# Folate Receptor–Positive Circulating Tumor Cell Detected by LT-PCR–Based Method as a Diagnostic Biomarker for Non–Small-Cell Lung Cancer

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**Introduction:** To investigate the diagnostic performance of folate receptor–positive circulating tumor cells in distinguishing non–small-cell lung cancer (NSCLC) from lung benign disease by using a novel ligand-targeted polymerase chain reaction (PCR) detection technique.

**Methods:** Circulating tumor cells were enriched from 3-ml peripheral blood by immunomagnetic depletion of leukocytes and then labeled with a conjugate of a tumor-specific ligand folic acid and a synthesized oligonucleotide. After washing off free conjugates, the stripped bound conjugates were analyzed by quantitative PCR.

**Results:** Seven hundred fifty-six participants (473 patients with NSCLC, 227 patients with lung benign disease, and 56 healthy donors) were randomly assigned to a training set and a test set. The circulating tumor cell (CTC) levels in patients with NSCLC were significant higher than those with lung benign disease ($p < 0.001$) and healthy donors ($p < 0.001$). Compared with carcinoembryonic antigen, neuron-specific enolase, and Cyfra21-1, CTCs displayed the highest area under the receiver operating characteristic curve (training set, 0.815; validation set, 0.813) in the diagnosis of NSCLC, with a markedly sensitivity (training set, 72.46%; validation set, 76.37%) and specificity (training set, 88.65%; validation set, 82.39%). The model combining CTCs with carcinoembryonic antigen, neuron-specific enolase, and Cyfra21-1 was more effective for the diagnosis of NSCLC than tumor makers alone (sensitivity and specificity in the training set, 84.21% and 83.91%; validation set, 88.78% and 87.36%, respectively). In addition, the CTC levels were higher in patients with stage III/IV NSCLC compared with those with stage I/II disease.

**Conclusion:** Ligand-targeted PCR technique was feasible and reliable for detecting folate receptor–positive CTCs in patients with NSCLC, and CTC levels could be used as a useful biomarker for the diagnosis of NSCLC.

**Key Words:** Circulating tumor cells, Non–small-cell lung cancer, Folate receptor, Polymerase chain.

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The concept of circulating tumor cells (CTCs) was not a new kid, which has been described a century ago by Paget.[1] Recent advances in technical approaches (i.e., CTC detection, enumeration, and characterization) have demonstrated that CTCs play an important role in the development of tumor metastasis[2] and could act as a potential biomarker for early cancer detection, diagnosis, evaluation of treatment efficacy, and prognosis in several solid tumors.[3–10] More recently, the potential value of CTC detection as a liquid biopsy has been established and become increasingly important, especially in those tumors where tissue is not easy to access, such as lung cancer. Conversely, the demand for tumor tissue is increasing because of the introduction of individual therapy in clinical practice. Thus, detection of CTCs may be potentially used to establish a diagnosis, as an alternative to invasive biopsies for early detection.

Currently, the CellSearch System (Veridex LLC, Raritan, NJ) is the first and unique technique that has been approved by the U.S. Food and Drug Administration for CTC detection and routine use in the clinical setting of breast, colorectal, and prostate cancers, but not in lung cancer so far. In brief, the CellSearch System is a semiautomated and immunomagnetic system for quantitative evaluation of CTCs by capturing an immune-bead antigen, epithelial cell adhesion molecule (EpCAM). However, this system is unable to detect the CTCs that present with nonepithelial characteristics (EpCAM-negative CTCs). For instance, the epithelial-to-mesenchymal transition, a common biologic process in lung cancer that changes epithelial cells to a mesenchymal phenotype, may result in escaping detection by CellSearch technique.[11–13] Although EpCAM-based CTC-Chip device has exhibited an ability to capture large numbers of CTCs,[14]

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the reasons similar with CellSearch System limited its further use in clinical setting.[15] Isolation by size epithelial tumor cells (ISET; RareCell Diagnostics) is a nonimmunologic-based, EpCAM-independent CTC isolation technique. Krebs et al. have demonstrated that ISET could detect higher numbers of CTCs including epithelial-negative CTCs in non–small-cell lung cancer (NSCLC) compared with CellSearch System.[16] However, ISET may miss cells less than 8 μm in size and needs further validation before routine clinical use.[17]

The folate receptors (FRs), a cell-surface receptor glycoprotein, are highly expressed in a variety of cancers, especially in lung cancer, in which 72% to 83% patients with NSCLC overexpress the FR on cell surface.[18–20] Previous studies have showed promising clinical value of detecting FR-positive CTC by a novel ligand-targeted polymerase chain reaction (LT-PCR) method in patients with NSCLC.[21,22] Herein, we conducted this large-scale, prospective, double-blind clinical trial to evaluate the feasibility of LT-PCR CTC detection method in the diagnosis of NSCLC. Furthermore, we compared the diagnostic yields between CTCs and traditional tumor makers (i.e., carcinoembryonic antigen [CEA], neuron-specific enolase [NSE], and Cyfra21-1) in patients with NSCLC.

## PATIENTS AND METHODS

### Patients

This was a prospective, single-center clinical trial conducted at Shanghai Pulmonary Hospital, Tongji University, China (trial registered number: ChiCTR-DDT-12003034). From September 15, 2012, to December 15, 2013, 756 participants were enrolled into this study (including 56 [7.4%] healthy volunteers, 227 [30%] patients with benign lung disease, and 473 [62.6%] patients initially diagnosed with NSCLC). The healthy participants also underwent low-dose spiral chest computed tomography (CT) scan to exclude the potential thoracic disease. Benign lung diseases were pneumonia, pulmonary tuberculosis, bronchiectasis, or pneumothorax diagnosed according to the results of thoracic CT scan, laboratory examination, or lung biopsy. The NSCLC patients had histologically or cytologically confirmed as adenocarcinoma (ADC) or squamous cell carcinoma (SCC) or others (large-cell carcinoma, no other specific). The clinical staging was based on the initial evaluation consisting of clinical assessment, the thoracic and abdomen CT scan, CT or magnetic resonance imaging of the brain, and bone scan. The International Association for the Study of Lung Cancer 7th Tumor, Node, Metastasis Staging System was used for clinical disease staging. The ethics committees of Shanghai Pulmonary Hospital approved the study, and an informed consent was obtained from each participant before study entry.

### CTC Analysis

CTC analysis was performed using CytoploRare method provided by GenoSaber Biotech Co. Ltd. (Shanghai, China) as described previously.[21,22] Blood sample (3 ml) from eligible individuals were collected in vacuum tubes containing the anticoagulant ethylenediaminetetraacetic acid for analysis after diagnosed before commencing treatment. All peripheral blood specimens were stored in 4°C refrigerator, and CTC analysis was performed within 24 hours.

In brief, CTCs were enriched by lysis of erythrocytes and immunomagnetic depletion of leukocytes (by anti-CD45-coated magnetic beads) from the blood according to the manufacture's protocol. Then it was labeled with a conjugate of a tumor-specific ligand folic acid and a synthesized oligonucleotide as described previously.[21,22] After that, the CTCs were collected for quantitative PCR analysis. Before amplification, the conjugate first annealed and extended on the reverse transcriptase primer. After immunofluorescence staining the enriched CTCs, CTCs were defined as cells expression folate ligands and cytokeratin (8, 18, and 19) and 4′,6-diamidino-2-phenylindole–stained nucleus. In this study, we used an arbitrarily defined CTC unit, which was defined as the number of CTCs detected in 3 ml of blood. A serial of standards containing oligonucleotides ($10^{-14}$ to $10^{-9}$ M, corresponding to 2 to $2 \times 10^5$ CTC units/3 ml blood) were used for CTC quantification.

To evaluate the recovery ratio in spiked cell assay, we used human nasopharyngeal cancer line KB cells that express FR on cell surface. To determine the linearity of the assay, the observed and spiked CTC count across all the data points were analyzed by linear regression, as described previously.[21]

### Analysis of Tumor Makers

Three milliliter anticoagulant blood sample of all recruitment patients was collected for analysis of tumor makers (CEA, NSE, and Cyfra21-1) by enzyme-linked immune sandwich assay method (Roche Diagnostics) at the day of sample collection.

### Statistical Analysis

The datasets were randomly assigned to training set and validation set by crossvalidation to confirm gender and age matched. First, training set was used to train a classifier. Then, the validation set was used to test the model obtained from the training set to be used as evaluation of performance of the classifier. The differences of CTC units between two groups were compared using the Mann-Whitney *U* test, and among three groups or more were compared using the Kruskal-Wallis test. Prism 5.0 (GraphPad software Inc., San Diego, CA) and MedCalc (version 13.0.0) were used to analyze receiver operating characteristic (ROC) curves to determine the threshold of specificity and sensitivity, and the area under the ROC curves (AUCs) were calculated for each index. The Youden index was used to identify the optimal cutoff point and diagnostic efficiency (when the threshold value for [sensitivity + specificity – 1] was maximized).

To identify the best diagnostic biomarkers, ROC and corresponding AUCs were calculated for all biomarker and for their combinations. We compared the specificity and sensitivity of joint diagnostic model (CTC in combination with tumor marker) with tumor markers only (CEA in combination with NSE and Cyfra21-1) by binary logistic regression analysis to determine whether CTC could improve the diagnostic accuracy. Statistical analysis was performed using SPSS software

**TABLE 1.** Patient Characteristics

| | Training Set | | | Validation Set | | |
|---|---|---|---|---|---|---|
| | | Controls | | | Controls | |
| | Lung Cancer | Benign Diseases | Healthy Controls | Lung Cancer | Benign Diseases | Healthy Controls |
| No. of patients | 236 | 113 | 28 | 237 | 114 | 28 |
| Age (yr) | | | | | | |
| Mean (range) | 59 (33–82) | 49 (15–85) | 51 (36–68) | 59 (23–83) | 49 (18–79) | 56 (28–72) |
| Gender | | | | | | |
| Male | 162 | 60 | 15 | 161 | 64 | 12 |
| Female | 74 | 53 | 13 | 76 | 50 | 16 |
| Smoking status | | | | | | |
| Former smoker | 128 | 64 | 19 | 127 | 62 | 17 |
| Current smoker | 108 | 49 | 9 | 110 | 52 | 11 |
| Pathology | | | | | | |
| ADC | 149 | | | 144 | | |
| SCC | 53 | | | 50 | | |
| Others | 34 | | | 43 | | |
| TNM stage | | | | | | |
| I | 6 | | | 12 | | |
| II | 2 | | | 3 | | |
| III | 66 | | | 61 | | |
| IV | 162 | | | 161 | | |

ADC, adenocarcinoma; SCC, squamous cell carcinoma; TNM, tumor, node, metastasis.

(18.0, SPSS Inc., Chicago, IL). All $p$ values were based on two-sided testing, where $p$ values less than 0.05 were considered significant.

## RESULTS

### Study Population

The characteristics of the patients in the training and validation sets are summarized in Table 1. With SPSS randomized software, a total of 756 participants were randomly assigned to the training set (total 377 participants [236 patients with NSCLC, 113 patients with lung benign disease, and 28 health donors]) and the validation set (total 379 participants [237 patients with NSCLC, 114 patients with lung benign disease, and 28 healthy donors]). The study groups were well matched with respect to baseline characteristics of enrolled patients, and exploratory analysis showed that there was no difference in patient baseline characteristics between the training set and validation set.

### CTC Levels in Patients with Lung Cancer and Benign Disease and Health Donors

The CTC units were presented as median ± interquartile range. On the basis of the ROC curve, the optimal cutoff threshold in differentiating patients with NSCLC from lung benign disease was 8.93 units, with a sensitivity of 74.4% and specificity of 86.6% (Youden index = 0.61; Fig. 1).

First, we compared the CTC unit levels among the different groups (NSCLC versus lung benign disease versus health donor; Fig. 2). In the training set, the CTC levels in patients with NSCLC (11.64±8.6 units) were significantly higher than those with lung benign disease (6.60±5.21 units; $p < 0.001$) and healthy donors (5.72±4.49 units; $p < 0.001$). While there was no notable difference between patients with lung benign disease and healthy donors ($p = 0.314$). In the validation set, the CTC

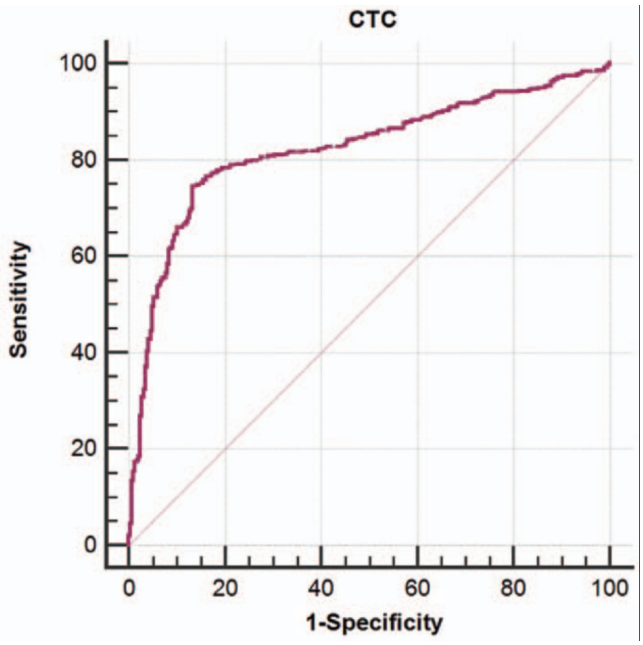


**FIGURE 1.** Receiver operating characteristic analysis between lung cancer and benign disease and healthy group. CTC, circulating tumor cell.

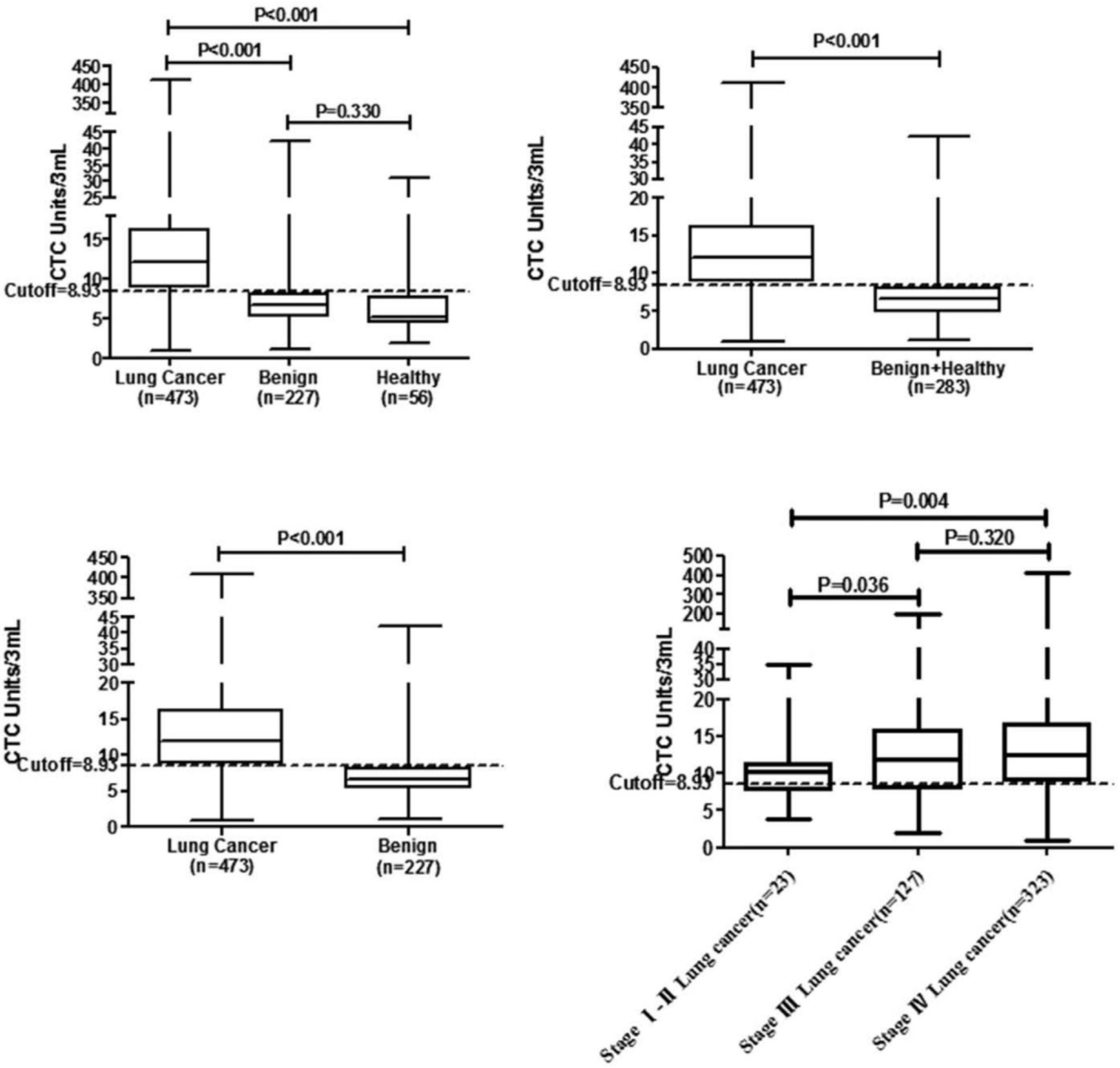


**FIGURE 2.** The level of CTC in lung cancer versus benign disease versus healthy donor. CTC, circulating tumor cell.

levels in patients with NSCLC (12.41±9.02 units) were also significantly higher than those with lung benign disease (6.95±5.45 units; $p < 0.001$) and healthy donors (5.95±4.57 units; $p < 0.001$). Consistently, there was no significant difference between patients with lung benign disease and healthy donors ($p = 0.335$).

Next, we analyzed the correlation between CTC level and the clinical characteristics of patients with NSCLC. The CTC levels in patients with stage I and II NSCLC (10.17±6.23 units) were significantly lower than those with stage III disease (11.96±8.23 units, $p = 0.036$) and stage IV disease (12.44±9.02 units, $p = 0.004$), whereas there was no significant difference between patients with stage III and IV NSCLC ($p = 0.320$; Fig. 2). In addition, we did not observe any relation between CTC level and other clinical characteristics, including age (≤60 years versus >60 years), gender (male versus female), smoking status (former smoker versus current smoker), and pathology (ADC versus SCC and others).

## ROC Analyses of CTC Level, CEA, NSE, and Cyfra21-1 and the Diagnostic Yield of Joint Diagnostic Models for NSCLC

The diagnostic efficiencies of the investigating clinical biomarkers, CTC level, CEA, NSE, and Cyfra21-1, in

distinguishing NSCLC from lung benign disease were compared by plotting ROC curves. The AUCs and 95% confidence interval (CI), sensitivity, specificity for CTC, CEA, NSE, and Cyfra21-1, and their positive predictive value and negative predictive value in the diagnosis of NSCLC are summarized in Table 2.

Among the four clinical biomarkers, CTC level displayed the highest AUC (training set, 0.815; 95% CI, 0.772–0.853; validation set, 0.813; 95% CI, 0.770–0.851; Table 2) in differentiating patients with NSCLC from lung benign disease, with a markedly sensitivity (training set, 72.46%; validation set, 76.37%) and specificity (training set, 88.65%; validation set, 82.39%). Among the four clinical biomarkers, CTC level (training set: AUC, 0.827; 95% CI, 0.775–0.870; validation set: AUC, 0.806; 95% CI, 0.752–0.852; Table 2) displayed comparable efficacy in differentiating patients with ADC from lung benign disease compared with CEA (training set: AUC, 0.819; 95% CI, 0.761–0.867; validation set: AUC, 0.817; 95% CI, 0.758–0.867; Table 2). Among the four clinical biomarkers, CTC level (training set: AUC, 0.794; 95% CI, 0.731–0.847; validation set: AUC, 0.822; 95% CI, 0.763–0.872; Table 2) displayed comparable efficacy in differentiating patients with non-ADC from lung benign disease compared with Cyfra21-1 (training set: AUC, 0.796; 95% CI, 0.727–0.855; validation set: AUC, 0.853; 95% CI, 0.790–0.903; Table 2).

To improve the diagnostic accuracy, a joint model (CTC in combination with tumor marker) and tumor markers model (CEA in combination with NSE and Cyfra21-1) for NSCLC diagnosis were conducted. Notably, the combination of CTC, CEA, NSE, and Cyfra21-1 could significantly improve the diagnostic efficacy in differentiating patients with NSCLC, ADC, and non-ADC from lung benign disease than tumor markers model alone, respectively.

The AUCs of the joint model and tumor markers model for NSCLC and benign disease were 0.883 (95% CI, 0.841–0.925) and 0.842 (95% CI, 0.794–0.889) in training set and 0.936 (95% CI, 0.906–0.965) and 0.870 (95% CI, 0.828–0.911; $p = 0.001$) in validation set, respectively. The same difference was shown in differentiating patients with ADC from lung benign disease ($p = 0.007$) or patients with non-ADC from lung benign disease ($p = 0.003$; Table 3 and Fig. 3).

## DISCUSSION

In this study, we found that CTC levels were significantly higher in 473 patients with NSCLC compared with 227 patients with lung benign disease and 56 healthy donors. CTC levels can effectively distinguish lung cancer from nonmalignant lung disease with a consistently high AUC of 0.815 in the training set and 0.813 in the validation set, which was significantly higher than the combination of plasma tumor makers (CEA, NSE, and Cyfra21-1). In addition, we also found that the diagnostic yield of CTC for NSCLC improved in this study when combined with plasma tumor markers.

Recently, several reliable and reproducible techniques to isolate, enumerate, and detect CTCs in peripheral blood have been established. However, there is still no standard procedure to detect CTCs in clinical practice for NSCLC, and each

**TABLE 2.** The Diagnostic Efficiency of CTC in Differentiating Patients with NSCLC and Benign Pulmonary Disease and Healthy People

| | Training Set | | | | | | | Validation Set | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Diagnostic Group | AUC | 95% CI | Sensitivity (%) | Specificity (%) | Positive LR | Negative LR | *p* | AUC | 95% CI | Sensitivity (%) | Specificity (%) | Positive LR | Negative LR | *p* |
| A arm | 0.815 | 0.772–0.853 | 72.46 | 88.65 | 6.39 | 0.31 | <0.0001 | 0.813 | 0.770–0.851 | 76.37 | 82.39 | 4.34 | 0.29 | <0.0001 |
| B arm | 0.814 | 0.770–0.854 | 72.46 | 88.50 | 6.30 | 0.31 | <0.0001 | 0.812 | 0.767–0.852 | 76.37 | 81.58 | 4.15 | 0.29 | <0.0001 |
| C arm | 0.818 | 0.776–0.862 | 72.88 | 89.29 | 6.80 | 0.30 | <0.0001 | 0.817 | 0.765–0.861 | 78.48 | 85.71 | 5.49 | 0.52 | <0.0001 |
| D arm | 0.562 | 0.476–0.645 | 71.68 | 50.00 | 1.43 | 0.57 | 0.3370 | 0.559 | 0.473–0.642 | 66.67 | 53.57 | 1.44 | 0.62 | 0.3477 |

A arm, NSCLC vs. controls (benign pulmonary disease and healthy people); B arm, NSCLC vs. benign disease; C arm, NSCLC vs. healthy people; CI, confidence interval; D arm, benign disease vs. healthy people; NSCLC, non–small-cell lung cancer.

**TABLE 3.** The Diagnostic Efficiency of Models in Differentiating Patients with NSCLC and Benign Pulmonary Disease

| | Training Set | | | | | Validation Set | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AUC (95%CI) | SN (%) | SP (%) | PPV (%) | NPV (%) | AUC (95% CI) | SN (%) | SP (%) | PPV (%) | NPV (%) |
| NSCLC vs. benign disease | | | | | | | | | | |
| CTC | 0.815 (0.772–0.853) | 72.46 | 88.65 | 93.02 | 60.65 | 0.813 (0.770–0.851) | 76.37 | 82.39 | 90.02 | 65.65 |
| CEA | 0.756 (0.703–0.804) | 52.86 | 89.66 | 91.43 | 47.66 | 0.771 (0.719–0.818) | 61.46 | 83.91 | 88.82 | 51.16 |
| NSE | 0.724 (0.670–0.774) | 50.24 | 87.36 | 89.24 | 45.67 | 0.612 (0.554–0.668) | 32.04 | 90.80 | 87.04 | 38.94 |
| Cyfra21-1 | 0.784 (0.733–0.830) | 79.15 | 70.11 | 84.68 | 61.69 | 0.800 (0.750–0.845) | 66.02 | 80.46 | 87.54 | 53.25 |
| CEA + NSE + Cyfra21-1 | 0.842 (0.794–0.889) | 81.82 | 78.16 | 88.67 | 67.31 | 0.870 (0.828–0.911) | 80.98 | 82.76 | 90.71 | 67.67 |
| CTC + CEA+ NSE + Cyfra21-1 | 0.883 (0.841–0.925)[a] | 84.21 | 83.91 | 91.61 | 71.79 | 0.936 (0.906–0.965)[a] | 88.78 | 87.36 | 93.59 | 78.93 |
| ADC vs. benign disease | | | | | | | | | | |
| CTC | 0.827 (0.775–0.870) | 73.83 | 88.50 | 89.43 | 71.95 | 0.806 (0.752–0.852) | 75.69 | 81.58 | 83.84 | 72.66 |
| CEA | 0.819 (0.761–0.867) | 60.15 | 93.10 | 92.00 | 63.92 | 0.817 (0.758–0.867) | 56.91 | 95.40 | 93.98 | 63.68 |
| NSE | 0.720 (0.655–0.778) | 50.00 | 87.36 | 83.91 | 56.99 | 0.591 (0.522–0.658) | 39.52 | 79.31 | 70.69 | 50.94 |
| Cyfra21-1 | 0.777 (0.717–0.830) | 79.85 | 70.11 | 77.89 | 72.53 | 0.766 (0.703–0.821) | 60.48 | 80.46 | 79.63 | 61.72 |
| CEA + NSE+ Cyfra21-1 | 0.869 (0.821–0.916) | 80.30 | 81.61 | 85.20 | 75.86 | 0.858 (0.807–0.909) | 69.11 | 93.10 | 92.67 | 70.47 |
| CTC + CEA+ NSE + Cyfra21-1 | 0.892 (0.849–0.934) | 86.36 | 85.06 | 88.40 | 82.55 | 0.929 (0.894–0.965)[a] | 87.80 | 88.51 | 90.61 | 85.17 |
| Non-ADC vs. benign disease | | | | | | | | | | |
| CTC | 0.794 (0.731–0.847) | 70.11 | 88.50 | 82.44 | 79.36 | 0.822 (0.763–0.872) | 77.42 | 81.58 | 77.42 | 81.58 |
| CEA | 0.647 (0.569–0.720) | 50.65 | 74.71 | 60.66 | 66.29 | 0.703 (0.628–0.771) | 52.44 | 83.91 | 72.67 | 68.38 |
| NSE | 0.732 (0.657–0.798) | 75.32 | 64.37 | 61.94 | 77.21 | 0.643 (0.566–0.715) | 39.02 | 90.80 | 77.58 | 64.60 |
| Cyfra21-1 | 0.796 (0.727–0.855) | 72.73 | 82.76 | 76.50 | 79.76 | 0.853 (0.790–0.903) | 60.98 | 95.40 | 91.54 | 74.98 |
| CEA + NSE + Cyfra21-1 | 0.809 (0.739–0.879) | 75.32 | 79.31 | 73.70 | 80.67 | 0.889 (0.839–0.939) | 69.51 | 94.25 | 90.79 | 79.12 |
| CTC + CEA + NSE + Cyfra21-1 | 0.883 (0.829–0.938)[a] | 85.71 | 82.76 | 79.29 | 88.27 | 0.955 (0.926–0.983)* | 95.12 | 86.21 | 84.91 | 95.59 |

[a]X X X.

ADC, adenocarcinoma; AUC, area under the receiver operating characteristic curve; CEA, carcinoembryonic antigen; CI, confidence interval; CTC, circulating tumor cell; NPV, negative predictive value; NSE, neuron-specific enolase; PPV, positive predictive value; SN, sensitivity; SP, specificity; NSCLC, non–small-cell lung cancer.



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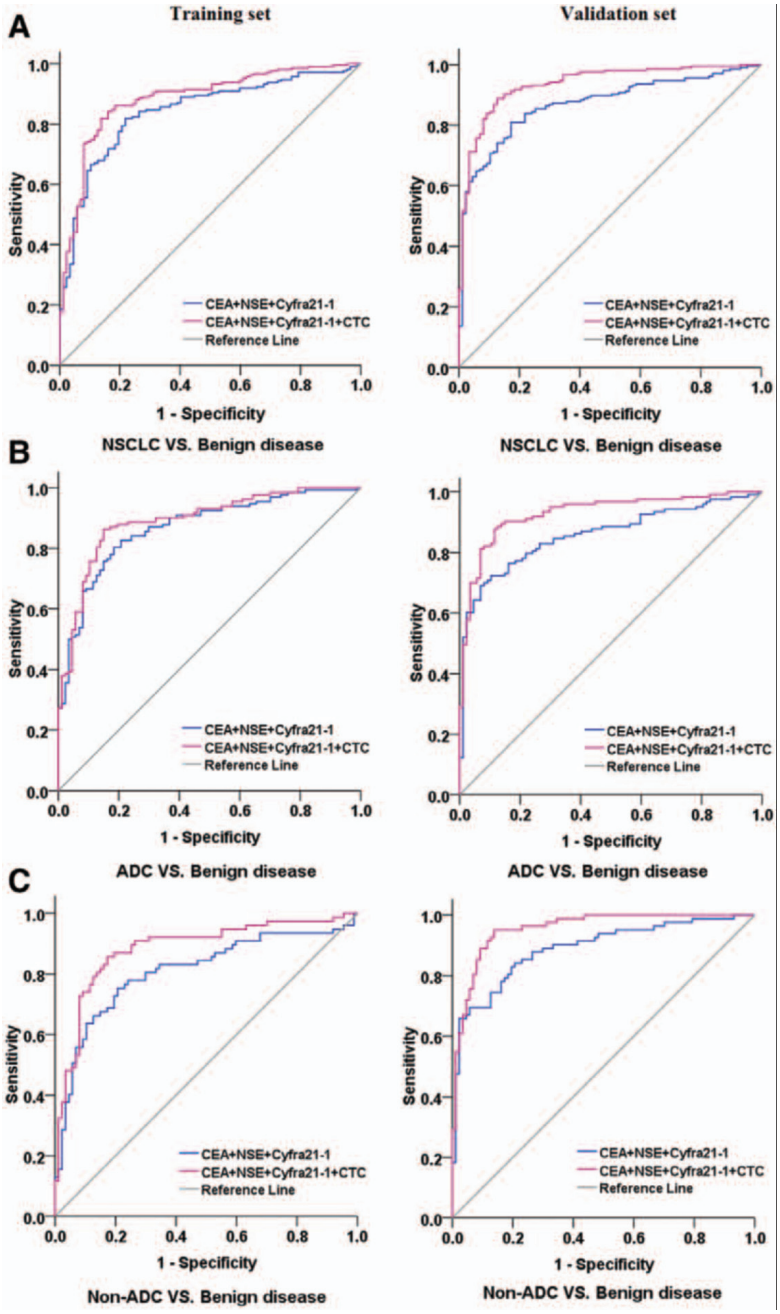


**FIGURE 3.** The receiver operating characteristic curve of circulating tumor cell in combination with tumor marker in pulmonary malignant tumor. ADC, adenocarcinoma; NSCLC, non–small-cell lung cancer.

technique has its own weakness.[23,24] The LT-PCR technique used in this study was based on the negative depletion of leukocytes followed by labeling with folate-linked oligonucleotide and quantification with quantitative PCR. ROC curve analysis identified 8.93 units as the optimal cutoff threshold in the diagnosis of NSCLC, with a sensitivity of 74.4% and specificity of 86.6%, which is in line with previous studies.[21,22] In a previous study with limited samples by Yu et al.,[22] the cutoff threshold in distinguishing NSCLC from controls (healthy donors and lung benign disease) was 8.64 CTC units, with a sensitivity of 73.2%, specificity of 84.1%, and AUC of 0.823. Moreover, the median CTC units in their study were 5.71, 6.74, and 10.82 in healthy donors, benign diseases, and patients with NSCLC, respectively, which was also very similar with the results of our study. In another small sample study, the identified cutoff threshold was 8.5 units.[21] Taken together, we demonstrated that LT-PCR technique was feasible, reliable, and reproducible for quantifying FR-positive CTCs in peripheral blood samples from patients with NSCLC.

Currently, majority of technologies just showed their ability to monitor CTC in patients with advanced lung cancer. For example, Krebs et al.[8] found that CTCs detected by CellSearch System act as a novel prognostic factor in patients with NSCLC; however, positive CTC counts were

not detected in patients with stage IIIA disease and were found only in 32%(19 of 60) of patients with stage IV NSCLC in their study. Thus, the enrolled patients mainly included patients with advanced NSCLC. Besides that, the patients with early-stage (stage I/II disease) NSCLC were also included as a small proportion (23 of 473, 4.9%). Our study showed that the median CTC of the patients with early-stage NSCLC were significantly higher than patients with lung benign disease, although it is lower than patients with advanced NSCLC. Consistent with our results, previous study also demonstrated that the FR is a potential surface marker in identifying CTC of patients with NSCLC, even in early stage,[21] which suggest that CTC could also be potential biomarkers for early diagnosis of lung cancer and predicting the staging of patients with NSCLC. Notably, Lou et al.[21] found that the CTC levels were significantly higher in EpCAM-negative fraction than in EpCAM-positive fraction in patients with NSCLC when using FR-based detective method. As EpCAM-dependent immunologic techniques tend to sacrifice sensitivity for the sake of specificity, CellSearch System showed an insufficient capability to distinguish patients with NSCLC from lung benign disease ($p = 0.122$).[25] More sensitive and specific analytical methods, such as LT-PCR–based method, are urgently needed as alternative techniques in clinical practice.

There were controversial reports about the FR expression of CTCs in different histologic types. In the study by Yu et al.,[22] the CTC levels in SCC were significantly higher than that in ADC ($p = 0.038$). However, this study included a large cohort of 473 patients with NSCLC, and there was also no significant difference of FR expression between ADC and SCC regarding CTC levels, which was consistent with the result of the study by Lou et al.[21] Previous studies demonstrated that the FR expression is much lower in SCC than that in ADC in primary tumors,[18,19,26] while genes expressed differently between primary tumor and CTC samples may partly explain the discrepant result.[27] Importantly, these inconsistent findings also raised an intriguing issue in clinical practice: Is the difference existed between primary tumors and CTCs regarding the driver genes, which has been already observed in breast cancer?[28] However, several studies have found that it is feasible to extract genomic DNA and perform mutational driver genes analysis such as epidermal growth factor receptor and anaplastic lymphoma kinase in CTCs in patients with NSCLC.[29,30] Thus, CTCs, serving as a "liquid biopsy" approach, might be potentially widely used in clinical practice in the future.

In addition, we also compared the diagnostic yields of CTC with traditional serum tumor marker in this study. As we know, serum tumor biomarkers such as CEA, NSE, and Cyfra21-1 were frequently used to help diagnose NSCLC in clinical practice. However, their sensitivity is still low, ranging from only 20% to 70%,[31–34] and lack specificity. Our results showed that CTC levels detected by LT-PCR–based method displayed the highest AUC compared with CEA, NSE, and Cyfra21-1 in the diagnosis of NSCLC. Thus, CTC levels could act as an effective auxiliary diagnostic marker for NSCLC. Furthermore, the diagnostic yield improved when CTC was combined with tumor makers in the diagnosis of NSCLC, suggesting that the combined model should be recommended in clinical practice in the future.

In conclusion, we found that LT-PCR technique was feasible and reliable for detecting FR-positive CTCs in patients with NSCLC in this large-scale, prospective clinical trial. More importantly, CTC levels could be used as a useful diagnostic biomarker for patients with NSCLC, especially in combination with serum tumor markers. Further study to investigate the prognostic or "liquid biopsy" value of CTCs detected by LT-PCR–based method in patients with NSCLC is warranted.

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