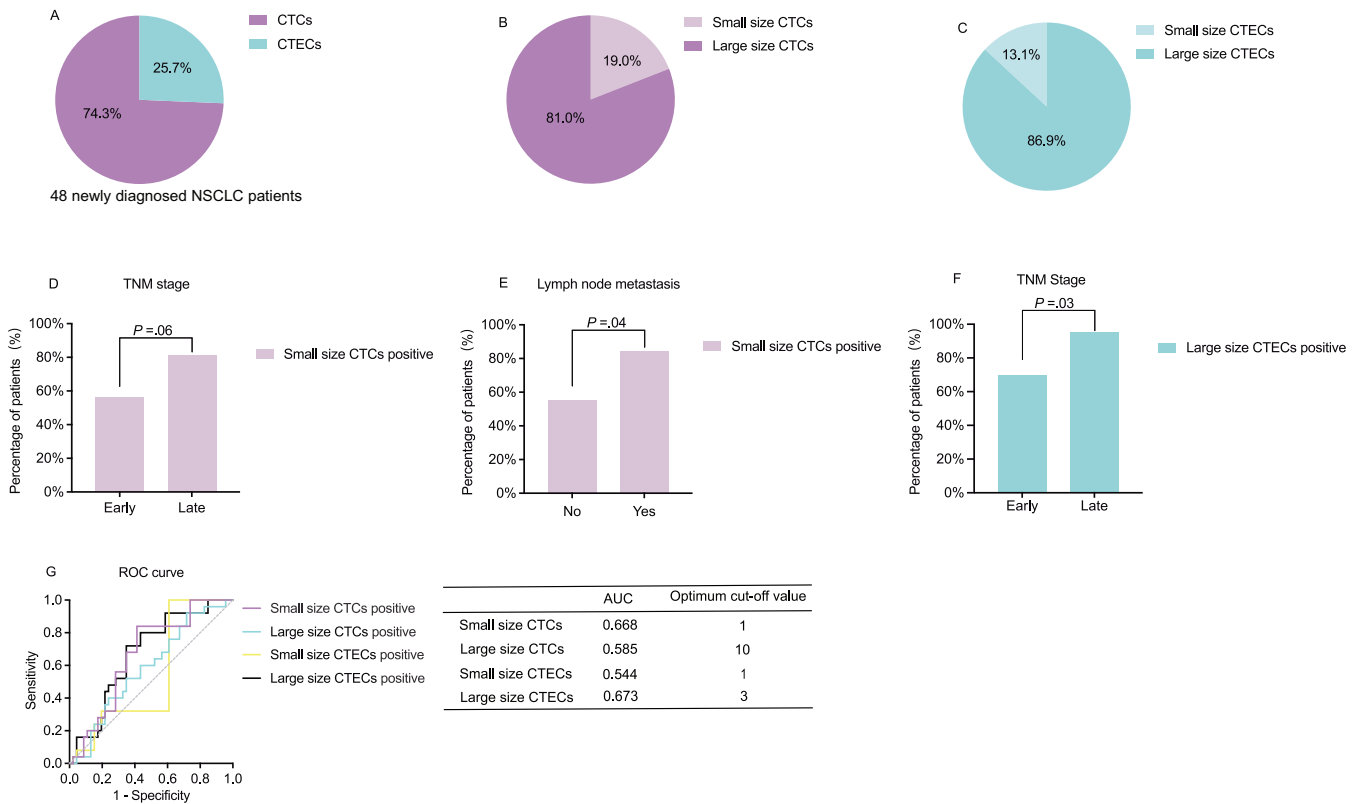


Figure 4. (A through C) Distribution of different subtypes of CTCs and CTECs. (A) Among all cells detected, CTCs accounted for 74.3% and CTECs for 25.7%. (B and C) Small-size CTCs accounted for 19.0% of CTCs, while large-size CTCs were in the majority (81.0%); the situation was similar for CTECs, with small-size CTECs being 13.1% and large-size CTECs being 86.9%. (D and E) Comparison of percentage of small-size CTCs between TNM stage and lymph node metastasis. Small-size CTCs were more positive in patients with late-stage compared to early-stage lung cancer ($P = .06$), but it was not statistically significant; the positivity rate of small-size CTCs was higher in patients with lymph node metastasis than in those without lymph node metastasis ($P = .04$). (F) Comparison of percentage of large-size CTECs between TNM stages. For large-size CTECs, the positivity rate was higher in late-stage patients than in early-stage patients ($P = .03$). (G) ROC curve analysis for CTCs and CTECs. Small-size CTCs and large-size CTECs had the highest diagnostic value and their AUC values were 0.668 and 0.673, respectively. When small-size CTCs were taken as 1 and large-size CTECs were taken as 3 for the cutoff value, they both had high sensitivities of 84.0% and 72.0%, respectively. Abbreviations: CTC, circulating tumor cell; CTEC, circulating tumor-derived endothelial cell; ROC, receiver operating characteristic; AUC, area under curve; NSCLC, non-small cell lung cancer; TNM, tumor node metastasis.

Our results suggested that the number of small-size CTCs was predictive of PFS in all patients with NSCLC, and in advanced patients, the median PFS was significantly shorter in patients with ≥ 1 small-size CTC (2.9 months) than in patients with < 1 small-size CTC (17.7 months). On top of that, survival univariate analysis (Table 3) showed that the following factors influenced prognosis: size, stage, lymph node metastasis, distant metastasis, and small-size CTCs. Cox multivariate survival analysis was performed revealing that small-size CTCs and stage were independent prognostic factors (Figure 6). The risk of disease progression in patients with small-size CTCs positive was 3.446 times higher than in patients with small-size CTCs negative ($P = .03$, 95% CI: 1.159–10.249). To rule out the influence of treatment on prognosis, we subgrouped patients (see Supplemental Table, supplemental digital content) and performed a multivariate analysis, which discovered that treatment was not a prognostically independent factor ($P = .39$).

DISCUSSION

Lung cancer incidence and mortality rates in China had been on the rise in the past few years. According to 2020 global cancer statistics,¹⁶ approximately 820 000 people

were diagnosed with lung cancer in China, and 715 000 people died from lung cancer. The 5-year survival rate for lung cancer patients is only 19%.³ Difficulty in early diagnosis and detection of metastases is an important factor in the low 5-year survival rate of lung cancer. Accordingly, it is important to find markers for disease diagnosis and disease progression monitoring. Low-dose computed tomography had been investigated in several randomized trials in the United States and Europe as a tool for early lung cancer detection and screening.¹⁷ In 2011, the American National Lung Screening Trial first reported a statistically significant reduction of mortality from lung cancer of about 20% in low-dose computed tomography compared to chest X-ray screening.¹⁸ At this stage, a relatively mature method for early screening of lung cancer existed. However, it was still tough predicting the prognosis and progression of lung cancer and in consequence, it was important to find indicators to predict the development of lung cancer as early as possible.

In this study, liquid biopsy was a noninvasive technique that allowed efficient, timely, and dynamic observation of changes in a patient's condition, with CTCs and CTECs being the cell types identified. It has been demonstrated that CTCs and CTECs are useful for the early screening of

Table 2. Various-Size Circulating Tumor Cell (CTC) and Circulating Tumor-Derived Endothelial Cell (CTEC) Status According to Clinical Characteristics of Non-Small Cell Lung Cancer

	Small-size CTCs			Large-size CTCs			Small-size CTECs			Large-size CTECs			
	Total	Positive ^a (n = 32)	Negative (n = 16)	Total	Positive (n = 45)	Negative (n = 3)	Total	Positive (n = 13)	Negative (n = 35)	Total	Positive (n = 39)	Negative (n = 9)	P Value
Age,y													
≤60	24	17	7	24	24	0	24	6	18	24	22	2	
>60	24	15	9	24	21	3	24	7	17	24	17	7	.06
Sex													
Female	25	15	10	25	23	2	25	4	21	25	19	6	
Male	23	17	6	23	22	1	23	9	14	23	20	3	.33
Smoking													
No	33	22	11	33	30	3	33	7	26	33	25	8	
Yes	15	10	5	15	15	0	15	6	9	15	14	1	.15
Family cancer history													
No	31	19	12	31	29	2	31	9	22	31	26	5	
Yes	17	13	4	17	16	1	17	4	13	17	13	4	.53
Size													
≤2 cm	24	14	10	24	22	2	24	5	19	24	18	6	
>2 cm	24	18	6	24	23	1	24	8	16	24	21	3	.27
Stage													
Early	27	15	12	27	24	3	27	6	21	27	19	8	
Late	21	17	4	21	21	0	21	7	14	21	20	1	.03 ^b
Lymph node metastasis													
No	29	16	13	29	26	3	29	6	23	29	21	8	
Yes	19	16	3	19	19	0	19	7	12	19	18	1	.05
Distant metastasis													
No	30	18	12	30	27	3	30	8	22	30	22	8	
Yes	18	14	4	18	18	0	18	5	13	18	17	1	.07

^a Positive: small-size CTCs ≥ 1, large-size CTCs ≥ 1, large-size CTECs ≥ 1; negative: small-size CTCs < 1, large-size CTCs < 1, large-size CTECs < 1.

^b P < .05.

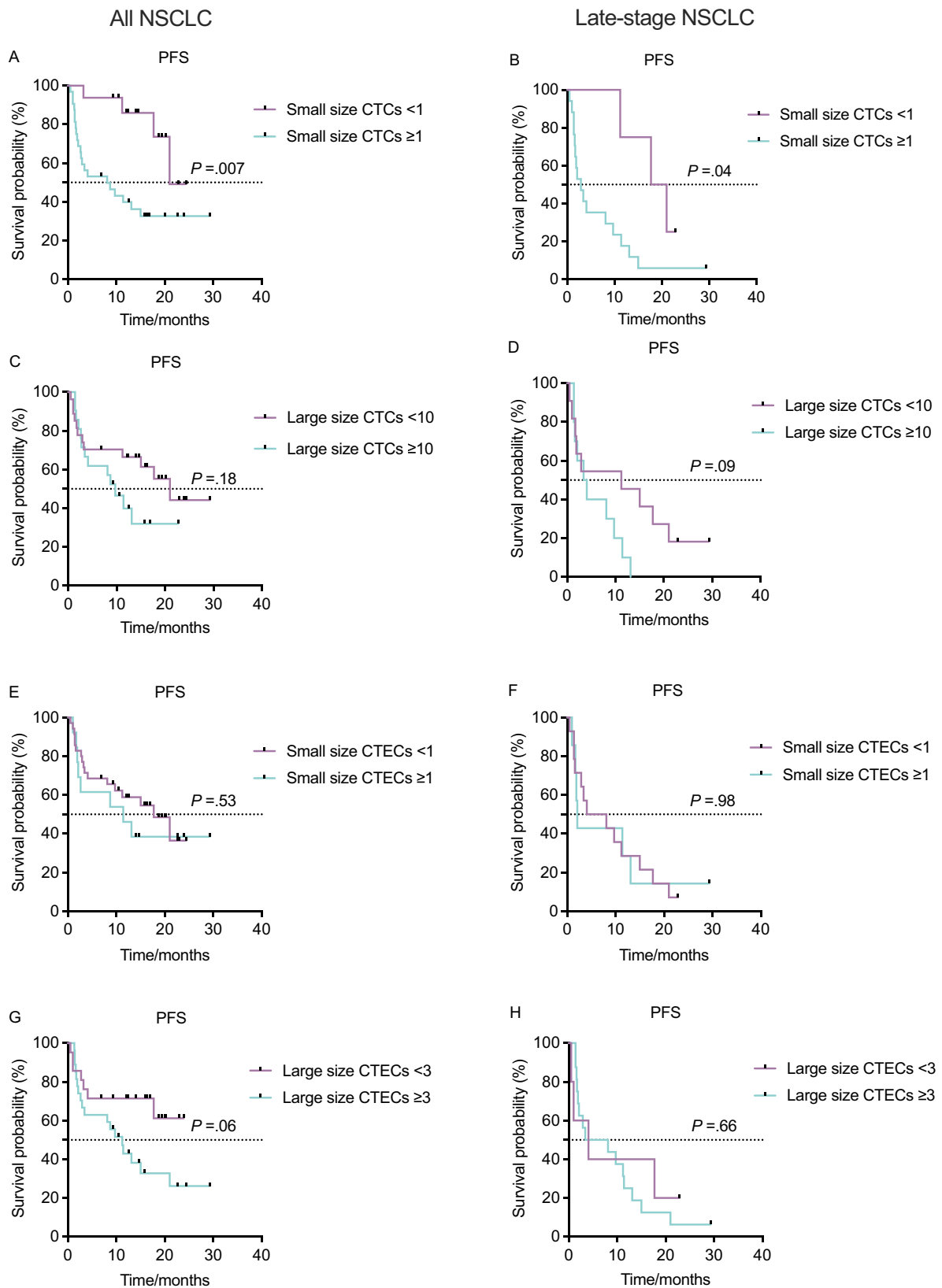


Figure 5. (A, C, E, and G) Kaplan-Meier survival curve analysis of PFS in NSCLC patients according to counts of small-size CTC and CTEC and large-size CTC and CTEC before any therapy. A count of ≥ 1 small-size CTCs was a significant risk factor for reducing the PFS of NSCLC patients ($P = .007$). Other counts of CTCs or CTECs had no significant influence on PFS. (B, D, F, and H) Kaplan-Meier survival curve analysis of PFS in late-stage NSCLC patients according to counts of small-size CTCs and CTECs and large-size CTCs and CTECs before any therapy. A count of ≥ 1 small-size CTCs was equally a significant risk factor for reducing the PFS of late-stage NSCLC patients ($P = .04$). Abbreviations: CTC, circulating tumor cell; CTEC, circulating tumor-derived endothelial cell; PFS, progression-free survival; NSCLC, non-small cell lung cancer.

Table 3. Univariate Analysis of Survival in All Patients

	Median PFS (mo)	95% CI	P Value
Age, y			
≤60	11.2	0.0–22.9	
>60	21.0	7.2–34.8	.21
Sex			
Female	17.7	—	
Male	13.1	2.1–24.1	.74
Smoking			
No	15.0	2.3–27.7	
Yes	21.0	8.7–33.3	.75
Family cancer history			
No	17.7	10.2–25.2	
Yes	3.2	1.5–4.9	.06
Size			
≤2 cm	—	—	
>2 cm	11.2	0.7–21.7	.04 ^a
Stage			
Early	—	—	
Late	4.1	0.0–11.9	<.001 ^a
Lymph node metastasis			
No	—	—	
Yes	8.1	0.0–17.8	<.001 ^a
Distant metastasis			
No	—	—	
Yes	4.1	0.0–13.9	<.001 ^a
Small-size CTCs			
Positive (≥1)	8.1	0.0–16.5	
Negative (<1)	21.0	—	.007 ^a
Large-size CTCs			
Positive (≥1)	15.0	7.1–22.9	
Negative (<1)	—	—	.88
Small-size CTECs			
Positive (≥1)	11.4	0.0–23.7	
Negative (<1)	17.7	9.3–26.1	.54
Large-size CTECs			
Positive (≥1)	13.1	6.3–19.9	
Negative (<1)	—	—	.18

Abbreviations: CTC, circulating tumor cell; CTEC, circulating tumor-derived endothelial cell; PFS, progression-free survival.

^a $P < .05$.

lung cancer and could determine the efficacy of treatment and prognosis. CTCs are formed after cells entered the bloodstream from the primary or metastatic tumor, and eventually, they could end up forming new metastatic foci through blood circulation, leading to tumor recurrence and metastasis. Tumor tissues contain abundant large blood vessels, and the cells lining the walls of these vessels are known as tumor-derived endothelial cells (TECs). Endothelialization of tumor cells and tumorization of vascular endothelial cells are the main mechanisms of TEC formation.¹⁹ When they enter the blood or lymph fluid, they form

CTECs, which re involved in blood and lymph node metastasis of tumors. Together, CTCs and CTECs constitute cell-based circulating tumor biomarkers, while they act as nonhematopoietic aneuploid circulating rare cells (apCRCs) and are distinguished from hematopoietic apCRCs by differences in CD45 expression and centromere probe (CEP).¹⁹ CEP is an important indicator for the diagnosis of malignant cells and aneuploid chromosome 8 is observed in neoplastic cells of almost all solid tumors, including lung cancer.²⁰ CD45 is expressed on all leukocytes. With the presence of CD45⁻/CEP8⁺, a diagnosis of nonhematopoietic apCRCs can be made. To further differentiate between CTCs and CTECs, attention was given to CD31 expression. CD31 is one of the most representative endothelial cell markers. The presence of DAPI⁺/CD45⁻/CD31⁻/CEP8⁺ indicated CTCs, and when DAPI⁺/CD45⁻/CD31⁺/CEP8⁺ was present, it was CTECs. SE-iFISH technology has been proven to be highly sensitive and specific for the detection of various CTCs and CTECs,²¹ and not only that, it takes into account the 3 elements of the intracellular biocontainment chain (nucleic acids, proteins, and cellular morphology) in a holistic manner, allowing for the 1-step counting of CTCs, karyotyping, phenotyping, and molecular typing, as well as detecting the expression of specific tumor markers, and identifying a variety of CTCs and CTECs of varying clinical significance. A rise in the number of CTCs after treatment compared with before treatment has been associated with a poorer prognosis in patients with advanced adenocarcinoma, and pretreatment vimentin⁺ CTECs were more likely to recur and had a shorter PFS.²² In patients with early-stage NSCLC, triploid circulating aneuploid cells and small-size circulating aneuploid cells were more likely to relapse and had shorter disease-free survival.²³ In advanced lung cancer, ≥2 small-size CTCs was associated with poorer PFS.²⁴ In advanced NSCLC patients undergoing anti-programmed death receptor-1 (anti-PD-1) therapy, the prognosis was worse with programmed cell death ligand-1⁺ CTECs and might be resistant to anti-PD-1 therapy.²⁵ Not only that, CTCs and CTECs also had an early diagnostic role: when small tetraploid CTCs and CTECs were present, early adenocarcinoma was more likely compared to patients with benign nodules, while when large polyploid CTCs and CTECs existed, they could distinguish between early and late stages of adenocarcinoma.²⁶ However, in spite of these previous studies, there was still a lack of reports on the prognosis related to different sizes of CTCs and CTECs in patients with all stages of NSCLC.

Many types of CTC and CTEC detection methods have been reported, which can be roughly categorized into 3 main groups.²⁷ The first one, the positive capture method based on epithelial antigen and polypeptide expression, led to a low detection rate caused by its dependence on epithelial antigen expression. The second one, the negative enrichment method by CD45 antibody, not only couldn't effectively remove most of the leukocytes in the blood but also caused damages to CTCs and CTECs because of the hypotonic cleavage method. The third one, a filtration method, was based on the size of the cells; although it was more convenient, small CTCs and CTECs with clinical significance were also filtered. Recently, a novel SE-iFISH technique has been reported.²⁸ This technique includes 2 steps: isolation and identification. The SE isolation method removes most of hematologic leukocytes by using special-immuno-magnetic beads that are conjugated to antibodies specific for multiple leukocytes' surface antigens. In the

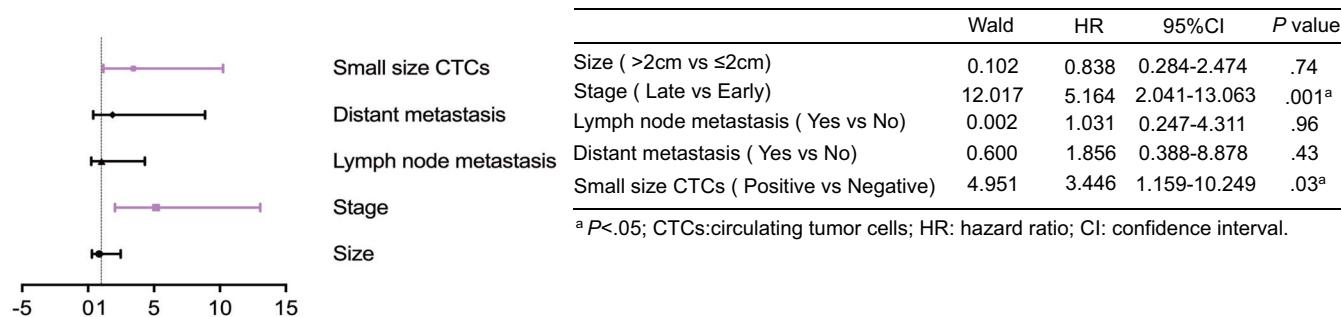


Figure 6. Forest plot of multivariate survival analysis for all patients. Small-size CTCs and stage were independent risk factors affecting the prognosis of NSCLC patients ($P < .05$); the risk of disease progression in NSCLC patients with small-size CTCs was 3.446 times higher than in patients without small-size CTCs ($P = .03$; 95% CI: 1.159–10.249). Abbreviations: CTC, circulating tumor-derived endothelial cell; HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer.

iFISH identification method, positive immunostaining of tumor markers combines with fluorescence in situ hybridization of chromosome 8 aneuploidy^{20,29} to subtype CTCs and CTECs. SE-iFISH considers the 3 elements of the intracellular biological chain (nucleic acids, proteins, and cell morphology) as a whole, and detects chromosomal karyotypes, tumor marker protein phenotypes, and cell morphology on cells synchronously and in situ. Compared with other techniques, it has 4 major advantages: it does not rely on the expression of tumor markers to isolate CTCs and CTECs from a variety of solid tumors, it can isolate small CTCs and CTECs, the cells can avoid damage, and cells are complete enough to be used for single-cell analysis. Compared to the traditional enrichment and identification method, SE-iFISH demonstrated higher sensitivity for CTC detection, showing a 92% positive rate in the identical population of lung cancer patients.³⁰ Similar high CTC positivity detected by SE-iFISH was also observed in gastric (90.5%) and esophageal (87%) carcinoma patients.^{30,31} Moreover, in contrast to conventional lengthy FISH protocol, which takes more than 20 hours, the time required for an iFISH experiment including antibody staining is as short as 3–4 hours.²⁰

In this study, we used the SE-iFISH technique to detect CTCs, CTECs, and their different sizes of cell subtypes to find predictive indicators related to prognosis. We found that CTCs were present in all patients and that the CTC positivity rate was higher in late-stage patients than in early-stage patients; this might be due to the fact that the increase in the number of CTECs allowed for more angiogenesis, which provided nutrients to the tumor, which in turn caused the tumor to be less susceptible to eradication and development of an advanced stage. Although there was no statistically significant difference in the number of CTCs and CTECs between early- and late-stage patients, the distribution of the number of CTCs and CTECs in early-stage patients was more in the 0–10 range, and the amount of patients with the number of CTCs or CTECs in the range greater than 10 increased in late-stage patients; this change may be correlated with the size of the tumor burden, which also suggested that even in the early stages of the disease, the escape of tumor cells into the circulation system already existed. Therefore, we believed that the analysis of CTCs and CTECs will be helpful for the prognosis of patients. We speculated that there was a correlation with patient survival. Next, we explored small- or large-size CTCs and CTECs. CTCs accounted for most of the total number of all cells, and large-size CTCs were more numerous among CTCs.

The positivity rate of small-size CTCs was higher in patients with advanced-stage lymph node metastasis, while this was not the case for large-size CTCs. Large-size CTECs were more positive in patients with late-stage compared to early-stage lung cancer. These results might suggest that patients with NSCLC positive for small-size CTCs and large-size CTECs might be more likely to develop lymph node metastases or advanced stages. To confirm this conclusion, Kaplan-Meier survival curves were performed. Ultimately, presence of ≥ 1 small-size CTC was found to be a risk factor for shorter PFS not only in all patients but also in patients with advanced disease. Furthermore, small-size CTCs were an independent prognostic factor. This also supported the previous statement that patients were more likely to relapse when they had a specific cell type—small-size CTCs, which led to disease progression. In terms of the findings of this study, we thought that early-stage patients might also show a similar trend, namely, the median PFS of early-stage patients with ≥ 1 small-size CTC was shorter than the patients with < 1 small-size CTC, and we will then follow up further with early-stage patients to determine the significance of small-size CTCs as a predictor of PFS in early-stage patients. Not only that, some studies had reported that when patients with advanced NSCLC had vimentin⁺ small-size CTCs were more likely to have liver metastasis and poor prognosis,²⁴ which might be since tumor cells with epithelial-mesenchymal transition properties were smaller in size than those without such properties,³² which made tumor cells invasive and metastatic, thus affecting patient prognosis. Given the above results, we believe that small-size CTCs may be a risk factor for NSCLC and have some value in predicting the early and late stages and prognosis of NSCLC patients. This finding would provide important information for clinical work as a way to individualize treatment. If combined with the detection of the expression of specific tumor markers, such as programmed cell death ligand-1⁺, epithelial cell adhesion molecule, etc, it might be possible to accurately determine the efficacy of a patient for a specific drug, or combined with single-cell gene sequencing, and it would be expected to explore new drug therapy targets. All of these tests could be done by the almost noninvasive SE-iFISH technology, which was believed to be of great help in the treatment of tumor patients.

There are a few limitations to this study. First, the findings are limited because of the lack of healthy population controls. Second, the sample size is relatively small. So, a prospective study with a larger cohort size is necessary to

validate the finding. Finally, the pathologic types are predominantly adenocarcinomas, with squamous carcinomas and other types remaining low, and more pathologic types need to be included in the future.

CONCLUSIONS

We utilized the SE-iFISH technique to detect CTCs, CTECs, and their subtypes in the peripheral blood of NSCLC patients. Presence of ≥ 1 small-size CTC was associated with poor PFS and an independent prognostic indicator; the presence of small-size CTCs was associated with advanced lung cancer and lymph node metastasis and was considered to be a predictive risk factor.

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