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Overcoming Obstacles in Pathological Diagnosis of Pulmonary Nodules through Circulating Tumor Cell Enrichment

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With the popularity of low-dose computed tomography (LDCT) in clinical examination of the lung, the prevalence of pulmonary nodules has significantly increased, thus significantly improving the early diagnosis of lung cancer, but also potentially contributing to overtreatment. This study aims to develop a noninvasive method to assist in diagnosing the pulmonary nodules. To do so, 3798 patients are recruited from the Department of Thoracic Surgery at Shanghai Pulmonary Hospital and peripheral blood samples are collected from them before surgery. From these samples, circulating tumor cells (CTC) are isolated using folate receptor (FR) positivity, and then enriched and analyzed in relation to cancer gene expression, stage, and level of invasion. The average CTC concentration of patients with lung disease is 11.97 functional unit (FU) in a 3 mL sample of blood. FR-positive CTC levels correlate with the expression of lung cancer driver genes tumor-node-matastasis (TNM) stage, and pleura invasion. The sensitivity of CTC levels to lung cancer diagnosis is 87.05%. Results from this study demonstrate that the determination of FR-positive CTC concentration is a convenient and time-saving strategy to improve the pathological diagnosis of pulmonary nodules.

1. Introduction

With the increasing use of low-dose computed tomography (LDCT) in the clinical examination of the lung, the prevalence of pulmonary nodules has significantly increased. Pulmonary nodules are focal, round imaging findings with increased density.[1] They can be single or multiple and with or without atelectasis, hilar enlargement, and/or pleural effusion. Pulmonary nodules may arise from multiple etiologies, including lung inflammation, tuberculosis, hemorrhage, or tumor.[2] While determining the nature of an identified nodule (i.e., benign or malignant) is key for subsequent clinical decision-making and treatment planning, an accurate diagnosis is not always clear given our lack of sensitive diagnostic indicators. Suspicious cases are currently *inferred* by the position, shape, and size of the lesion, and are generally recommended for immediate surgery; in some cases, the lesion that is removed is benign,[3] meaning the patient was improperly subjected to the risks of major surgery for no clinical benefit. Therefore, there is an urgent need to improve our ability to accurately diagnose pulmonary nodules screened by LDCT examination.

One factor limiting our ability to identify new diagnostic factors in this area is a lack of access to patients and samples. The collection of lung tissue is invasive and not very convenient or easy; a practical alternative would be a biological fluid, like peripheral blood. This would be feasible, given that peripheral blood contains

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a small number of tumor cells, known as circulating tumor cells (CTCs).[4] CTCs, discovered more than 150 years ago, have become increasingly studied as promising diagnostic or screening indicators for many different types of cancer; however, due to the scarcity of CTCs in the peripheral blood, their identification and enrichment has been a technical barrier that has slowed the adoption of CTC analysis in the clinic. Recently, technical advancements in the field, for instance, the development of immunomagnetic beads, has allowed researchers to reevaluate the inclusion of CTC in the screening and diagnostic workflows.[5,6] As such, the correlation between CTCs and various types of tumors has been gradually confirmed as the number of studies increases. In 2010, the analysis of CTCs was written into the American Joint Committe on Cancer (AJCC) Cancer Staging Manual as a new indicator to complement pathological staging, namely the use of CTC for tumors staging between M0 and M1. Given that they carry all the genomic information of a patient's tumor burden, CTCs have a great clinical significance in tumor diagnosis, treatment, and detection.[7] The number of CTCs has also been shown to be positively correlated with tumor pathology and can be used to assess the prognosis of advanced breast cancer. In the current study, we developed and validated a simple and easy-to-use CTC enrichment and detection protocol to improve lung lesion diagnosis, based on the use of immunomagnetic beads to isolate and concentrate folate receptor (FR)-positive CTCs from peripheral blood.

2. Results and Discussion

2.1. Circulating Tumor Cell Enrichment

The integration of CTC analysis into the diagnostic workflow for pulmonary nodules has some obvious advantages, in terms of its ease of collection, accuracy, cost effectiveness, and timeliness; however, it requires two preconditions: The first is the identification and validation of key markers that can enrich for the CTC population in whole blood. The technical difficulties of enriching for CTCs are primarily due to their rarity and heterogeneity. Compared with 10^9 erythrocytes and 10^6–10^7 neutrophils in 1 mL of peripheral blood, patients with advanced tumors will typically only have about 10 CTCs per milliliter of peripheral blood, and the number of CTCs in early stage patients is even less. Furthermore, CTCs are highly variable in terms of their size, morphology, and biochemical composition, thus furthering the difficulty of enrichment.

At present, there are four main methods used to enrich CTCs: The first is through physical means. Physical separation can be accomplished through one of two strategies: a) Screening based on cell size, given that the diameter of CTCs is larger than most leukocytes and erythrocytes. This is most often performed by using a mesh of a certain pore size. b) Screening based on cell density, given that CTC density varies with volume. A second method used to enrich CTCs is their capture and isolation using immunomagnetic beads. This process can also be achieved through two methods: a) Forward separation, in which epidermal cells (including CTCs) are separated by their binding to the epithelial cell adhesion molecule (EpCAM), which is attached to the surface of the magnetic beads. b) Negative separation, in which CTCs are enriched after removing leukocytes (through their binding to the leukocyte universal antigen, cluster of differentation 45 (CD45)) and rupturing erythrocytes. A third method is nucleotide-based detection, in which the presence of CTCs is confirmed by recognizing tumor-specific DNA or mRNA. Finally, CTCs can be enriched using microfluidic chips, although these practices have not been well applied yet to clinical practice.

These various methods have unique advantages and disadvantages.[8–10] Given that the cellular membrane of CTCs is malleable and easily deformed, cell-size-based screening can very often permit CTCs to slip through a given mesh.[11] This is particularly true for those CTCs that are <8 μm in diameter, but have retained their cancer stem cell properties and, thus, would have significance in clinical diagnosis, treatment, and prognosis. Furthermore, the size of CTCs is often similar to leukocytes, which reduces their separation coefficient and, thus, reduces the sensitivity of downstream analytical tests.[12] There are also problems that arise when forward-separating CTCs using immunomagnetic beads. Although the products tend to be highly pure, the procedure's recovery rate is poor since EpCAM is only expressed on about 30% of CTCs.[13,14] In contrast, negative separation procedures produce CTCs with relatively low purity but at a high recovery rate. Nucleotide-based amplification, on the other hand, is extremely sensitive; however, it lacks specificity because it may capture normal cells and produce false-positive signals.

Based on the above comprehensive analysis, we chose to utilize the leukocyte-based negative enrichment strategy to capture CTCs, given this procedure's high recovery efficiency. Our patient cohort included 3798 patients who were admitted to the Department of Thoracic Surgery, Shanghai Pulmonary Hospital (Shanghai, China). Of these, 57.79% (2195) were female and the age of the patients spanned from 17 to 87 years of age. A minority (14.35%; 545) of the patients had a history of smoking, and only four had a past family history. Peripheral blood (3 mL) was collected from all patients before they underwent surgery; blood samples were immediately processed for CTC enrichment. After erythrocyte rupture, leukocytes were removed through absorption by the leukocyte universal antigen, CD45, leaving a mixture of CTCs and residue white blood cells (**Figure 1**). This protocol has the highest recovery rate, which we found to be essential for addressing CTC heterogeneity.

2.2. Folate Receptor as a Sensitive Marker of CTCs in Peripheral Blood

The second precondition for using CTCs is the identification of a CTC-specific marker that has robust sensitivity and specificity across a population of patients. This marker should be unique to tumor cells and must have a high expression rate. Folic acid, an essential vitamin, is endocytosed into cells through its binding to the FR located on the cell surface. FR is minimally expressed in the fallopian tubes, renal tubules, alveolar parietal cells, choroid, and uterus, and is not expressed in blood cells and only weakly on the surface of activated macrophages. FR, however, is highly expressed in several type tumor cells,[15] and in more than 78% of lung cancer cells.[16,17] Furthermore, FR can be used to recognize live CTCs and its abundance is not affected by epithelial to mesenchymal transition. We thus chose to utilize FR as a CTC-specific marker in blood samples. In order to utilize real-time

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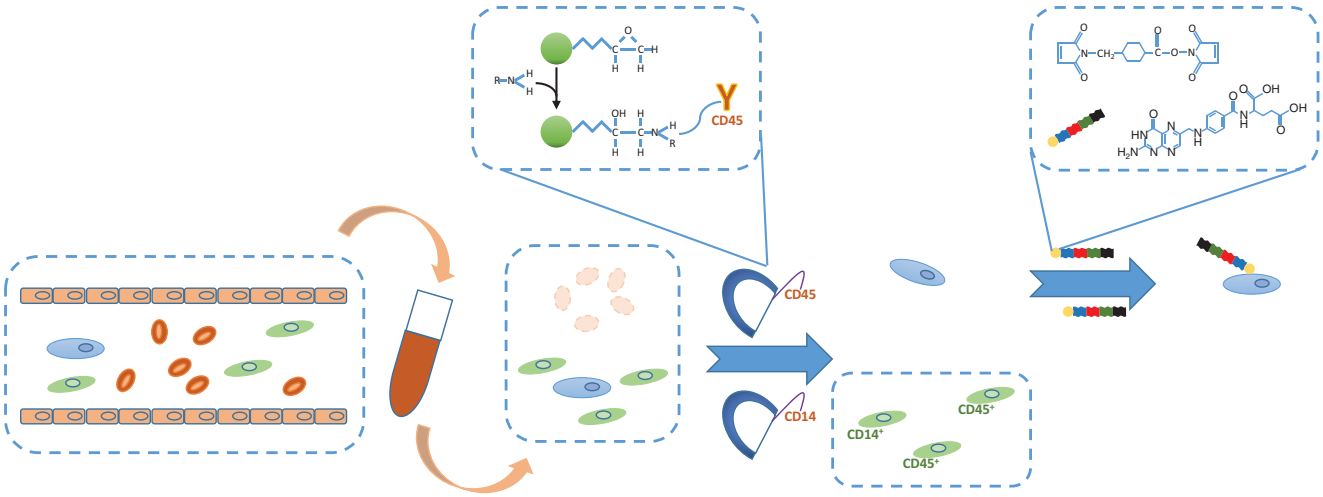

Figure 1. The schematic of FR-assisted CTC concentration analysis.

polymerase chain reaction (PCR) as the diagnostic platform, we designed a DNA probe to the FR ligand (Figure 1). When applied to our patient cohort, we characterized an average CTCs concentration of 11.97 FU in a 3 mL sample of peripheral blood.

2.3. The Relationship between FR-Positive CTC Levels and Clinicopathological and Genetic Indicators

We next determined the correlation between the level of FR-positive CTCs and other clinicopathological markers (leukocyte, osteocalcin, alpha-fetoprotein, carcinoembryonic antigen, neuro-specific enolase, pro-gastrin-releasing peptide, Beta2 microglobulin, carbohydrate antigen 242, carbohydrate antigen 153, CYFRA21-1, carbohydrate antigen 50, carbohydrate antigen 199, carbohydrate antigen 724, squamous cell carcinoma antigen, and ferritin). As shown in **Figure 2**, while we found no association between the levels of CTCs and any of these markers, we did find a correlation with the expression of three major lung cancer driver genes (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), anaplastic lymphoma receptor tyrosine kinase (*ALK*) and

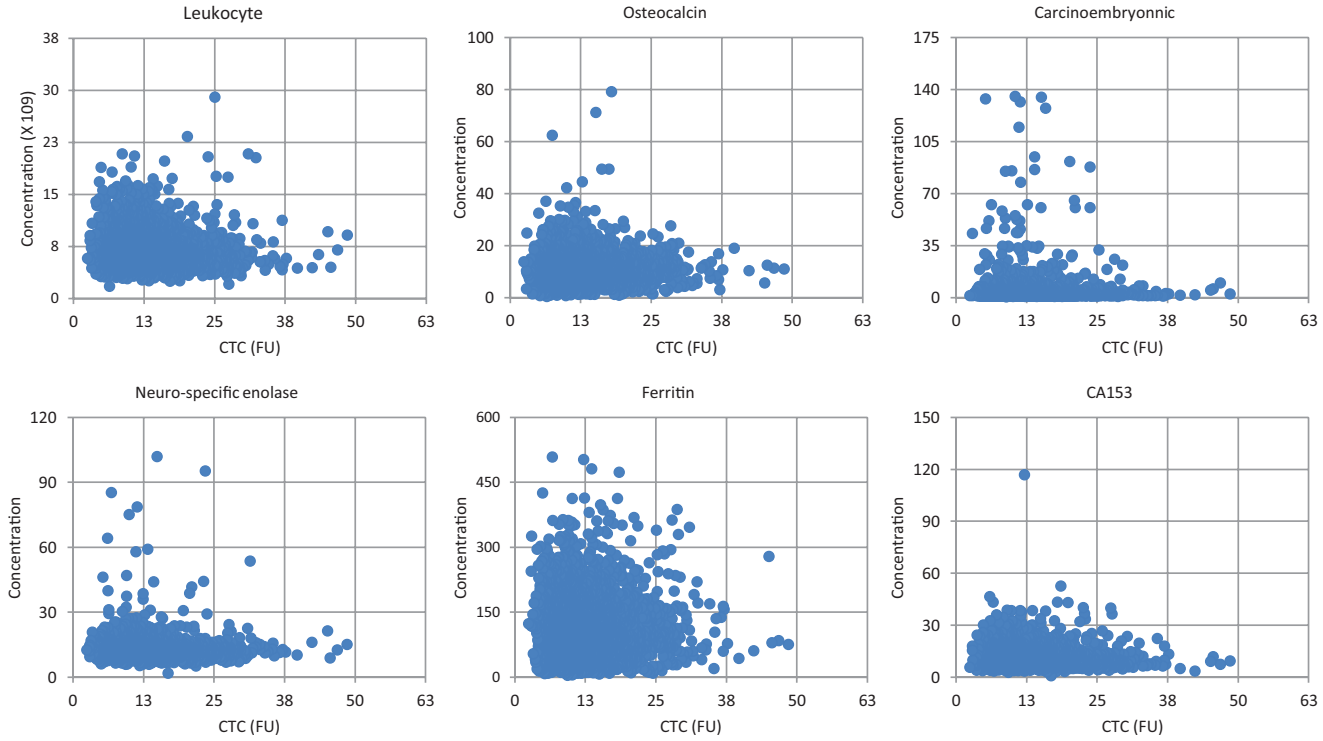

Figure 2. The correlation between CTC concentration and tumor biomarkers.

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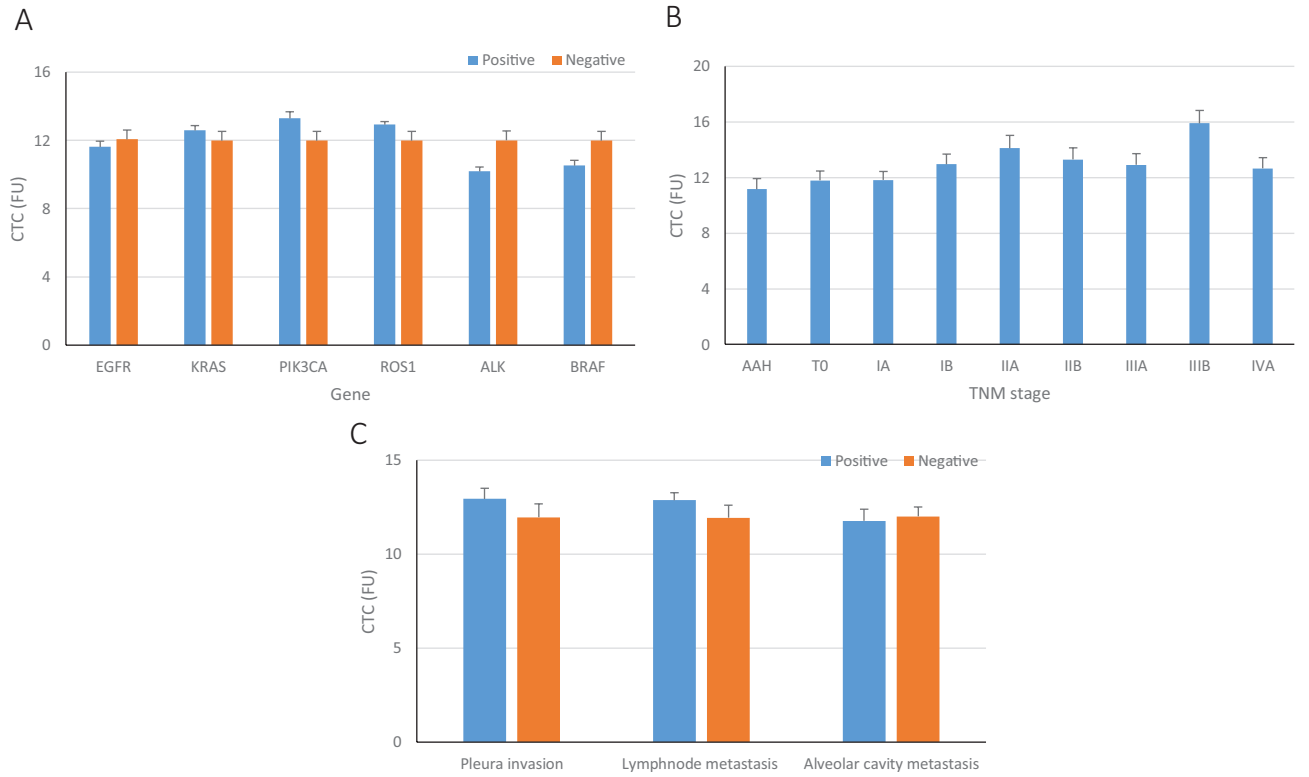

Figure 3. The CTC concentration in patients with different genotypes (A), TNM stage (B), and clinicopathological features (C).

B-raf proto-oncogene, serine/threonine kinase (*BRAF*)), the tumor-node-matastasis (TNM) stage of the lung lesion, and also the presence of pleura invasion (**Figure 3**).

2.4. A Diagnostic Threshold for FR-Positive CTC Levels and Lung Cancer

We then analyzed the diagnostic potential of characterizing FR-positive CTC levels. Based on our previous CTC analysis in patients with benign lung disease and lung cancer, we set the cutoff diagnostic threshold for lung cancer at 8.7 functional unit (FU) from a 3 mL blood sample. In our cohort, 2835 samples had a CTC level above this diagnostic threshold; of those, 2468 (87.05%) had subsequent pathological confirmation of lung cancer. These results indicate that the additional knowledge of FR-positive CTC levels strongly correlate with lung cancer diagnosis. We further compared the clinicopathological features of patients with FR-positive CTC levels higher or lower than 8.7 FU/3 mL. As shown in **Figure 4**, a combined signature of white blood cell (WBC), pro-gastrin-releasing peptide (ProGRP), CA242, and CA199 predicted the above- or below-threshold levels of FR-positive CTCs. This evidence supports the notion that additional biological indicators may be combined with FR-positive CTCs to improve lung lesion screening and diagnostic procedures.

3. Conclusion

In the current study, we combined leukocyte-based negative enrichment and FR-specific analysis to enable the assessment

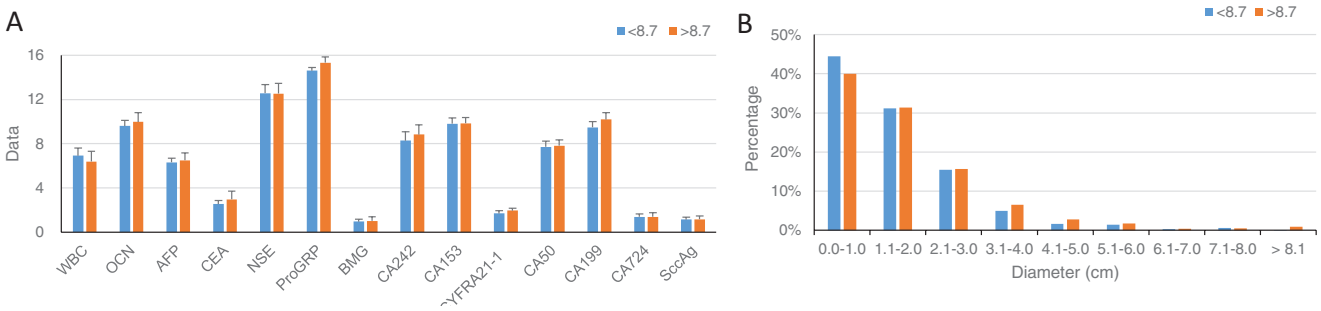

Figure 4. The clinicopathological features of patients (A: biomarkers; B: tumor diameter) with CTC concentration higher or lower than a clinically meaningful diagnostic threshold (8.7 FU/3 mL blood).

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of CTCs in order to improve the diagnostic workflow for pulmonary nodules. Our findings in a large patient population suggest that this strategy has satisfactory performance and can improve the sensitivity of pulmonary nodules diagnosis.

4. Experimental Section

Ethical Approval: This study was conducted in accordance with the amended Declaration of Helsinki and approved by the Institutional Review Board (IRB) at the Department of Thoracic Surgery, Shanghai Pulmonary Hospital. Written, informed consent was obtained from all participants.

Sample Collection: Participants in this study were consenting patients admitted for primary lung cancer into the Department of Thoracic Surgery, Shanghai Pulmonary Hospital. After enrollment, a vacuum blood collection tube (containing anticoagulant EDAT) was used to collect peripheral venous blood from each patient. The blood sample was processed immediately as follows: first, the erythrocytes were lysed with the addition of lysing buffer and, then, leukocytes were removed by anti-CD45/*-cluster of differentation 14 (CD14)* immunomagnetic beads.

Anti-CD45/-CD14 Immunomagnetic Beads: The polystyrene beads which was coated with a cross-linked layer of hydrophilic polyether were used as the magnetic beads. The assembled anti-CD45/-CD14 immuno-magnetic beads were superparamagnetic beads (4.5 μm in diameter) which could enriched the leukocytes as CD45 expressed on all human leucocytes and CD14 expressed mainly on human monocytes.

Determination of FR-Positive CTCs: A specific detection probe was designed to not only recognize cells that positively express FR, but also to quantitatively determine the cell number. It contained the folic-acid-targeting region of the FR, as well as an oligonucleotide for PCR amplification (5′-CTCAA CTGGT GTCGT GGAGT CGGCA ATTCA GTTGA GGGTT CTAA-3′). The samples without erythrocytes and leukocytes were incubated with detection probes (10 μL) for 40 min and eluted to remove the excess probe. Quantitative PCR (qPCR) (ABI 7300 Real-Time PCR System) was used to quantitatively analyze the samples. The primer sequences were as follows: forward, 5′-TATGA TTATG AGGCA TGA-3′; reverse, 5′-GGTGT CGTGG AGTCG-3′; TaqMan probe, 5′-FAM-CAGTT GAGGG TTC-MGB-3′. The quantity of FR-positive CTCs of each sample was calculated through a comparison the amplification curve of sample and standard reference. The optimal cutoff threshold for lung cancer diagnosis was 2.9 FU mL^{-1}.

Statistical Analysis: The CTC level was calculated as the mean ± the standard deviation (SD). One-way analysis of variance (ANOVA) were used to determine the statistically significant difference (SPSS 17.0 for Windows, SPSS, Chicago, IL). *P*-value < 0.05 was considered as statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

circular tumor cells, folate receptor, lung cancer, pulmonary nodules, qPCR

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