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Original Research

# The value of circulating tumor cells with positive centromere probe 8 in the diagnosis of small pulmonary nodules

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ABSTRACT

Circulating cancer cells (CTCs) can serve as a non-invasive liquid biopsy and provide opportunities for early cancer diagnosis and evaluation. However, the value of CTCs for diagnosis or prognosis of small pulmonary nodules (SPNs) is unclear. Fifty-three patients diagnosed with SPNs with a diameter less than 30 mm by CT examination were enrolled in the study. The CTC numbers, CT examination features, serum tumor marker concentrations, and histopathological characteristics were analyzed. Centromere probe 8 (CEP8) was used as a marker for CTC identification. The CTC numbers were significantly different in patients with malignant and benign SPNs and with early (0/Ia) and advanced (Ib/II/III) lung cancer stages. ROC analysis showed that the CTC numbers was effective on malignant SNP diagnosis. The combined use of CTCs and the density features of the nodules determined by CT further improved the overall screening, the diagnostic effectiveness for malignant SNPs, and determination of the pTNM (≤Ia vs.>Ia) stage. The CT morphology revealed that large, single, and solid SPNs were associated with significant CTC numbers and the CTC numbers were correlated with malignant histopathology. Using CEP8 as a marker resulted in detection of more CTC numbers in 22 patient samples triple stained for CEP8, EpCAM, and CKs. The CTCs determined by CEP8-positive staining could serve as potential screening and diagnostic markers for malignant SPNs.

## Background

Lung cancer is the leading cause of cancer related deaths worldwide [1], and non-small cell lung carcinoma (NSCLC) is the dominant lung cancer type. Pathologically, NSCLC takes the form of adenocarcinomas and squamous cell carcinomas. The 5-year survival rate for NSCLC is only about 19% because most patients are not diagnosed until at an advanced stage [1]. This is unfortunate because early diagnosis and treatment of NSCLC can significantly increase patient survival, for example, can increase the 5-year survival rate to 70% for patients with stage I disease [2]. For patients in stage IV at the time of diagnosis, the 1-year survival rate is just 15–19%, according to the 2014 report of the UK Office for National Statistics [2].

Recently, chest computed tomography (CT) examinations have uncovered millions of patients with small pulmonary nodules (SPNs), which are smaller than 30 mm (the largest diameter) and consist of single or multiple ground-glass or partial-solid nodules [3,4]. However, a Chinese population study has indicated that only about 30% of these SPNs are malignant [5]. Most SPNs are benign growths arising from conditions like typical interstitial pneumonia, idiopathic pulmonary fibrosis, and tuberculosis [6]. Several guidelines are available for SPN management, such as optimized surgical resection and CT surveillance, and these can significantly increase the patient survival rate. However, repeated CT exams (even low-dose CTs) increase the patient's radiation exposure and can, in turn, increase the cancer risk, as well as impose mental stress and financial burdens [7].

Acceptable forms of management for patients with SPNs include sub-lobar resection, typically used for noninvasive adenocarcinomas, like adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA) that are considered indolent. Segmentectomy is considered

*Abbreviations:* CTCs, circulating tumor cells; SPNs, small pulmonary nodules; CT, computed tomography; CEP8, centromere probe 8; pTNM, pathological tumor nodal metastasis; EpCAM, epithelial cancer-associated marker; CKs, cytokeratins; FISH, fluorescence in situ; NSCLC, non-small cell lung carcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IA, invasive adenocarcinoma; FDA, Food and Drug Administration; ROC analysis, receiver operating characteristic analysis; AUC, the area under the curve; CEA, carcinoembryonic antigen; AFP, alpha-fetoprotein; FR, folate receptor; MTD, maximum tumor diameter.

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appropriate for invasive adenocarcinoma (IA) and stage Ia NSCLC. The use of limited resection based on precise diagnosis greatly preserves lung functions without increasing the risk of cancer recurrence [8,9]. Therefore, precisely identifying the malignant SPNs in the lungs and further determining the progression stage and invasiveness could strongly favor patient survival.

Metastasis and cancer-related death are strongly influenced by circulating cancer cells (CTCs), which are detached invasive cancer cells that float freely through the vascular system [10]. These CTCs are also recognized as an acceptable non-invasive tool for early cancer diagnosis, evaluation, and progression. The study of CTCs began more than a hundred year ago; however, advancements in the development and use of CTCs for diagnosis or prognosis have only emerged in recent years [11,12]. Currently, CellSearch® is the only method approved by the US Food and Drug Administration (FDA) for clinical determination of CTCs, although various methods and technologies are used in academic studies and clinical diagnosis [13]. Most of these methods involve collection of CTCs from peripheral blood cells. The first step in CTC collection is to deplete the blood of lymphocytes, which are CD45 positive cells and the most abundant cell types in the blood. Different cell membrane proteins that are expressed at high levels in cancer cells are then used as markers to identify and enrich the CTCs. For example, cells positive for epithelial markers like epithelial cancer associated marker (EpCAM+) and cytokeratins (CKs+) are identified as CTCs from epithelial-originating cancers, including lung and breast cancer [14,15]. Another well studied marker for the CTCs of lung cancer is the folate receptor [16,17]. Recently, Zhou et al. reported a correlation between folate receptor-positive CTCs and indeterminate lung nodules, and they also suggested that these CTCs had prognostic value [4].

Other methods used to identify CTCs have included epithelial cell molecule-independent strategies, such as chromosome centromere probe 8 (CEP8) and CEP10, for the diagnosis of early and advanced lung cancer [18, 19]. However, none of the current methods for CTC enrichment are fully satisfactory for cancer diagnosis and prognosis, and new specific markers are urgently needed for identification of CTCs that originate from different types of cancers. To date, numerous studies have shown correlations between CTCs and cancer stages, invasiveness, and metastasis in several cancers, including lung cancer [20,21]. However, patients with NSCLC have only very limited numbers of CTCs in their peripheral blood (a median of 2 cells in a 3.2 mL blood sample; range: 0–32 cells/sample in 174 patients); therefore, the likelihood of detecting CTCs in the peripheral blood of patients with SPNs remains questionable [22]. Nevertheless, collecting CTCs from these patients and analyzing the cell characteristics would be expected to significantly improve the sensitivity and specificity of the diagnosis of lung cancer, as well as identifying appropriate therapeutic strategies.

The present study examined patients who were diagnosed with SPNs by CT scans and who subsequently underwent surgery. The histopathological diagnoses of the patients were also available for statistical analysis. Peripheral blood was collected before lung resection (pre-surgery blood) and subjected to CTC detection using CEP8 as a marker. Correlations between CTC numbers and the results from CT scans, serum marker analysis, and histopathological examinations were then statistically analyzed. We also compared the positive rate of different CTC markers, including CEP8, EpCAM, and pan-CKs. Our findings suggested that CTCs could be enriched from 3.2 mL peripheral blood samples from SPNs patients, and that the CTC number correlated with the SPN histopathological characters. We conclude that CTCs could serve as potential markers that can assist in the diagnosis of SPNs.

## Materials and methods

### *Patients*

This study included 53 patients who had been diagnosed with SPNs by CT examination at Nanjing First Hospital from May to November in 2019. In detail, single or multiple lung nodules with the diameter ≤30 mm detected by CT were defined as SPNs. Tissue sections of the nodules from lung surgery underwent histopathological diagnosis by two pathologists. The histological types of NSCLC and the invasiveness of lung adenocarcinoma, including AIS, MIA, and IA, were determined according to World Health Organization (WHO) classification of tumors of the lung. The stages of lung cancer (pTNM stage) were determined according to the American Joint Committee on Cancer (AJCC) stage manual (8th· Version). Among the 53 patients, 41 patients (41/53) were diagnosed with malignant SPNs (lung cancer); most of these (38/41) were lung adenocarcinoma. In total, 12 of the 53 patients (12/53) had benign SPNs that included fibrosis, hyperplasia, and tuberculosis (Supplementary Table S1). Patients with or without clinical pulmonary symptoms, such as cough and chest pain, were included, whereas patients with histories of chemotherapy, radiotherapy, or lung cancer-related disease or surgery were excluded. The Ethics Committee of Nanjing Medical University approved the study. Informed consent was obtained from all the participants.

### *CTC isolation and identification*

Before each patient's lung surgery, we collected peripheral venous blood (3.2 mL) into customized acid citrate dextrose (ACD) anticoagulant tubes (Becton Dickinson, NJ, USA). The CTCs were enriched and determined by the Cyttel method (Cyttel, Jiangsu, China), as previously reported [21]. Briefly, a red blood cell (RBC) lysis buffer was used to deplete the RBCs from the whole blood, and the leukocytes were pulled down by anti-CD45 antibody-conjugated immunomagnetic beads, followed by centrifugation. The supernatant containing CTCs was smeared onto slides for subsequent analysis.

We used a fluorescence in situ hybridization (FISH) method to detect centromere probe 8 (CEP8), as previously reported [23]. CEP8 staining indicated the copy number of chromosome 8 in the cells. We then used Alexa Fluor 594 conjugated anti-human CD45 antibody (Cyttel, Jiangsu, China) and DAPI (Vector Laboratories, Burlingame, CA, USA) to stain leukocytes and nuclei, respectively. Most of the cancer cells were multiploid; therefore, cells with more than one CEP8 copy number (CEP8 ≥2) were defined as cancer cells (CEP8+ cells). In this study, cells with CEP8+/CD45-/DAPI+ staining were considered CTCs. The CTC identification was conducted blindly [20].

Several slides (22/53) were subjected to immunofluorescence analysis against an additional two tumor markers, EpCAM and pan-CKs. Cells smears were incubated with primary anti-EpCAM (BAF960, R&D system) or anti-pan-cytokeratin (anti-pan-CKs) (ab7753, Abcam) antibodies, followed by incubation with secondary antibody Alexa Fluor 488 or Alexa Fluor 555 (ab150129 and ab105106, Abcam). Imaging and data analysis were done using an Olympus BX63 microscope equipped with IMSTAR high content screening device (IMSTARSA, France).

### *SPN identification by CT*

We classified SPNs according to their CT characteristics as small (<15 mm) or large (≥15 mm, ≤30 mm) SPNs based on the nodule size; as single ($n=1$) or multiple ($n>1$) SPNs based on the nodule number; and as solid (high density, cord-like appearance, or with globular mass) or non-solid (low density, or ground glass-like appearance) SPNs based on the nodule density. Two independent radiologists interpreted the CT images.

### *Tumor marker analysis in serum from patients with SPNs*

We separated the serum from peripheral venous blood in anticoagulant-free blood collection tubes. The tumor markers CEA, CYFRA 21–1, and AFP were detected by chemiluminescence microparticle immunoassays (CMIAs) (Reagent kit #7K68, 2P55, 3P36) using ARCHITECT-i2000sr (Abbott Laboratories, USA). CA 19–9 was detected

with an automatic electrochemiluminescence immunoassay (Reagent kit #11,776,193) using Cobas e602 (Roche Ltd, Germen) [16, 21].

### *Statistical analysis*

The Mann-Whitney *U* test and the Student's *t*-test were used to compare the different groups. The chi-square tests or Fisher's exact tests were used to compare the categorical variables. Receiver operating characteristic (ROC) analysis was performed, and the area under the curve (AUC) was calculated to assess the histopathology of SPNs. A value of $p < 0.05$ was considered statistically significant. The data were analyzed using SPSS 18.0 and Prism 6.0 software.

## Results

### *CTC numbers were correlated with SPN malignancy*

Several studies have reported the detection of CTCs in patients with advanced cancer, including lung cancer. However, whether CTCs could be detected in the peripheral venous blood of patients with SPNs is unknown. We collected peripheral blood prior to surgery from patients who were diagnosed as having SPNs based on CT examinations. We then enriched the CTCs, examined the serum tumor markers, and collected the histopathological diagnosis reports of these patients. The subsequent statistical analysis was conducted on 53 patients who had data for CTCs, CT, serum markers, and histopathology. The demographic and clinic pathologic characteristics of those patients are displayed in Supplementary Table S1.

We found that 41/53 patients had lung cancer, mostly (38/41) in the form of adenocarcinomas. Since adenocarcinoma invasiveness provides critical guidelines for the resection strategy and is correlated with its prognosis; we divided the patients into AIS, MIA, and IA groups. We also divided the patients into two sub-groups (≤Ia and >Ia) based on the pTNM stage, as the ≤Ia stage is recommended for limited surgical resections, such as segmentectomy, sub-lobar or lobectomy resection, and this would both increase the survival rate and maximally preserve the lung function [24–27]. Benign pulmonary diseases, such as fibrosis, hyperplasia, and tuberculosis, were present in 12 of the 53 patients. Representative hematoxylin & eosin (H&E) staining of these tissues is shown in Fig. 1.

We also analyzed the correlation between CTC level and SPN malignancy. The CEP8+/CD45-/DAPI+ samples were identified as CTCs. Representative staining is shown in Fig. 2A. The numbers of CTCs were counted in each sample and were significantly higher in patients with malignant SPNs than with benign SPNs according to the Mann-Whitney *U* test ($p < 0.05$) (Fig. 2B). Similarly, the average CTC number was significantly higher in patients with malignant SPNs than with benign SPNs according to Student's *t*-test (Supplementary Figure S1A). The CTC numbers were significantly lower for the >Ia stages (Ib/II/III) than for the ≤Ia stage (0/Ia) of lung cancer (Fig. 2C). In all, 38/41 of the malignant SPNs were adenocarcinomas (Supplementary Figure S1B); therefore, we analyzed the correlation between the CTC levels and cancer invasiveness. However, no significant difference was found in the CTC numbers between the AIS, MIA, and IA groups (Fig. 2D). Given our limited sample size, we pooled the MIA and AIS patients to create a noninvasive group, since MIA is considered to have a noninvasive pathology. We did not observe any statistical difference in the CTC numbers between the noninvasive and invasive groups (Supplementary Figure S1C).

The ROC analysis showed a sensitivity and specificity for using CTCs as a diagnostic marker to distinguish malignant SPNs from benign SPNs of 92.7% and 50%, respectively (Table 1). The area under the curve (AUC) was statistically significant ($p < 0.05$), indicating that CTCs could serve as a potential diagnostic marker for SPNs (Fig. 2E). Previous reports have suggested that ≥2 CTCs/sample can be used in ROC analysis for lung cancer diagnosis. We therefore redefined our samples as positive for ≥2 CTCs/sample and as negative for <2 CTCs/sample, based on the finding that the median CTC count in the malignant group was 2 CTCs/sample compared to 0.5 CTCs/sample in the benign group (Fig. 2B). The specificity increased to 75%, but the sensitivity decreased to 70.7% with a significant AUC of 0.729 ($p < 0.05$) (Table 1). However,

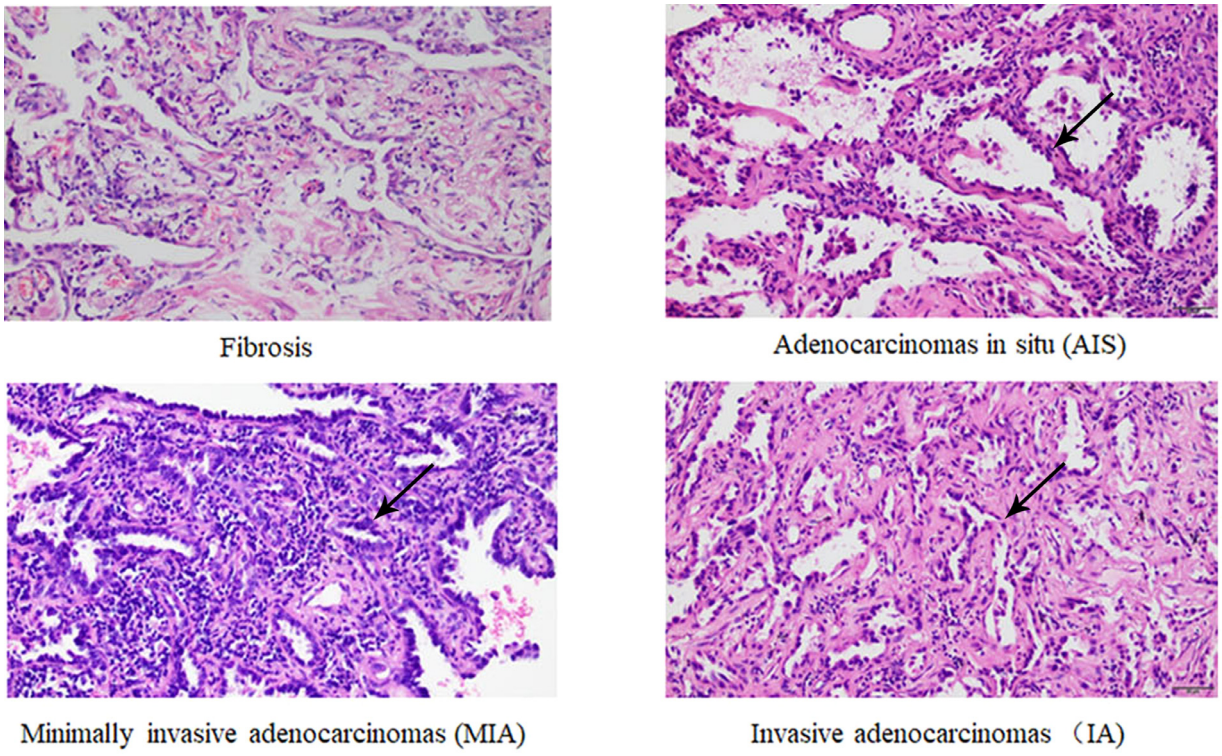


**Fig. 1.** Representative histopathology of small pulmonary nodule (SPN) tissues stained with Hematoxylin–Eosin (H&E). Magnification, 200×.

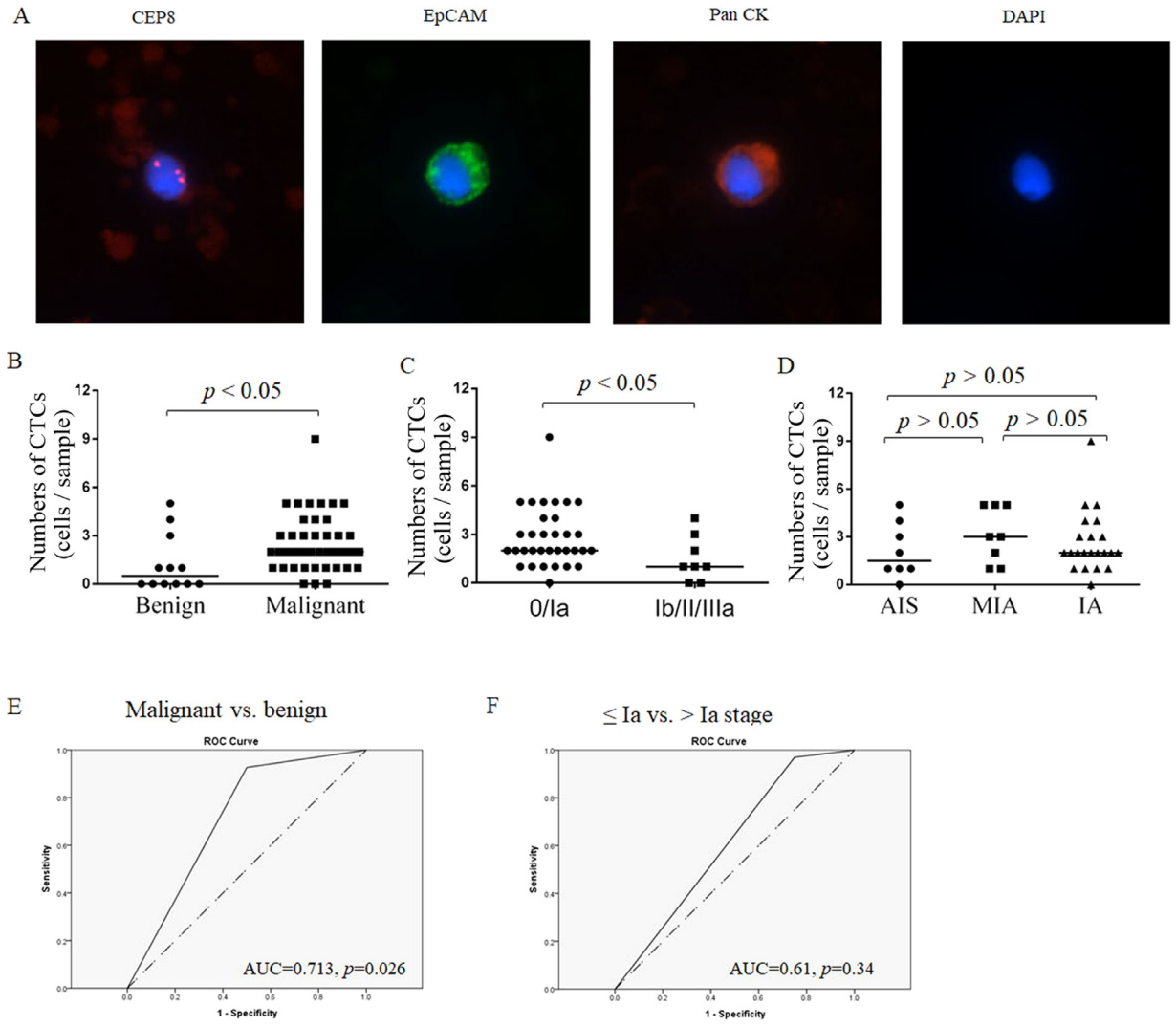


**Fig. 2.** Statistical analysis of the correlation between circulating cancer cells (CTCs) and the histopathological characteristics of small pulmonary nodules (SPNs). A, Representative images of circulating tumor cells. CEP8 FISH, EpCAM, pan-CKs, DAPI staining of cells enriched from 3.2 mL peripheral venous blood of patients with SPNs. Magnitude, 200×. B-D, The Mann-Whitney *U* test of the CTCs level between different groups as indicated. E and F, The ROC curve of the CTC number to distinguish malignant from benign SPNs (E), or ≤ Ia from > Ia stages (F).

**Table 1**
results of ROC analysis (malignant versus benign).

| | AUC (95% CI) | *p* value | Cut-off value | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|
| CTCs | 0.713 (0.525, 0.902) | 0.026 | 0.5 | 92.7 | 50.0 |
| CTCs | 0.729 (0.564, 0.893) | 0.017 | 0.5 | 70.7 | 75.0 |

the CTC numbers showed no significant difference in distinguishing the (≤Ia) from the (>Ia) lung cancer stages (Fig. 2F), invasive from non-invasive SPNs (Supplementary figure S1D), and adenocarcinoma from other cancer types (Supplementary figure S1B and S1E). These findings suggest that the numbers of CTCs might be higher in patients with malignant SPNs than with benign SPNs and that the number of CTCs can serve as a potential screening and diagnosis marker for malignant SPNs.

### *The combined use of CTC numbers and density features in CT identified malignant SPNs*

Currently, the growing use of thin-section CT scans is allowing the diagnosis of millions of patients annually with SPNs; however, only 30% of these SPNs are identified as malignant. Thus, more methods are urgently needed to assist in diagnosis. We analyzed the correlation between the CT morphology and the SPN clinicopathological characteristics, based on the three most frequently used criteria, namely the nodule size, number, and density. As shown in Table 2, no significant correlation was detected between the nodule size and malignancy or between the nodule number and malignancy, whereas the nodule density had a significant correlation with malignancy (Table 2). No statistical correlation was found between the nodule size, number, or density and the cancer stage or type (Supplementary Table S2).

The nodule number, but not the size, displayed a significant correlation with adenocarcinoma invasiveness (Supplementary table S3), but no correlation was apparent between nodule density and invasiveness (Supplementary Table S3). One reason could be the limited sample size; therefore, we pooled the AIS and MIA samples and repeated the

**Table 2**
the correlation between CT morphology and histology of SPNs.

| CT parameters Total | n 53 | Histology Malignant | Benign | $X^2$ value | p value |
|---|---|---|---|---|---|
| Size | | | | | |
| <15 mm | 34 | 27 | 7 | 0.018 | 0.892 |
| ≥15 mm | 19 | 14 | 5 | | |
| Number | | | | | |
| Single | 30 | 23 | 7 | 0.019 | 0.891 |
| Multiple | 23 | 18 | 5 | | |
| Density | | | | | |
| Solid | 32 | 21 | 11 | 4.77 | 0.029 [a] |
| Non-solid | 21 | 20 | 1 | | |

Note: a, adjust chi-square test, $1<T<5$.

comparison. This resulted in a significant correlation between density and invasiveness (Supplementary Table S4), in agreement with other reports showing that a high nodule density is an essential indicator of malignancy.

We also analyzed the correlation between the CTC numbers and CT examinations. We did not observe any significant difference in the CTC numbers between the groups with small (<15 mm) and large (≥15 mm) SPNs based on the CT examinations (Fig. 3A). We also evaluated the CTC number and the nodule size determined by CT as predictors of SPN malignancy. A shown in Fig. 3B, the CTC level in the group with large nodules, but not with small size nodules, was significantly higher in malignant than in benign SPNs.

No significant difference was apparent in the CTC numbers between the single and multiple nodule groups, but the numbers of CTCs were significantly higher in the patients with malignant SPNs than with benign SPNs (Fig. 3C and D). No difference was noted for the CTC numbers between the solid and non-solid nodule groups, but the CTC numbers were higher for patients with solid malignant SPNs than with benign SPNs (Fig. 3E and F). The limited number of benign SPN samples was an obstacle in performing a comparison with the non-solid group.

We also used ROC analysis to examine the sensitivity and specificity of CTC numbers combined with CT characteristics to predict SPN malignancy. The AUC of the density and the CTC number, but not the nodule size, showed a statistical significance in recognizing SPN malignancy (Fig. 3G). Moreover, the combined use of the CTC number and the density features had a higher AUC than was obtained using each independently. As shown in Table 3, the CTCs showed the highest sensitivity (92.7%), while the density showed the highest specificity (91.7%) when they were used alone to identify SPN malignancy. The combined use of these two features increased the sensitivity to 95% but did not increase the specificity.

We also analyzed the ability of CTC numbers, nodule size, and density features to differentiate lung cancers ≤ Ia stage from > Ia stage. The ROC curve showed that the AUC of each feature did not display any statistical significance; however, the combined use of CTC numbers and density increased the AUC to 0.735 compared to each parameter assessed independently (0.61 for CTCs and 0.648 for density) (Fig. 3H). Table 4 shows that the combined CTC number and density was significantly correlated with the SPN stage ($p = 0.041$). In addition, the sensitivity for density increased from 54.5 to 97%, and the specificity for CTC numbers increased from 25% to 50%.

These findings suggest that the combined use of the density revealed by CT examination and the number of CTCs can serve as a potent marker for the screening and diagnosis of SPNs.

*Serum tumor markers were not correlated with SPN malignancy, whereas the CEA tumor marker levels were correlated with CTC numbers*

Examination of the serum tumor markers in patients with SPNs revealed, surprisingly, that most patients were negative based on the standard criteria (<5.0 ng/mL for CEA, <7.0 ng/mL for CYFRA 21–1, < 7.29 ng/mL for AFP, and < 27.0 Unit/mL for CA 19–9). This may possibly have reflected the fact that most of the participants in this study were at an early lung cancer stage or had benign tumors. The average concentrations of the markers did not show any significant difference between the malignant and benign SPN groups (Fig. 4A). Analysis of these markers between the two groups of CTC positive and negative patients, revealed that only the CEA concentrations showed a difference between the CTC+ and CTC- groups (Fig. 4B), suggesting that the CEA concentrations differ in patients who possess CTCs.

*CEP8, EpCAM, and pan-CKs markers aided CTC identification*

Currently, no CTC-specific markers have been conclusively identified, as the same populations have shown opposing results for CTC numbers depending on the measurement technique used [28, 29]. In the present study, the CTCs were identified by a FISH assay using CEP8. We then compared commercially available markers, such as EpCAM and pan-CKs, for CTC detection using immunofluorescence staining (Fig. 2A). In total, 22 slides (1 slide for each patient) were tested for all three markers. The CTC levels determined by EpCAM+, pan-CKs+, or CEP+ were 0, 0.5, 2 cells/sample, respectively. Several patients had cells that showed dual-positive or triple-positive staining. The CTC numbers showed no significant differences between the pan-CKs-positive and EpCAM-positive samples. However, the CEP8-positive cell numbers were statistically higher than the numbers showing EpCAM or pan-CKs positivity (Supplementary figure S2). These findings indicated that different markers could detect different subpopulations of CTCs, possibly reflecting the heterogeneity of the cancer cells.

## Discussion

Lung cancer is the most prevalent cancer-related cause of death worldwide [30]. Early diagnosis and treatment can dramatically increase the patient survival rate [4]; therefore, early lung cancer diagnosis has attracted significant research interest and has made significant advances in recent years. The ability to enrich CTCs from peripheral blood means that they can serve as a kind of liquid biopsy that is both non-invasive and patient friendly [31]. However, developing CTCs as a diagnostic marker is challenging because of the scarce number of CTCs in the peripheral blood. Studies using different detection methods have reported different CTC numbers in different blood volumes [32]. For example, Teixeira et al. used the CellSearch system to enrich and sequence the CTCs collected from 7.5 mL of peripheral blood from patients with pre-invasive squamous cell lung cancer lesions [33].

Various studies have reported a correlation of CTCs with different types of cancers or different clinicopathological characteristics of cancer, but the enrichment of CTCs from SPNs at very early cancer stages has not been reported. Recently FR+ (folate receptor) CTCs were detected and used to distinguish invasive and pre-invasive lung adenocarcinomas [4]. The CEP8+ CTC count has also been reported to show a correlation with lung cancer [19]. The present study is the first to report the detection of CTCs from 3.2 mL of peripheral venous blood from patients with SPNs. We found that the number of CTCs was higher in group with malignant SPNs than with benign SPNs. The ROC analysis also showed that the CTC number was able to distinguish malignant SPNs from benign SPNs with high sensitivity and specificity using ≥2 CTCs/sample as the definition of a positive CTC level. A previous study using the standard CellSearch method reported that CTC levels of ≥5 CTCs/7.5 ml of blood in patients with metastatic breast cancer and prostate cancer and ≥3 CTCs in patients with colorectal cancer were associated with poor prognosis [34]. Our findings suggest that the CTC number could be developed as a potential marker to assist in the diagnosis of SPNs.

Surprisingly, more CTCs were detected in a very early stage (0/Ia) of lung cancer than in the advanced stages (Ib/II/III). The CTCs are cancer cells that have detached from their tumors of origin and have

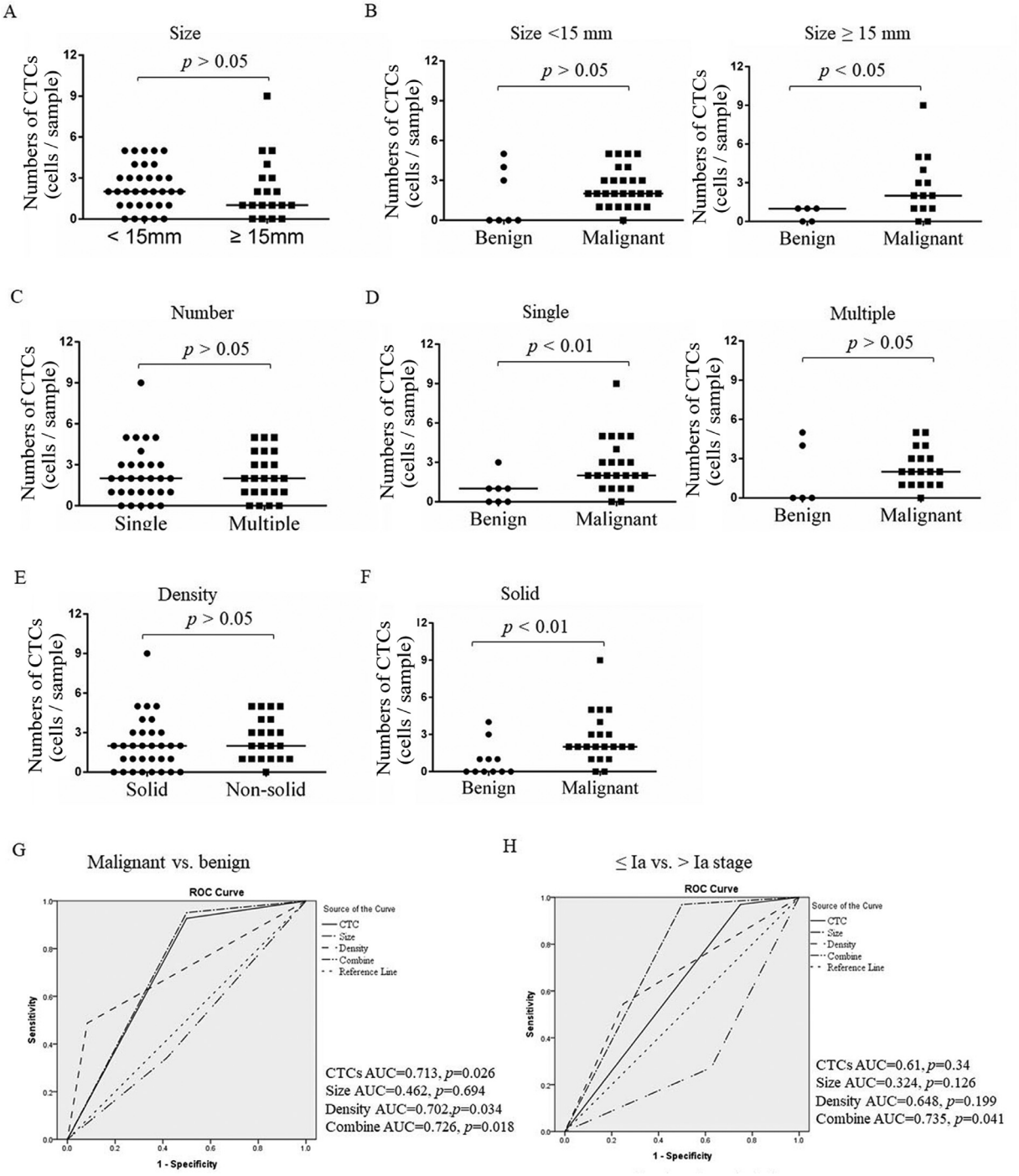


**Fig. 3.** Statistical analysis of the correlation between the numbers of circulating cancer cells (CTCs), computed tomography (CT) morphology, and the histopathological characteristics of small pulmonary nodules (SPNs). A-F, The Mann-Whitney *U* test of the CTC numbers between different groups as indicated. G and H, The ROC curve of the CTC number combined with density to distinguish malignant from benign SPNs (G), or ≤ Ia from > Ia stages (H).

**Table 3**
results of ROC analysis (malignant versus benign).

| | AUC (95% CI) | *p* value | Cut-off value | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|
| CTCs | 0.713 (0.525, 0.902) | 0.026 | 0.5 | 92.7 | 50.0 |
| Size | 0.462 (0.274, 0.651) | 0.694 | 0.5 | 34.1 | 58.3 |
| Density | 0.702 (0.551, 0.853) | 0.034 | 0.5 | 48.8 | 91.7 |
| CTCs+Density | 0.726 (0.537, 0.914) | 0.018 | 0.5 | 95.1 | 50.0 |

**Table 4**
results of ROC analysis (≤ Ia stages versus > Ia stages).

| | AUC (95% CI) | *p* value | Cut-off value | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|
| CTCs | 0.610 (0.369, 0.851) | 0.34 | 0.5 | 97.0 | 25.0 |
| Size | 0.324 (0.128, 0.540) | 0.126 | 0.5 | 27.3 | 37.5 |
| Density | 0.648 (0.441, 0.855) | 0.199 | 0.5 | 54.5 | 75.0 |
| CTCs+Density | 0.735 (0.369, 0.851) | 0.041 | 0.5 | 97.0 | 50.0 |

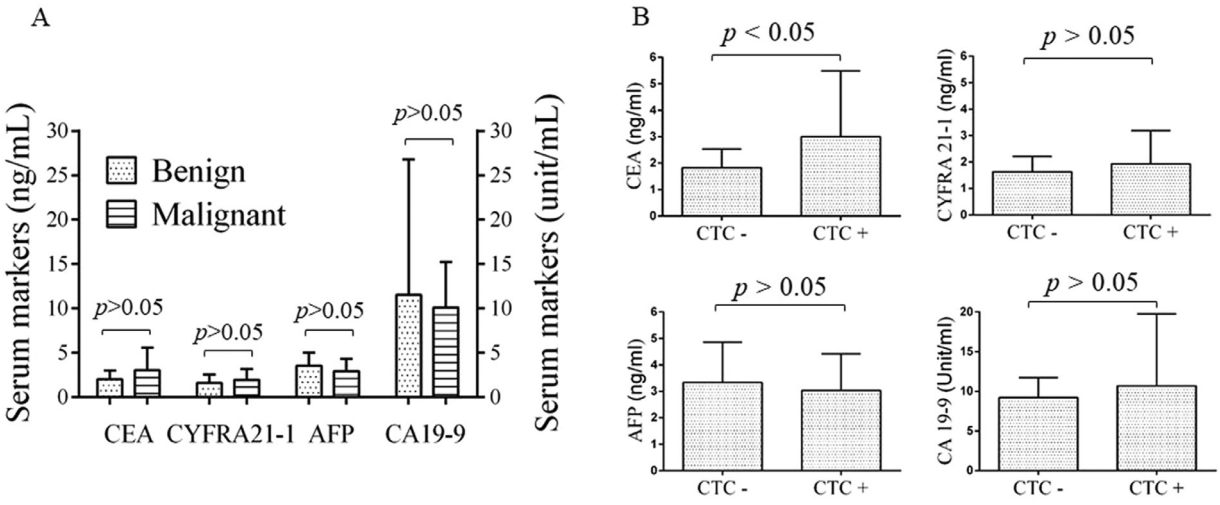


**Fig. 4.** Statistical analysis of the correlations between the numbers of circulating cancer cells (CTCs), serum markers, and the histopathological characteristics of small pulmonary nodule (SPNs). A, The Student *t*-test of the serum markers between malignant and benign SPNs. B, The Student *t*-test of the serum markers between positive and negative CTCs.

invaded the circulatory system. Therefore, higher CTC counts were expected with more advanced stages of lung cancer. However, possible explanations for the higher CTC counts at an early stage could be developmental changes, such as the formation of fibroblasts or inflammatory cells at later stages, or abnormal tumor environments in some subtypes of lung cancer. These differences in tumor composition could restrict the escape of cancer cells from late-stage tumors into the circulation. This possibility is worth investigating in the future. However, in our work, we considered this finding of early-stage CTC release to reflect a bias caused by the sample sizes because most of the patients with SPNs were in early stages and only 9 patients presented at the >Ia stage.

Currently, most SPNs are first identified by CT examinations. The prevalence of chest CT, and especially thin-section CT, has significantly increased the detection rate of SPNs (≤30 mm); however, most patients are later diagnosed with non-cancer–related diseases, inflammation, hyperplasia, fibrosis, tuberculosis, or even stress reactions [35]. These patients undergo CT examinations for common clinical symptoms, such as coughing, chest pain, minor discomfort, or as part of an annual physical examination. Thus, once diagnosed with SPNs, indicating a risk of lung cancer, they can be offered several choices, ranging from CT surveillance to CT-guided biopsy or direct surgery.

Cancer diagnosis based on CT morphology has undergone some significant recent advances. For example, radiomics nomograms have made significant progress in distinguishing malignant from benign SPNs, in determining the invasiveness of lung adenocarcinomas, and even in distinguishing ground-glass nodules less than 10 mm [36, 37]. This progress has allowed the establishment of critical guidelines for appropriate treatment and has led to a noticeable improvement in lung cancer prognosis [38]. In the current study, we also observed that the nodule density, but not the size, could distinguish malignant from benign SPNs when using the AUC in the ROS curve. The combined use of CTCs and density increased the AUC significantly and was even capable of distinguishing the 0/Ia from the Ib/II/III stages of lung cancer. However, we do not consider the latter finding correct at this point in time because of our limited sample size. Further studies with larger patient numbers are required for revealing the truth.

We observed a significant increase in the CTC level in patients with a nodule size larger than 15 mm and in patients with solitary nodules. These findings indicate that when CT scans are used to diagnose patients with SPNs, an elevated CTC number can suggest malignancy if the nodules are larger than 15 mm or if they are solid nodules. Therefore, the use of CTC numbers together with CT examinations might be more successful than either approach alone in predicting malignant SPNs.

Another issue in this study is that the CT features, and especially the density, displayed higher sensitivity and specificity than was observed for the numbers of CTCs. We suspect that the limitations of the study design are a root cause of this finding. The patients included in the study were required to have a histopathological diagnosis; therefore, only those with CT morphology, indicating a high risk of cancer, and who had been assigned for surgery could be recruited in the study. This strategy significantly increased the specificity and diagnostic value of the SPN morphology. In the CT diagnostic process for identifying malignancy, the density feature typically received more attention than other features, and this could have caused some bias in this study. This bias might also be the reason why the combined use of CTC numbers and density did not further increase the specificity of the malignancy diagnosis in the ROC analysis, since the density exhibited the highest specificity on its own. A multicenter and broad investigation reported by Lindsay et al. indicated that the CTC number could be an independent prognostic marker for advanced NSCLC [39]. Because of the limited sample size and the possible bias in our study, we are cautious in concluding that the CTC number could also be a diagnostic marker that could assist in the diagnosis of SPNs. Further studies are needed for verification.

The use of specific serum tumor markers helped to increase the sensitivity of malignancy diagnosis; however, the specificity was not satisfactory. Some serum markers are indicative of cancer origins, such as CEA for colon cancer, AFP for liver cancer, and CYFRA 21–1 for lung cancer [40]. In this study, we did not observe any significant alterations in the

serum markers based on the criteria used for clinical diagnosis. We also found no difference in the average serum concentrations between the malignant and benign groups. We identified an increased CEA concentration in the group with SPNs who also had detectable CTCs, indicating that CEA concentration and CTCs may be correlated. Overall, these findings indicate that changes in the serum tumor markers might not be significant in the very early stage of lung cancer.

We also compared several CTC markers in this study. EpCAM positivity has been widely used to identify CTCs, and CellSearch is the only method approved by the FDA; however, better methods for CTC detection are still under development [41]. Initially, the EpCAM marker is highly expressed in epithelial cells; this would cause a rise in EpCAM positive cells due to detachment of the epithelial and endothelial cells and their release into the bloodstream [42]. However, some cancer cells do not express EpCAM due to heterogeneity or because they have undergone the epithelial-mesenchymal transition (EMT), a process that causes cancer cells to lose their epithelial features and increase their migratory or invasive capacities [43]. The same issues arise when using CKs as a CTC marker [15]. In the present study, we used CEP8 as a CTC marker, and we subjected 22 samples to triple staining for CEP8, EpCAM, and pan-CKs. We observed higher numbers of CEP8+ CTCs than EpCAM+ or pan-CKs+ CTCs, but this did not suggest that CEP8+ staining was better than the other two markers because more false-positive circulating cells were also detected. Some triple-positive or dual-positive CTCs were also detected, and this would strongly support cancer cell heterogeneity. These findings suggest that different subpopulations of CTCs express different markers and further emphasize the urgent need for the establishment of new markers that can identify CTCs.

## Conclusion

We detected CTCs using CEP8 as a marker in 3.2 mL peripheral venous blood samples from patients with SPNs detected by CT scanning. The CTC numbers were correlated with SPN malignancy. The combined use of CTCs and CT morphological density features increased the sensitivity for discriminating malignant from benign SPNs. Our findings suggest that the number of CEP8-positive CTCs could serve as a potential screening and diagnostic marker that can assist in the diagnosis of malignant lung cancer in patients with SPNs.

## Ethical approval

Nanjing Medical University's ethics Committee approved the current study. Informed consent was obtained from all the participants.

## Consent for publication

All authors are responsible for the submission of this article and accept the conditions of submission.

## Availability of data and materials

The dataset(s) supporting the conclusions of this article are included within the articles and its additional files.

## Declaration of Competing Interest

All the authors have declared no conflicts of interest.

## CRediT authorship contribution statement

**Caidong Liu:** Conceptualization, Investigation, Formal analysis, Resources, Writing - review & editing. **Hongling Chen:** Investigation, Visualization, Formal analysis, Writing - review & editing. **Tong Sun:** Visualization, Funding acquisition, Writing - review & editing. **Haibo Wang:** Methodology, Investigation, Writing - review & editing. **Baoan Chen:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Xuerong Wang:** Conceptualization, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101052.

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