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Folate Receptor–Positive Circulating Tumor Cells as a Novel Diagnostic Biomarker in Non–Small Cell Lung Cancer[1]

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Abstract

The study aims to determine the efficacy and feasibility of a novel folate receptor (FR)–based circulating tumor cell (CTC) detection method in the diagnosis of non–small cell lung cancer (NSCLC). CTCs were collected from 3 ml of blood based on negative enrichment by immunomagnetic beads and then labeled by a conjugate of a tumor-specific ligand folate and an oligonucleotide. After washing off redundant conjugates, the bound conjugates were removed and analyzed by quantitative polymerase chain reaction. The captured cells were validated as tumor cells by immunofluorescence staining. In the evaluation of clinical utility, the results showed that the CTC levels of 153 patients with NSCLC were significantly higher than the controls (49 healthy donors and 64 patients with benign lung diseases; $P < .001$). With a threshold of 8.64 CTC units, the method showed a sensitivity of 73.2% and a specificity of 84.1% in the diagnosis of NSCLC, especially a sensitivity of 67.2% in stage I disease. Compared with the existing clinical biomarkers such as neuron-specific enolase (NSE), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), cyfra21-1, and squamous cell carcinoma antigen (SCC Ag), the method showed the highest diagnostic efficiency (area under the curve, 0.823; 95% confidence interval, 0.773-0.874). Together, our results demonstrated that FR-positive CTCs were feasible diagnostic biomarkers in patients with NSCLC, as well as in early-stage tumors.

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Introduction

Lung cancer is the leading cause of cancer-related death in the world, and the non–small cell lung cancer (NSCLC) subtype accounts for about 80% of all cases; the overall 5-year survival rate remains at approximately 15%, and the majority of patients still present with advanced disease [1]. Early diagnosis and treatment could improve the 5-year survival rate by three-fold to four-fold, with the possibility of cure [2], which need more sensitive and specific methods. Low-dose computed tomography is an effective mean for early diagnosis and screening, which could reduce the mortality of lung cancer by 20% [3]. However, a high rate of false positives to small nodules and central tumors still represents a distinct clinical entity [4].

Circulating tumor cells (CTCs) have recently emerged as important potential biomarkers of diagnosis, evaluation of treatment effect, and prognosis in several epithelial cancers including lung cancer. Because of epithelial-to-mesenchymal transition, the recent methods of epithelial cell adhesion molecule (EpCAM)-based CTC analysis showed limitations to detect CTCs in patients with malignant tumors [5]. CTCs in patients with NSCLC often present with nonepithelial characteristics [6]. A prospective study demonstrated that only 32% of patients (19/60) with distant metastatic NSCLC had positive CTC counts at baseline by using CellSearch System [7].

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The folate receptors (FRs) are highly expressed in a variety of cancers, especially in ovarian and lung cancers, whereas most normal tissues express low to negligible levels [8]. Therefore, FR may be a potential target for capturing CTCs in patients with NSCLC. A novel FR-based CTC analysis method had been developed on the basis of this fact. This study aims to determine the efficacy and feasibility of the FR-based CTC detection method in the diagnosis of NSCLC, especially for early-stage tumors.

Materials and Methods

Patients and Control Sample Selection

The current prospective study was approved by the Institutional Review Board at Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences (Approval No. 13-031/707), and informed written consent was obtained from all subjects before the study. This study was registered with the Chinese Clinical Trial Registry (ChiCTR-DDT-13003251). One hundred fifty-three consecutive patients with lung adenocarcinoma (ADC) or squamous cell carcinoma (SCC) undergoing treatment were enrolled into the study, from May 2013 to July 2013. All patients were established on the diagnosis by cytologic or pathologic examinations. None of the patients had received any anticancer therapies or with a history of other cancers before this study. Disease stages were based on the seventh edition of the American Joint Committee on Cancer staging manual.

In addition, peripheral blood samples were obtained from 49 healthy donors who exhibited no evidence of any clinically detectable disease after health checkups and 64 patients who were diagnosed with benign lung disease based on imaging or pathologic examinations. Patient data are presented in Table 1.

Table 1. Patient Characteristics.

Characteristics	No.
NSCLC (n = 153)	
Age, years (mean, range)	59.4 years (25-85 years)
Sex (male/female)	99/54
Smoking	88 (57.5%)
MTD (mean, range)	3.4 cm (0.8-11 cm)
T stage	
T1	43 (28.1%)
T2	89 (58.2%)
T3	13 (8.5%)
T4	8 (5.2%)
N stage	
N0	82 (53.6%)
N1	34 (22.2%)
N2	34 (22.2%)
N3	3 (2%)
M stage	
M0	145 (94.8%)
M1	8 (5.2%)
Grade	
Well differentiated (G1)	16 (10.5%)
Moderately differentiated (G2)	88 (57.5%)
Poorly differentiated (G3)	49 (32%)
TNM stage	
I	67 (43.8%)
II	36 (23.5%)
III	42 (27.5%)
IV	8 (5.2%)
Histopathologic subtype	
ADC	102 (66.7%)
SCC	51 (33.3%)
Healthy donors (n = 49)	
Age, years (mean, range)	38.3 years (23-75 years)
Sex (male/female)	26/23
Smoking	10 (20.4%)
Benign diseases (n = 64)	
Age, years (mean, range)	51 years (23-77 years)
Sex (male/female)	32/32
Smoking	29 (45.3%)

Processing of Blood Samples

To prevent the contamination of epithelial cells, a catheter was used and the first 5 ml of blood was discarded. Following this, peripheral blood samples were collected in the 6-ml EDTA-containing tubes (BD Diagnostics, Sparks, MD). Samples were stored at 4°C and processed within 24 hours of blood withdrawal. Blood samples were taken from an antecubital vein in all patients who were scheduled for surgery. Technologists who operated the detection of CTCs were blinded to the sample groups. All samples had been de-identified before technologists received them.

Detection of CTCs

The CytoploRare Circulating Lung Cancer Cell Kit was provided by GenoSaber Biotech Co Ltd (Shanghai, China). The fundamental principles of this kit was designed as previously described [9,10] and had been modified for detection of CTCs in patients with lung cancer.

In short, the method comprises two components: one is for CTC enrichment that is based on negative enrichment by immunomagnetic beads, and the other is for CTC detection and quantification that is based on ligand-targeted polymerase chain reaction (PCR). The primer sequences were listed as follows: reverse transcription (RT) primer (a oligonucleotide that is conjugated to the tumor-specific ligand folic acid), 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGTTCTAA-3′; forward primer, 5′-TATGATTATGAGGCATGA-3′; reverse primer, 5′-GGTGTCGTGGAGTCG-3′; TaqMan probe, 5′-FAM-CAGTTGAGGGTTC-MGB-3′.

Following the manufacturer's instruction manual, 3 ml of whole blood samples was lysed of erythrocytes with red cell lysis buffer (vol/vol, 1:4) for 15 minutes at 4°C and then depleted of leukocytes with 150 μl of anti-CD45 magnetic beads and macrophages with 50 μl of anti-CD14 beads for 30 minutes at 4°C. Following that, enriched CTCs were incubated with 10 μl of labeling buffer that contained conjugates of a tumor-specific ligand folic acid and a synthesized oligonucleotide for 40 minutes at room temperature. The samples were then washed three times with 1 ml of wash buffer and, then at 500 rpm for 10 minutes at 4°C to remove the unbound conjugates. The specific ligand–oligonucleotide conjugates were removed with 120 μl of stripping buffer for 2 minutes at 4°C, collected by centrifugation, and neutralized by 24 μl of neutralization buffer for further RT-PCR analysis.

In the phase of PCR analysis, RT-PCR and data collection were performed with an ABI 7300 StepOne System (Life Technologies, Carlsbad, CA). The following reaction conditions were used on the ABI 7300 instrument: denaturation at 95°C for 2 minutes, annealing at 40°C for 30 seconds, extension at 72°C for 30 seconds, and then cooling at 8°C for 5 minutes; 40 cycles of denaturation at 95°C for 10 seconds, annealing at 35°C for 30 seconds, and extension at 72°C for 10 seconds.

A self-defined measurement, named CTC unit, which was defined as the number of CTCs detected in 3 ml of blood, was used in our study. If one CTC was detected in 3 ml of blood, it was defined as one CTC unit. A series of standards containing oligonucleotides

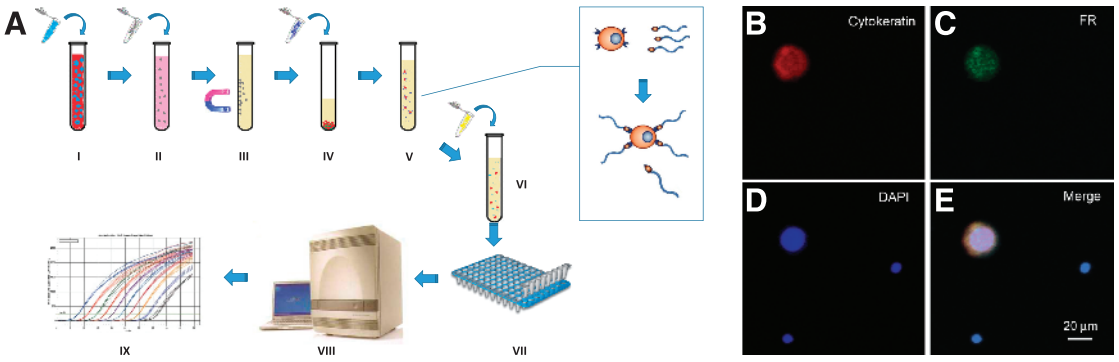

Figure 1. (A) The flowchart of CytoploRare Circulating Lung Cancer Cell Kit: I, lysis of erythrocytes; II, treatment with anti-CD45 and anti-CD14 magnetic beads; III, immunomagnetic depletion of leukocytes and macrophages; IV, incubation with conjugates of a tumor-specific ligand folic acid and a synthesized oligonucleotide; V, removal of the unbound conjugates; VI, dissection of oligonucleotides from specific bound conjugates and collection; VII, addition of the prepared samples into the PCR System; VIII, PCR amplification; IX, data analysis. (B–E) Confocal microscopic analysis of an isolated CTC from a patient with NSCLC. Cytokeratin (B), folate (C), and nucleus (D) images are merged on E. In contrast, the leukocytes cannot be stained by red and green fluorescein (B–C) and with only small nuclei (D–E).

(from 10^{-14} to 10^{-9} M, corresponding to from 2 to 2×10^5 CTC units) is used for CTC quantification. All blood samples were tested in duplicates with six standards and three quality controls (Figure 1*A*).

Immunofluorescence Staining of Enriched CTCs

The enriched CTCs from 3 ml of blood sample were fixed by methanol for 10 minutes at −20°C. Then, these cells were washed with phosphate-buffered saline for 1 minute and stained with phycoerythrin-conjugated pan-cytokeratin monoclonal antibody (sc-8018PE; Santa Cruz Biotechnology, Dallas, TX) and Alexa Fluor 488–conjugated folic acid that was synthesized by WuXi AppTec Co (Shanghai, China), on the basis of the previously reported literature [9], for 1 hour at room temperature. After washing with phosphate-buffered saline, samples were finally mounted with 4′-6-diamidino-2-phenylindole–containing mounting media (D3571; Life Technologies). Imaging was carried out with an inverted Nikon ECLIPSE TE2000-S microscope (Nikon, Tokyo, Japan).

Statistical Analysis

The CTC units were presented as median and interquartile range. We compared the CTC unit between two groups using the Mann-Whitney *U* test and among three groups or more using the Kruskal-Wallis test. The most efficient cutoff values to discriminate patients with cancer from non-cancer controls were identified using receiver operating characteristic (ROC) curve analysis, and the area under the ROC curve (AUC) was calculated for each index. The Youden index was used to identify the optimal cutoff point and diagnostic efficiency. All *P* values were based on two-sided testing, and statistical analyses were performed with SPSS 18.0 software (SPSS Inc, Chicago, IL) or the Prism 5.0 (GraphPad Software Inc, San Diego, CA).

Results

Patient Characteristics

CTC levels in clinical samples. To evaluate the specificity of FR expression on CTCs, immunofluorescence staining was performed at first. As shown in Figure 1, *B* to *E*, the heterocyst is labeled by red (phycoerythrin-conjugated anti–pan-cytokeratin) and green (Alexa Fluor 488–folate), with a giant and multishaped nucleus (4′-6-diamidino-2-phenylindole). In contrast, the leukocytes cannot be

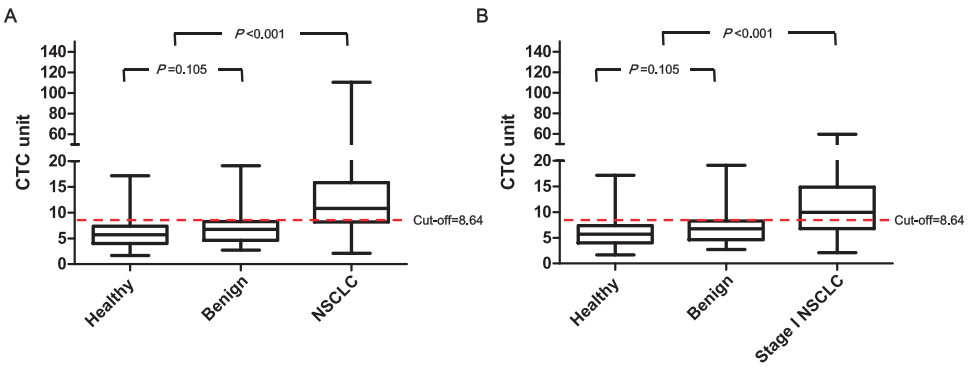

Figure 2. (A) The CTC levels of healthy donors, patients with benign lung diseases, and patients with NSCLC. (B) The CTC levels of healthy donors, patients with benign lung diseases, and patients with stage I NSCLC.

Table 2. The CTC Levels in Patients with NSCLC.

Characteristics	CTC Unit, Median (Interquartile Range)	*P* Value
Age		
≤60 years	11.29 [8.35-15.85]	.551
>60 years	10.24 [8.14-15.62]	
Sex (male/female)		
Male	10.7 [7.65-15.91]	.78
Female	10.87 [8.99-15.22]	
Smoking		
Yes	10.83 [7.72-16.41]	.975
No	10.82 [8.96-15.17]	
MTD		
≤3 cm	9.92 [6.8-14.1]	.001
>3 cm	12.49 [9.78-18.06]	
Grade		
G1	10.31 [8.79-18.78]	.94
G2	10.57 [7.76-15.8]	
G3	11.43 [8.16-15.84]	
TNM stage		
I and II	9.95 [7.23-15.2]	.007
III and IV	12.4 [10-19.56]	
Histopathologic subtype		
ADC	10.28 [7.49-15.15]	.038
SCC	12.21 [9.46-17.28]	

stained by red and green fluorescein (Figure 1, *B* and *C*) and only with small nuclei (Figure 1, *E* and *F*).

The median CTC units were 5.71 [4.03-7.38], 6.74 [4.64-8.27], and 10.82 [8.21-15.84] in healthy donors, benign diseases, and patients with NSCLC, respectively. Compared with patients with NSCLC, the CTC levels were significantly lower in healthy donors and benign diseases ($P < .001$). However, there was no significant difference between the two control groups ($P = .105$; Figure 2*A*).

To further evaluate the efficacy and feasibility of FR-based CTC detection method in early diagnosis, we analyzed the diagnostic values of the CTC unit in patients with stage I NSCLC. The median CTC units were 5.71 [4.03-7.38], 6.74 [4.64-8.27], and 9.95 [6.8-14.88] in healthy donors, benign diseases, and patients with stage I NSCLC, respectively. Compared with control groups, the CTC levels were significantly higher in patients with stage I NSCLC ($P < .001$; Figure 2*B*).

To investigate the significance of CTC levels in patients with NSCLC, the clinical and pathologic characteristics of individuals, including age at diagnosis (≤60 years *vs* >60 years), sex (male *vs* female), history of smoking, maximum tumor diameter (MTD, ≤3 cm *vs* >3 cm), grade of tumor (G1 *vs* G2 *vs* G3), TNM stage (I-II *vs* III-IV), and histopathologic subtypes (ADC *vs* SCC) were also tested for association with CTC levels, and the results are shown in Table 2. The CTC levels of patients with bigger tumor or advanced stage diseases or SCC were higher than those with smaller tumor or early-stage diseases or ADC (all at $P < .05$; Figure 3, *A*–*C*).

ROC analysis. ROC curves were plotted to determine the diagnostic efficiency of CTC levels for NSCLC (ADC and SCC), and the Youden index was used to identify the cutoff point, which meant to choose the "optimal" threshold value when the threshold value for sensitivity + specificity − 1 was maximized. Figure 1 shows that the cutoff value between control groups and NSCLC group is 8.64 CTC units, with the sensitivity of 73.2%, the specificity of 84.1%, and the AUC of 0.823 [95% confidence interval (CI), 0.773-0.874].

With the cutoff point of 8.64 CTC units, the sensitivity was 67.2% (45/67) in the diagnosis of patients with stage I NSCLC by using the FR-based CTC detection method. In patients with stage II, III, and IV NSCLC, the sensitivities were 69.4% (25/36), 80.9% (34/42), and 100% (8/8), respectively. In patients with different histopathologic subtypes, the sensitivities for ADC was 70.6% (72/102),

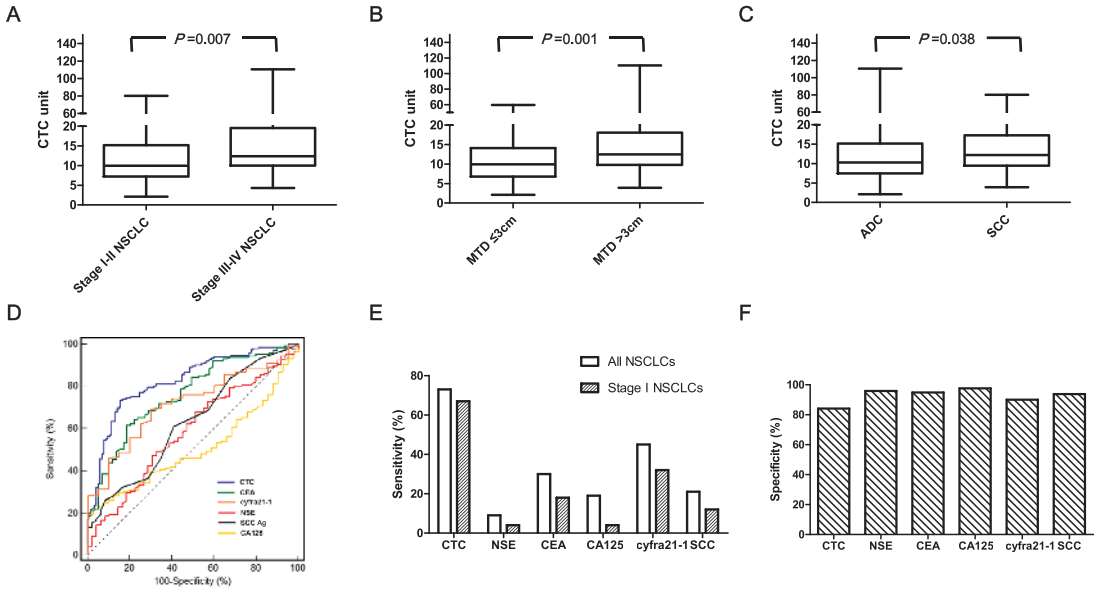

Figure 3. (A) The CTC levels between patients with stage I and II NSCLC and those with stage III and IV NSCLC. (B) The CTC levels between patients with MTD ≤ 3 cm and those with MTD > 3 cm. (C) The CTC levels between patients with ADC and those with SCC. (D) ROC curve analyses of the use of CTC, neuron-specific enolase (NSE), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), cyfra21-1, and squamous cell carcinoma antigen (SCC Ag) to differentiate patients with NSCLC and controls. (E) The sensitivity of different markers in all NSCLCs and stage I NSCLCs. (F) The specificity of different markers in controls.

Table 3. The Diagnostic Efficiency of the FR-Based CTC, NSE, CEA, CA125, Cyfra21-1, and SCC Ag in Patients with NSCLC and Early-Stage NSCLC.

Diagnostic Method	Cutoff Point	Sensitivity (%)	Specificity (%)	Youden Index	Sensitivity of Stage I NSCLC (%)	AUC (95% CI)
CTC	8.64 CTC units	73.2	84.1	0.573	67.2	0.823 (0.773-0.874)
NSE	18 ng/ml	9.6	95.9	0.055	4.6	0.585 (0.495-0.675)
CEA	5 ng/ml	30.1	94.9	0.25	18.5	0.76 (0.69-0.83)
CA125	35 U/ml	19.9	97.6	0.175	4.6	0.505 (0.417-0.592)
Cyfra21-1	3.3 ng/ml	45.2	90	0.352	32.3	0.716 (0.615-0.816)
SCC Ag	1.5 ng/ml	21.2	93.8	0.15	12.3	0.624 (0.535-0.713)

and for SCC, it was 78.4% (40/51), respectively. The sensitivities for ADC and SCC at stage I were 66.7% (36/54) and 69.2% (9/13), respectively.

Comparison of the existing clinical biomarkers. To further analyze the diagnostic efficiency of the FR-based CTC detection method in patients with NSCLC, we compared it with the existing clinical biomarkers (NSE, CEA, CA125, cyfra21-1, and SCC Ag). These tumor markers were analyzed with an Elecsys immunoassay analyzer (Roche Diagnostics, Bavaria, Germany).

As shown in Figure 3, *D* to *F*, and Table 3, the FR-based CTC detection method displays the highest AUC (0.823; 95% CI, 0.773-0.874) and Youden index (0.573) compared with these five biomarkers and could satisfactorily discriminate patients with NSCLC from controls, even in early-stage NSCLC.

Discussion

Sensitive and specific detection and enumeration of CTCs remain a challenge in patients with NSCLC; however, it is still a developing field, with no universal method of detection suitable for all types of cancer [6]. The CellSearch System (Veridex, Raritan, NJ) is the pioneer in developing a standardized commercial system for CTC detection, which has gained approval from the US Food and Drug Administration for providing CTC enumeration tests in advanced breast, prostate, and colon cancers [11]. However, this technique relies on the use of epithelial markers to identify CTCs, loss of these markers during epithelial-to-mesenchymal transition in lung cancer can render these methods ineffective [6,12]. In addition, it is difficult to detect CTCs in NSCLC using EpCAM-based CTC chip devices for similar reasons. Isolation by size of epithelial tumor cells is another CTC detection technology based on morphologic features without involving immunolabeling of cell surface markers. Previous studies found that more CTCs and higher positive rate were identified by the isolation by size of epithelial tumor cells method compared with CellSearch in patients with lung cancer [13]. However, lack of subsequent classic cytopathologic assessment of the captured cells and the possibility of false-positive diagnosis stress the need for using immunophenotypic characterization methods to improve this approach [14].

The FR, a cell-surface receptor glycoprotein, which is overexpressed mainly in ovarian and lung cancer tumors, has become an important potential drug target for patients with NSCLC [15]. A study found that FR expression was upregulated in about 75.7% of patients with NSCLC and that higher levels of FR expression were seen in ADC than SCC [16]. Although some normal tissues including normal lung tissues can also express FRs, there are no normal cells in blood except a subgroup of activated macrophages that could express functional FRs [9]. However, these activated macrophages are barely detectable in the blood samples from healthy donors or benign diseases [10]. In the present study, we used the FR-based CTC detection method in patients with NSCLC, and lower CTC levels were found in the control groups compared with NSCLC groups ($P < .001$).

Ligand-targeted PCR is another important technology in our CTC detection method, which is based on negative selection of CTCs, followed by labeling with folate-linked oligonucleotide and subsequent quantification with RT-PCR. Parker et al. identified that NSCLC samples expressed an average level of FR at 6.11 pmol FR/mg solubilized membrane protein by using a quantitative radioligand binding assay, which meant that there were about more than 100,000 FRs in the surface of one NSCLC cell [8]. One CTC in the peripheral blood of patients with NSCLC was increased by almost 100,000 order of magnitude after the binding of folate-linked oligonucleotide and FR, and these oligonucleotides could be further amplified by using RT-PCR. On the basis of the two-step method of enlargement, a small amount of CTCs could be detected in as low as 3 ml of blood samples. Our results showed that the sensitivity was 73.2% and the specificity was 84.1% by using the FR-based CTC detection method for the entire cohort (Table 3).

Previously, oncologists thought that the development of cancer was a stepwise progression accompanied by accumulation of genetic and epigenetic alterations. During local progression, aggressive cells were selected and dissemination initiates; the cells became increasingly malignant until they form metastases in distant organs and killed the patient. Long ago, shedding of the tumor cells into the blood was considered as a terminal event according to the linear progression model, but this theory had difficulty in explaining the distant metastases that occur in some early-stage cancers or even before diagnosis of primary tumors [17]. The parallel progression model believed that CTCs were released at an early stage of cancer progression, and the primary tumor and metastasis grew in parallel, long before the first symptoms of cancer appear [18]. A recent study confirmed that CTCs could be detected in very early stage during tumor development, even at a premalignant stage in a pancreatic cancer model [19], which implied that CTCs could be potential biomarkers for early diagnosis of cancer. Our results also showed that the sensitivity was 67.2% in diagnosing patients with stage I NSCLC by using the FR-based CTC detection method. The sensitivities were 66.7% and 69.2% in patients with stage I ADC and SCC, respectively.

Subjects in our study also accepted detection of the existing lung tumor biomarkers, such as NSE, CEA, CA125, cyfra21-1, and SCC Ag, and we compared diagnostic efficacies of FR-based CTC detection with these five biomarkers. FR-based CTC detection method showed the highest AUC and Youden index compared with other biomarkers (Table 3). In the diagnosis of early-stage NSCLC, five clinical biomarkers showed a significantly reduced diagnostic efficacy except cyfra21-1, but FR-based CTCs still maintained a satisfactory diagnostic sensitivity. Compared with five clinical biomarkers, the specificity of FR-based CTCs was lowest but acceptable.

Studies on CTC detection in NSCLC had identified a correlation between disease stages and number of CTCs [7], and our results also found that the number of CTCs was higher in patients with stage III/IV NSCLC compared with patients with stage I/II disease. We also investigated the correlation between CTC levels and primary tumor load. Significantly lower CTC levels were found in patients with maximum diameter of primary tumor ≤3 cm compared with those >3 cm, which meant that the quantification of CTCs might be a good surrogate of tumor load. However, previous studies reported that FR expression was much lower in SCC than in ADC [8], and our results found that the CTC levels of SCC were higher than those of ADC (Figure 3*C*; $P = .038$). A probable explanation to the contradictory finding is that gene expression between primary tumors and CTC samples is different [20], which leads to the different expression level of FR between them. However, the hypothesis needs to be verified in the further study.

In conclusion, our results suggested that FR-positive CTCs were feasible diagnostic biomarkers in NSCLC, even in early-stage tumors. Further investigation on the correlation between the preoperative CTC levels and the prognosis of patients with NSCLC is required.

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