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

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Abstract
Background Circulating cancer cells (CTCs) provide opportunities for early diagnosis and evaluation of cancer stage as a more acceptable non-invasive liquid biopsy. The advanced development and use of CTCs for diagnosis or prognosis just started in recent years.
Methods Fifty three patients, diagnosed as SPNs with a diameter less than 30 mm by CT examination, was enrolled for statistical analysis based on their CTCs level, CT examination features, serum tumor marker concentrations, and histopathological characteristics. Centromere probe 8 (CEP8) was utilized as a marker for CTCs determination.
Results The CTCs level was significantly different between malignant and benign SPNs, as well as between early (0/Ia) and advanced (Ib/II/III) stages of lung cancer. For the malignancy judgment and the pTNM stage diagnosis of SPNs, ROC analysis results showed that combined use of CTCs level or density feature of CT morphology significant improved diagnostic effect compared to use of these two markers solely. Moreover, in bigger, single, and solid SPNs based on CT morphology, the CTCs level significantly correlated with the malignant histopathology. Additionally, triple staining (CEP8, EpCAM and CKs) results using samples from 22 out of 53 patients showed that more CTCs was detected when CEP8 was used as a marker.
Conclusion The CTCs determined by CEP8 positive would be a potential adjuvant diagnostic marker for the malignance and stage of lung cancer for patients with SPNs.

Introduction
Lung cancer is the most lethal cancer type in the world [1]. Non-small cell lung carcinoma (NSCLC) is the major type of lung cancer. It mainly contains pathologically adenocarcinomas and squamous cell carcinomas. The 5-year survival rate of NSCLC is only approximately 19% for the reason that most of the patients were diagnosed at advanced stage [1]. However, early diagnosis and treatment can significantly increase the survival rate and improve the prognosis of the disease [2]. For instance, the 5-year disease-free survival for early NSCLC (stage I) reaches 70–90% after proper treatment [2]. In recent years, with the prevalence of chest computed tomography (CT) examination, millions of patients are diagnosed as small pulmonary nodules (SPNs), which are smaller than 30 mm (the
largest diameter), solitary or multiple, ground-glass or partial-solid nodules [3, 4]. However, only approximately 30% of such SPNs are malignant lung cancer [5]. Most of them are benign diseases, such as usual interstitial pneumonia, idiopathic pulmonary fibrosis, tuberculosis [6]. There are several guidelines for management of SPNs, such as optimized surgical resection and CT surveillance, which significantly increases the survival rate; however, it also results in overtreatment, radiation exposure, or increases the risk of cancer [7]. For example, in the management of nodules, noninvasive adenocarcinoma (AIS and MIA) is considered to be indolent and subjected to sublobar resection, which preserves the function of the lung, while invasive adenocarcinoma (IA) is different [8, 9]. Therefore, identifying the malignant SPNs and further distinguishing the stage and invasiveness of lung cancer could dramatically benefit the patients.

Circulating cancer cells (CTCs) are cancer cells circulating in the blood that detached from original or metastatic tumors and invaded to the vascular system [10]. They are the main causes of cancer metastasis and cancer-related death [11]. However, they also provide opportunities for early diagnosis and evaluation of cancer stage as a more acceptable non-invasive liquid biopsy. The study of CTCs began more than a hundred year ago, but the advanced development and use of CTCs for diagnosis or prognosis just started in recent years [12, 13]. Even though CellSearch is the only method that approved by FDA (Food and Drug Administration) of U.S. for clinical determination of CTCs, there are many methods and technologies used in academic study and clinical adjuvant diagnosis [14]. Commonly, CD45 positive cells are depleted from peripheral blood cells as lymphocytes. Epithelial markers, such as EpCAM (epithelial cancer associated marker) and CKs (cytokeratins), positive cells are determined as CTCs from epithelial-originated cancers, including lung and breast cancer [15, 16]. Folate receptor, another marker for CTCs of lung cancer, has also been well studied [17]. Very recently, Zhou et al. reported the correlation between folate receptor-positive CTCs with indeterminate lung nodules and suggested its prognostic values [4]. Currently, no method is fully satisfied for diagnosis and prognosis. Thus, specific markers are urgently needed for determination CTCs from different cancer types. To date, numerous studies have shown the correlation between CTCs and cancer stages, invasiveness, and metastasis, including lung cancer [18,
However, for the reason that CTCs are extremely rare in NSCLC, whether CTCs could be detected in the peripheral blood of SPNs patients are quite unknown [20]. Therefore, collecting CTCs and analyzing characteristics of these CTCs would greatly improve the sensitivity and specificity of the lung cancer diagnosis, and provide proper subsequent therapeutic strategies.

In this study, we collected patients diagnosed as SPNs by CT examination and expected to undergo surgery. We detected the CTCs in their peripheral venous blood before lung resection using centromere probe 8 (CEP8) as the selection marker. After we collected the pathological diagnosis of these SPNs, such as benignancy, malignancy, cancer type, stage and invasiveness, the level of CTCs were subjected to statistical analysis for their correlation with CT, serum markers, and histopathological characteristics. We also compared the positive rate of different markers for defining CTCs, including CEP8, EpCAM, and pan CKs. Our findings suggest that CTCs could be enriched from 3.2 mL peripheral blood of SPNs patients, and its level is correlated with the histopathological characteristics of SPNs. CTCs is a potential adjuvant diagnostic marker for SPNs.

Materials And Methods

Patients

This study included 53 patients with SPNs detected by computed tomography (CT) examination at Nanjing First Hospital from May to November in 2019. CT-detected single or multiple lung nodules with the diameter ≤30 mm were defined as SPNs. Diagnosis was determined by histopathological examine of tissues from lung surgery. Forty-one out of 53 patients were malignant SPNs, and most of which (38/41) were adenocarcinomas. Twelve out 53 patients were benign SPNs, which include fibrosis, hyperplasia and tuberculosis. Patients with or without pulmonary clinical symptoms, such as cough, chest pain were included. Patients with chemotherapy, radiotherapy, and lung cancer-related disease or surgery were excluded. The histological types of NSCLC and the diagnosis of adenocarcinoma of in situ (AIS), minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma (IA) of the lung were determined according to WHO classification of tumors of the lung. The pathological tumor nodal metastasis (pTNM) stage was determined according to AJCC stage manual (8th. Version). Histopathology diagnosis was obtained and determined by two pathologists.
The study was approved by the Ethics Committee of Nanjing Medical University. Informed consent was obtained from all the participants.

Isolation and identification of CTCs

Peripheral venous blood (3.2 mL) from each patient before lung surgery was collected in customized Acid Citrate Dextrose (ACD)-anticoagulant tubes (Becton Dickinson, NJ, USA). CTCs was enriched and determined by the Cyttel method following the instruction of the manufacturers (Cyttel, Jiangsu, China) as previously reported [19]. Briefly, red blood cell (RBC) lysis buffer was used to deplete the RBCs from the whole blood. The leukocytes were removed by centrifugation after incubated with anti-CD45 antibody-conjugated immuno-magnetic beads. The resulting solution containing CTCs was smeared on the slide for subsequent analysis.

Centromere probe 8 (CEP8) in the cells on the slide was detected by fluorescence in situ hybridization (FISH) method as previously reported [21]. CEP8 staining indicated the copy numbers of chromosome 8 in the cell. Then, Alexa Fluor 594 conjugated anti-human CD45 antibody (Cyttel, Jiangsu, China) and DAPI (Vector Laboratories, Burlingame, CA, USA) were used to stain leukocytes and cell nucleus. Cells with features of CEP8 ≥2/ CD45¬/ DAPI+ were considered CTCs. Identification of CTCs was performed by independent researchers in a blinded manner [18].

Some slides (22/53) were subjected to immunofluorescence for two more tumor markers, EpCAM and pan CKs for further analysis. Cells on the slides were incubated with primary anti-EpCAM (BAF960, R&D system) or anti-pan Cytokeratins (pan CKs) (ab7753, abcam) antibodies, followed by secondary antibody Alexa Fluor 488 or Alexa Fluor 555 (ab150129 and ab105106, abcam). Fluorescent images were captured with an Olympus BX63 microscope using the IMSTAR high content screening device (IMSTARSA, France).

Identification of SPNs by CT

The features of SPNs were classified into different groups, small (<15 mm) and big (≥15 mm, ≤30 mm) based on the nodule size, single (n=1) and multiple (n>1) based on the nodule number, or solid (high density, cord-like appearance, or with globular mass) and non-solid (low density, or ground glass-like appearance) based on the nodule density. Two independent radiologists read the CT image.
Examination of the tumor markers in the SPNs patients’ serum

Serum samples (from peripheral venous blood) were collected in anticoagulant-free blood-collecting tubes. Tumor markers, such as CEA, CYFRA 21-1, and AFP, were detected via the CMIA (chemiluminescence microparticle immunoassays) methods (Reagent kit #7K68, 2P55, 3P36) using ARCHITECT-i2000sr (Abbott Laboratories, USA). CA 19-9 was detected via automatic electrochemiluminescence immunoassay (Reagent kit #11776193) using Cobas e602 (Roche Ltd, Germen).

Statistical analysis

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

Results

The level of CTCs was correlated with malignant SPNs.

There are amounts of studies detected CTCs in advanced cancer patients, including lung cancer. However, whether CTCs could be detected from the peripheral venous blood of SPNs patients is unknown. We first enriched the CTCs from patients diagnosed as SPNs by CT examination. We collected the histopathologic diagnosis of the SPNs if the patient underwent surgery. We also examined the serum tumor markers before the surgery. Fifty-three patients which successfully completed CTCs, CT, serum markers, and histopathology examinations were included for the subsequent statistically analysis. The demographic and clinic pathologic characteristics of the patients were displayed in Supplementary table S1.

We found that 41/53 patients were lung cancer, and most of which (38/41) were adenocarcinomas. Since the invasiveness of adenocarcinomas provide critical guidelines for the resection strategy and is correlated with the prognosis, we classified the patients into 3 groups, AIS, MIA, and IA. We also
divided the patients into two groups (≤1a and >1a) based on the pTNM stage, given that ≤1a stage was recommended for limited surgical resection, such as segmentectomy, sublobar or lobectomy resection, which would both increase the survival rate and maximally preserve the lung function [22-25]. Twelve out of 53 patients were benign pulmonary diseases, such as fibrosis, hyperplasia, and tuberculosis. The representative hematoxylin & eosin (H&E) staining of these tissues were shown in Fig. 1.

Then, we analyzed the correlation between CTCs level and malignant SPNs. CEP8+/ CD45−/ DAPI + were determined as CTCs. Representative staining was shown in Fig. 2A. The level of CTCs was presented by the number of CTCs in each sample. We found that the CTCs level was significantly higher in malignant SPNs than benign SPNs by Mann-Whitey U test (p < 0.05) (Fig. 2B). Similarly, the average CTCs numbers of malignant SPNs were significantly higher than benign SPNs by the student t test (Supplementary figure S1A). Moreover, the CTCs levels were significantly lower in >1a stages (Ib/II/III) of lung cancer than ≤1a stage (0/1a) of lung cancer (p < 0.05) (Fig. 2C). Since 38/41 malignant SPNs were adenocarcinomas (Supplementary figure S1B), we analyzed the correlation between the CTCs levels and cancer invasiveness. However, no significant difference of the CTC levels was observed among AIS, MIA and IA groups (Fig. 2D). Considering the limited sample size, we pooled MIA and AIS to make up noninvasive group compare with invasive group (IA), since MIA was considered as noninvasiveness pathologically. We did not observe any statistical difference in the CTCs levels (Supplementary figure S1C).

Furthermore, the ROC analysis showed that the sensitivity and specificity for the CTCs as a diagnostic marker to distinguish malignant SPNs from benign SPNs were 92.7% and 50%, respectively (Fig. 2E, Table 1). The area under curve (AUC) of the CTC levels was 0.713 with a statistical significance, indicating it would be an independent diagnostic marker for malignancy (p < 0.05). However, the CTCs levels showed no significant difference to distinguish ≤1a stage lung cancer from >1a stage lung cancer (Fig. 2F), as well as to distinguish invasive from non-invasive SPNs (Supplementary figure S1D) and adenocarcinoma from other types (Supplementary figure S1B and S1E). These findings suggest that the levels of CTCs were increased in malignant SPNs compared to benign SPNs, albeit very early
stage of lung cancer released more CTCs than late stage disease. The level of CTCs would serve as a potential liquid biopsy marker for the diagnosis of malignant SPNs.

|               | 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            |

Combined use of CTCs level and density features of the CT examination identified malignant SPNs. Currently, with the growing use of CT examination, especially thin-section CT scan, millions of SPNs were detected annually; however, only 30% were finally identified as malignant. Thus, more adjuvant diagnostic methods were urgently needed. We firstly analyzed the correlation between the CT morphology and the clinicopathological characteristics of SPNs, based on the most frequently used three criteria, such as size (the largest diameter), number, and density of the nodules. As shown in Table 1, no significant correlation was observed between size and malignancy of the nodules, neither between the nodule number and malignancy. However, the density of the nodules showed significant positive correlation with the malignancy (Table 2). Statistical analysis also showed that there had no correlation between size, number, or density of the nodules and the cancer stages, neither any correlation with cancer types were observed (Supplementary table S2). Moreover, the number of the nodules displayed significant correlation with invasiveness of adenocarcinomas, but no correlation exhibited between the size and the invasiveness (Supplementary table S3). Furthermore, no obvious correlation was observed between the density and the invasiveness (Supplementary table S3). Considering this may be caused by limited sample size, we pooled AIS and MIA together and observed a significant positive correlation between density and invasiveness (Supplementary table S4). These findings were consistent with other reports that the high density of the nodules was an important indicator for malignancy.
Then, we analyzed the correlation between the CTCs levels and CT examination. We did not observe any significant difference of the CTCs level between the small (< 15 mm) and big (≥ 15 mm) SPNs group based on the CT examination (Fig. 3A). We investigated the prediction of SPNs malignancy by considering both the CTCs level and the size of CT morphology. As shown in Fig. 3B, the CTCs level was significantly higher in malignant than in benign SPNs in big SPNs group, but not in small group. Moreover, although no significant difference in CTCs level was exhibited between the number of single and multiple groups, the level of CTCs showed a significant increase in malignancy compared with benign SPNs when only single SPNs are considered (Fig. 3C and D). No difference of the CTCs level was observed between solid and non-solid group, albeit the level of CTCs was obviously higher in malignancy than benign SPNs when only solid SPNs are considered (Fig. 3E and F). We did not analyze non-solid group for the reason of lacking enough sample number in benign SPNs group.

Next, we analyzed the sensitivity and specificity of the CTCs level combined with CT characteristics for prediction of malignancy of SPNs by ROC analysis. The AUC of the density, the CTCs levels, but not the size, showed statistical significance (Fig. 3G). Moreover, combined use of the CTCs levels and the density features exhibited higher AUC than each single item. As shown in Table 1, the CTCs showed the highest sensitivity (92.7%) and the density showed the highest specificity (91.7%), when they were solely used for identifying malignancy. Combined use of these two features did further increase the sensitivity but not the specificity.

Additionally, we analyzed the CTCs level, size and density features of the nodules in differentiating the ≤ Ia stages from > Ia stages of lung cancer. The ROC curve showed that the AUC of each feature.
did not display any statistical significance; however, combined use of CTCs level and the density increased the AUC to 0.735 compared to each single one (0.61 for CTCs and 0.648 for density) (Fig. 3H). As shown in Table 3, combined use of CTCs level and density significantly correlates with SPNs stage ($p = 0.041$) and, with a sensitivity of 97% and specificity of 50%, this combined diagnostic strategy improved the overall diagnostic effect compared with when solely CTCs level or density was considered.

|               | 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            |

These findings suggest that combined use of the density feature of the CT examination and the CTCs level could be developed as a potent adjuvant quality and stage diagnosis of SPNs.

The serum tumor markers did not show any correlation with malignant SPNs, but the CEA levels were correlated with the CTCs levels.

We also examined the serum tumor makers of the SPNs patients. To our surprise, most of the patients showed negative results based on the normal 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), possible due to most of the subjects in this study are early lung cancer or benign diseases. Moreover, the average concentrations of the each marker did not show any significant difference between malignant and benign SPNs groups (Fig. 4A).

We analyzed these markers between the two groups in CTCs positive or negative patients. As shown in Fig. 4B, among the 4 serum markers, only the CEA concentrations exhibited difference between CTC + and CTC- groups. These findings indicate that the concentration of CEA would be different if the patient has CTCs.

The comparison the CEP8, EpCAM, and pan CKs for CTCs identification.

Currently, the specific markers of CTCs have not been well identified. Even the same population could show opposite results for CTCs levels when different methods were used [26, 27]. In this study, CEP8
positive cells by FISH assay was defined as the CTCs. We then compared other frequently used markers such as EpCAM and pan CKs for the detection of CTCs using immunofluorescence staining in the same slides following CEP8 examine (Fig. 2A). We found that among the 22 samples that successfully completed all three markers staining, only 5 patients (1 slide for each patients) had dual positive cells EpCAM+/pan CKs + with a detection rate of 22.7%, which was consistent with others report [28]. And there were some cells showed triple positive staining. The level of CTCs showed no significant difference between pan CKs and EpCAM positive cells. However, CEP8 positive cell numbers were higher than EpCAM or pan CKs with a statistical significance (Supplementary figure S2). These findings indicated that different markers detected different subpopulations of CTCs due to the heterogeneity of cancer cells.

Discussion
Lung cancer is the most prevalent cancer and the leading cause of cancer-related death worldwide [29]. Early diagnosis and treatment dramatically increased the survival rate of the patient [4]. So, early diagnosis of lung cancer attracted great research interests and developed fast in recent years. CTCs could be enriched from peripheral blood, a kind of fluid biopsy, noninvasive and more acceptable for patients [30]. But it is difficult to develop CTCs as a diagnostic marker, because the number of CTCs was extremely low in the peripheral blood. Different detection methods obtained different CTC numbers in different volumes of blood [31]. Recently, Teixeira et al. enriched and sequencing the CTCs collected from 7.5 mL peripheral blood of patients with pre-invasive squamous cell lung cancer lesions using Cellsearch system [32]. Even though large amount of studies reported the correlation of CTCs with different types of cancers or different clinicopathological characteristics of cancer, whether CTCs could be enriched from SPNs, a very early stage of cancer, are quite unknown. Recently FR+ (folate receptor) CTCs were detected and used to distinguish invasive and preinvasive lung adenocarcinomas [4]. In this study, we for the first time detected CTCs from 3.2 mL of the peripheral venous blood of SPNs patients. It showed that the level of CTCs was higher in malignant group than benign group. Furthermore, the ROC analysis also showed that the the method of measuring CTCs level was able to distinguish malignant SPNs from benign SPNs with high
sensitivity. These findings suggest that CTCs level could be developed as a potential adjuvant diagnostic marker for SPNs. To our surprise, more CTCs were detected in very early stage (0/Ⅰa) of lung cancer than advanced stages (Ⅰb/Ⅱ/Ⅲ). Since CTCs are escaped cancer cells that invaded to the circulating system and survived, we suspected that in the advanced stages of lung cancer, the tumor microenvironment was formed, therefore, CTCs was stuck in the tumor. It is possible a sample bias because most of the SPNs patients were in early stages.

Currently, most of the SPNs were first identified by CT examination. With the prevalence of chest CT, especially thin-section CT, the detection rate of small pulmonary nodules (≤ 30 mm) significant increased. However, most of the patients were lately diagnosed as non-cancer-related diseases, just inflammation, hyperplasia, fibrosis, tuberculosis, or even a stress reaction [33]. These patients take the CT examination for some light clinical symptoms, such as cough, chest pain, minor uncomfortable, or merely for annual physical examination. Thus, as soon as be diagnosed as SPNs, which indicated the risk of lung cancer, they were subjected to several choices, such as CT surveillance, CT-guided biopsy, or direct surgery. Recently, diagnosis of cancer by CT morphology was greatly developed. Various radiomics nomogram made great progress in identifying malignancy from benign SPNs, determining the invasiveness of lung adenocarcinomas, and even distinguishing ground-glass nodules less than 10 mm [34, 35]. These progresses provided critical guidelines for appropriate treatment which resulted in obviously improvement for the lung cancer prognosis [36]. In the current study, we also observed that the density of the nodules, but not the size of the nodules, distinguished malignant SPNs from benign SPNs according to AUC in the ROS curve. Combined use of CTCs and density increased AUC significantly. Moreover, combined use of CTCs and density also distinguished 0/Ⅰa stages of lung cancer from Ⅰb/Ⅱ/Ⅲ stages. On another hand, we observed significant increase in the CTCs level of patients with the nodule size bigger than 15 mm and the patients with solitary nodules. These findings indicate that when a patient was diagnosed as SPNs by CT examination, elevated CTCs level suggested malignancy if the nodules was bigger than 15 mm or the nodules was solid.

Therefore, CTCs is a potential adjuvant diagnostic marker in collaboration with CT examination. Another issue of this study is that the patients who were finally included in the study were those who
took the surgery and diagnosed by histopathology, based on the CT morphological changes. Surgeries were suggested only for those with risky CT morphological changes. In the CT diagnostic process of identifying malignancy, the density feature gained higher weight than other features, which would cause some bias in this study. This might also be the reason that combined use of CTCs and density did not further increase the specificity of malignancy diagnosis in the ROC analysis, since that the density exhibited the highest specificity alone. Lindsay et al reported that the CTCs could be an independent prognostic marker for advanced NSCLCs based on a multicenter, large size investigation [37]. We are carefully to say that it could be an independent diagnostic marker for SPNs based on our findings that the CTCs level was obviously correlation with malignancy, because of the limited samples size and the possible bias in the study.

Specific serum tumor markers were helpful in increasing the sensitivity of malignant diagnosis; however, the specificity is not satisfied. Some serum markers are indicative for the origin of the cancer types, such as CEA for colon cancer, AFP for live cancer, and CYFRA 21 – 1 for lung cancer [38]. In this study, we did not observed any significant alterations of serum markers based on the criteria used for clinical diagnosis. Neither did us observed any difference of average serum concentrations between malignant and benign groups. We identified increased CEA concentration in CTCs positive group of SPNs, indicating that these two factors may be correlated. These findings indicate that changes of the serum tumor markers might not be significant in very early stage of lung cancer.

We also compared several CTCs markers in this study. Even though EpCAM positive was acknowledged by most of the researchers for CTCs, and CellSearch was the only approved method for detection of CTCs, finding better methods for CTCs detection are still on the way [39]. Firstly, EpCAM was a highly expressed marker in epithelial cells, this would cause the issue that EpCAM positive cells may include some circulating epithelial cells and endothelial cells dropped physiologically into the blood stream [40]. Secondly, some cancer cells did not express EpCAM due to heterogeneity or epithelial-mesenchymal transition (EMT), a process that cancer cells lose their epithelial features to increase migratory or invasive capacities [41]. Therefore, not all cancer cells expression EpCAM.
Same issues existed for using CKs as a marker for CTCs [16]. Folate receptor (FR) positive cells as a CTCs marker was reported to distinguish malignant from benign diseases [4, 42]. Compared with FR (folate receptor) and MTD (maximum tumour diameter) combined system reported [4], our CEP8 and CT combined SPNs diagnostic system exhibited much higher sensitivity (95.1% versus 78.6%-82.7%). In addition, FR and MTD combined system could only differentiate noninvasive cancers from invasive cancers while our system could distinguish the nature of SPNs, i.e. between benignancy and malignancy, which would be used as a further upstream diagnostic guidance in the treatment of SPNs.

In this study, we used CEP8 as a CTCs marker. Notably, we successfully stained 22 samples with CEP8, EpCAM, and pan CKs. Even though this study was the first to use Cyttee method for CTCs detection in SPNs, we observed that the level of CEP8 + CTCs was higher than EpCAM + and CKs + cells. However, there also observed some triple positive cells. The detection rate of cells with dual EpCAM+/CKs + staining (22.7%, 5 out of 22 patients) was consistent with other reports (approximately 20%) [28]. These findings suggest that different subpopulations of CTCs express different markers, and more new markers for identifying CTCs are urgently needed.

In summary, we detected the CTCs using CEP8 as a marker in the 3.2 mL peripheral venous blood from patients with SPNs diagnosed by CT examination. The elevated level of CTCs was correlated with the malignancy and stages of SPNs. Combined use of CTCs and density feature of CT morphological diagnosis increased the sensitivity in distinguishing malignant SPNs. Our findings suggest that CTCs level combined with CT diagnosis would be used as a novel marker for determining the nature of SPNs at an early stage.

Declarations
Ethics approval and consent to participate

The study was approved by the Ethics Committee of Nanjing Medical University.

Consent for publication

Informed consent was obtained from all the participants.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding
author on reasonable request.

Competing interests

The authors declare no competing of interest.

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Authors’ Contributions

CL, HC and TS conducted sample collection, the experiments, and the statistical analysis. BC and HW were involved in study design and manuscript writing. XW were responsible for study design, data analysis, and manuscript writing. All authors reviewed the manuscript.

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Figures

![Histopathology of SPNs tissues with HE staining.](image1)

**Figure 1**

The representative histopathology of SPNs tissues with HE staining. Magnitude, 200×.
Statistical analysis of the correlation between CTCs and histopathological characteristics of 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 CTCs level to distinguish malignancy from benign (E), or ≤ Ia from > Ia stage (F).
Statistical analysis of the correlation among CTCs, CT morphological and histopathological characteristics of SPNs. A-F, The Mann-Whitney U test of the CTCs level between different groups as indicated. G and H, The ROC curve of the CTCs level combined with density to distinguish malignancy from benign (G), or ≤ Ia from > Ia stage (H).
Figure 4

Statistical analysis of the correlation among CTCs, serum markers and histopathological characteristics of SPNs. A, The Student t test of the serum markers between malignant and benign SPNSs. B, The student t test of the serum markers between positive and negative CTCs.

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