Original Article

Efficient isolation and quantification of circulating tumor cells in non-small cell lung cancer patients using peptide-functionalized magnetic nanoparticles

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Background: Circulating tumor cells (CTCs) carry a wealth of information on primary and metastatic tumors critical for enhancing the understanding of the occurrence, progression and metastasis of non-small cell lung cancer (NSCLC). However, the low sensitivity of traditional tumor detection methods limits the application of CTCs in the treatment and disease surveillance of NSCLC. Therefore, CTCs isolation and detection with high sensitivity is highly desired especially for NSCLC patients, which is significant because of high occurrence and mortality. While it is very challenging because of the lower expression of CTC positive biomarkers such as epithelial cell adhesion molecules and cytokeratins (EpCAM and CKs), herein we report a method based on peptide-functionalized magnetic nanoparticles with high CTC capture efficiency, which demonstrates superiority in NSCLC clinical applications.

Methods: For analysis and comparison of the peptide-functionalized magnetic nanoparticles (TumorFisher, Nanopep Corp.) and the antibody-modified magnetic beads (CellSearch, Janssen Diagnostics, LLC), two NSCLC cell lines, A549 and NCI-H1975 were chosen to measure the binding affinity and capture efficiency. In order to compare the effect of the clinical application of these two detection systems, 7 early stage patients with NSCLC were enrolled in this study. To further explore the clinical utility of CTC counting in different stages, 81 NSCLC patients in stage I–IV were enrolled for CTC enumeration and statistical analysis.

Results: The binding affinities of the recognition peptide to A549 and NCI-H1975 are 76.7%±11.0% and 70.1%±4.8%, respectively, which is similar with the positive control group (anti-EpCAM antibodies). CTCs were captured in 5/7 (71.4%) of early stage NSCLC patients with NSCLC in TumorFisher system, which is higher than CellSearch, and the false negative of TumorFisher is much lower than CellSearch. In a larger clinical cohort, the CTC numbers of NSCLC patients varied in different stages and the overall detection rate of TumorFisher was 59/81 (72.8%), with the similar proportion in stage I (21/29, 72.4%), II (17/22, 77.3%) and III (16/21, 76.2%).

Conclusions: Highly efficient CTC isolation technique based on peptide-magnetic nanoparticles was firstly applied in NSCLC patients. Compared with the antibody-based the technique, the higher CTC detection rates (71.4%) in NSCLC patient blood samples were demonstrated for the patients in different stages, I–IV, especially in early stages. This indicates the feasibility of the clinical utility of this new technique

in early stage screening, prognosis and therapy evaluation of NSCLC.

Keywords: circulating tumor cell (CTC); peptide (Pep); non-small cell lung cancer (NSCLC); nanoparticle; liquid biopsy

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Introduction

Lung cancer is the most common malignant tumor with the highest morbidity and mortality (1). Approximately 80% of lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC), with 92% 5-year overall survival in IA1 and only 0% in IVB. Only early detection and diagnosis of lung cancer could reduce mortality and improve quality of life (2). Several tumor markers have been used in clinic but with low sensibility and high false negatives such as carcinoembryonic antigen (CEA) (3). The National Lung Screening Trial (NLST) reported that in the low-dose CT group, the positive predictive value for positive screening results overall was only 3.8% while the value increased from 0.5% to 41.3% as the diameter of the nodule increased from 4 to 6 mm to more than 30 mm. Overall, with low-dose CT, the negative predictive value was 99.9% (4). Due to the high rate of false positives or false negatives, and considering the risk of radioactive exposure, the traditional tumor detection methods are not ideal for the detection of lung cancer in the early stage. Therefore, we need to discover new tumor markers for the early detection and diagnosis of lung cancer.

Cells from early, low-density lesions displayed more stemness features, migrated more and founded more metastases than cells from dense, advanced tumors have been found in breast cancer and lung cancer (5). In recent years, more and more attention has been paid to the role of "liquid biopsy" in early detection, disease progression, recurrence and metastasis, and prognosis monitoring of tumors. Assessing circulating tumor cells (CTCs), which CTCs were first discovered in peripheral blood of a patient with metastatic cancer in 1869 (6), counts using liquid biopsies as a whole, or as specific subgroups, could enhance understanding of disease progression and has been shown to carry prognostic information in several cancer types, including lung cancer CTCs are cells that have shed into the blood circulation system from a primary tumor (7-13). It spreads to the different body parts through blood circulation, proliferates in the appropriate environment, and leads to recurrence and metastasis of tumors (14). Many researches reveal that CTCs play an important role in the occurrence, progression and metastasis of breast cancer, colorectal cancer, prostate cancer and other cancers.

CTCs can be isolated and quantified at all stages in lung cancer (15). In early stage lung cancer, CTCs analysis might provide prognostic information in resectable patients (16,17). In advanced NSCLC, CTCs have been proposed to be an independent prognostic marker and the higher CTC numbers is associated with worse prognosis (18,19). It has been reported that after chemotherapy for advanced lung cancer, CTC numbers can reflect subtle metastases and recurrent tumors which are difficult to detect by imaging (20). However, due to the limited detection of CTCs in NSCLC at present, there are not many studies and applications of CTCs, which can facilitate treatment and disease monitoring in NSCLC (21).

The extensive applications of CTC detection play a vital role in tumor study. The gold standard of CTC detection is the CellSearch® (Veridex, Raritan, NJ, USA) System, which is the current clinical standard for CTC enumeration in breast, prostate and colorectal cancer. This system is based on the principle of specific immunological recognition of epithelial cell adhesion molecule (EpCAM)-positive cells. The sensitivity of CellSearch was quite low in every cancer species, around 10% to 60% (prostate cancer was 54.1%; breast cancer was 49.2%) (22-28). The low sensitivity limits the use of CellSearch system in NSCLC is also reported in late stage NSCLC from 31.7% to 78% (12,29-32). Only few researches revealed that the sensitivity of early stage lung cancer varies from 30% to 40% (17,33). Therefore, for more accurate and informative studies on NSCLC, a novel and more effective detection system for CTCs is crucial.

Previously, our team reported an innovative CTC detection method (TumorFisher) based on the iron oxide magnetic nanoparticles functionalized with EpCAM recognition peptide (Pep). The rate of capture efficiency and

purity were up to 90% and 93% in breast cancer, prostate cancer and liver cancer cell lines and patients (34-36), but in lung cancer remain unknown. In this study, we applied this technique to lung cancer cell lines and all stage lung cancer patients and compared with CellSearch.

Method

Patients and blood sample collection

Eighty-eight consecutive patients who underwent surgery or biopsy for NSCLC between August 2016 and November 2017 at the Department of Thoracic Surgery, Peking Union Medical College Hospital (PUMCH) were included in our study. The patients received the necessary information concerning the study, and consent was obtained from each of them. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the PUMCH ethics committees (JS-1263). Morphologic classification was assigned according to WHO and AJCC criteria (37). The tumors were staged according to the 8th edition of the international tumor-node-metastasis (TNM) system (2). All the patients were drawn fasting blood samples (2 mL) in the morning within 24 h before surgery or biopsy with treatment naïve.

Cell culture

Two typical NSCLC derived A549 and NCI-H1975 cell lines were purchased from the Cell Resource Center of Chinese Academy of Medical Sciences (Beijing, China). The two cell lines were selected to evaluate the binding affinity and capture efficiency between the two systems prior the clinical experiment. The two cell lines were cultured in a flask containing RPMI-1640 medium with 10% heat-inactivated FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. The cell culture incubator was set to a humidified atmosphere with 5% CO_2 at 37 ℃. Media was renewed every three days. The cells were harvested till the confluence reached about 80-90%. After washed twice with PBS buffer, 1 mL of 0.25% trypsin-EDTA was added for 3 min under 37 ℃ for trypsinization in order to detach the cells from the bottom of the flask. Then, 2 mL of RPMI-1640 complete medium was added to neutralized the trypsin. The cells were finally separated by centrifuging at 1,000 rpm for 5 min. Finally, the supernatant was discarded and the suspended cells were enumerated.

Preparation of functionalized Pep@MNPs and anti-EpCAM@MNPs

Specifically, 2 mg iron oxide magnetic nanoparticles modified with streptavidin (MNPs) with diameter of 150±30 nm and concentration of 5 mg/mL were incubated with 0.5 mg biotin-conjugated Pep or 0.1 mg biotin-conjugated anti-EpCAM antibody in 1 mL PBS for 1 h with continuous shaking at room temperature. Then, the functionalized Pep@MNPs and anti-EpCAM@MNPs were washed twice with PBS, and dispersed in 1 mL of PBS, and finally stored in PBS (containing 0.05% NaN_3) at 4 ℃. Anti-EpCAM@MNPs was used as positive control for Pep@MNPs.

The binding affinity measurement by flow cytometry

Flow cytometry was used for analyzing binding affinity of Pep and anti-EpCAM antibody to typical NSCLC A549 and NCI-H1975 cells. Harvested cells were resuspended in a 2 mL EP tube and fixed with 4% paraformaldehyde for 15 min. After washed with PBS two times, cells were blocked with 4% bovine serum albumin (BSA) for 30 min. Then 15 μL 1 mg/mL FITC-labeled Pep was added to cell suspension with about 10^5 suspended tumor cells for 1 h with continuous shaking at room temperature. After washed with PBS 3 times, the fluorescence signal was detected using a C6 Accuri flow cytometer (BD Biosciences, USA). FITC-labeled anti-EpCAM monoclonal antibody and mouse IgG2bκ-FITC were used as positive controls and isotype controls, respectively. Fluorescent distributions were measured by C6 flow cytometer for calculation of the binding affinity between Pep and tumor cells (numbers of FITC-labeled tumor cells/numbers of the total tumor cells added).

Evaluation of capture efficiency in cell lines

Added separately 10 μL Pep@MNPs and anti-EpCAM@MNPs into 2 mL EP tubes with 1 mL PBS containing about 1,000 cells. The above system was incubated for 1 h with continuous shaking at 37 ℃ and washed under a 116 mT magnetic field for 3 times. The captured cell samples were incubated with phycoerythrin (PE)-labeled anti-CD45 and AF488-labeled anti-CK for 1 h at 37 ℃ and followed by PBS washing at least 3 times. Then 1 μL DAPI at concentration of 100 μg/mL was added for cell nuclear staining. The Olympus IX 73 fluorescence microscopy was employed to count and measure the capture efficiency (numbers of identified tumor cells/numbers of added tumor

cells). The above same procedures were applied for both A549 and NCI-H1975 cell lines.

CTC capture and detection

Blood samples were collected in 10 mL CellSave (Veridex) preservative tubes, stored at room temperature, and processed within 96 h of collection, according to the manufacturer's instructions. CTCs from NSCLC patients' blood samples were captured and enumerated by TumorFisher and CellSearch technologies, respectively. The TumorFisher technology refer to the prior established Pep@MNPs CTC enumeration system. Briefly, 10 μL Pep@MNPs were added into 2 mL blood samples and incubated for 1 h at 37 ℃. The captured cells were gently washed with PBS at least 3 times under a high magnetic field (116 mT). The dying proceeding was the same as for cell lines above. CTC were detected by further quantifying expression levels of CK and CD45 in individual cells. CTCs exhibit strong CK expression and negligible CD45 signals. In contrast, white blood cells (WBCs) present low CK and high CD45 expression levels. DAPI staining validates that the captured cells retain intact nuclei. The combined information was utilized to identify CTCs ($DAPI^+/CK^+/CD45^-$) from WBCs ($DAPI^+/CK^-/CD45^+$) and cellular debris. CTCs was counted by identifying the number of captured cells ($DAPI^+/CK^+/CD45^-$) in 2 mL blood.

The CellSearch isolation procedures were conducted according to the manufacture's instruction. Approximately 6.5 mL of dilution buffer was added and mixed with 7.5 mL of blood sample. The mixture then was transferred into CellTracks AutoPrep system. The anti-EpCAM antibodies coated magnetic beads were used in the remaining isolation procedures, which conducted automatically by the instrument.

Statistical analysis

Statistical analysis was performed using SPSS software, version 24.0 (SPSS Inc., Armonk, USA). Binding affinity and capture efficiency were compared using a two-tailed Student's *t*-test. A two-sided $P<0.05$ was considered to be statistically significant.

Result

Binding affinity and capture efficiency

EpCAM is a widely used biomarker for CTC detection in breast, liver, lung carcinoma and other metastatic cancers. In the previous studies, we have reported the recognition Peps specifically binding with EpCAM with relatively high binding affinity (35). The schematic illustration of the TumorFisher (left side) and CellSearch (right side) methods are presented in *Figure 1* for demonstrating the different mechanisms for capture of CTCs from blood. In the middle zoom-in images, it is clearly observed that much more Peps (the white and light blue strings) interact with EpCAM molecules (brown rough spheres), while much less anti-EpCAM antibody interacts with EpCAM molecules. The different density of surface modification could be ascribed to the huge difference between molecular weight of Pep (2.7 KDa) and antibody (around 150 KDa). The bundle-like Peps as shown in zoom-in image (C in *Figure 1*) could be attributed to the self-assembly behavior of the N-terminal Pep segments, Asn-Asn-Cys-Asn-Asn-Gly-Gly-Cys between cysteine disulfide linkage and the hydrogen bonds between sidechains on Asn (37-39). Thus, more Pep-magnetic nanoparticles (TumorFisher, fuzzy spherical particles denoted as B in *Figure 1*) could be adsorbed onto the CTC surface due to the stronger interaction between EpCAM highly expressed CTCs and Pep-magnetic nanoparticles than that between antibody-magnetic beads and CTC. In contrast, it could be observed that less magnetic beads functionalized with anti-EpCAM (D in *Figure 1*, yellow sphere with randomly oriented Y-shaped antibodies) are adsorbed on the CTC cell surface possibly because less antibody density on bead surface.

The flow cytometry was used to calculate the binding affinity in cell lines. As shown in *Figure 2A*, the binding affinities of Pep to A549 and NCI-H1975 are 76.7%±11.0% and 70.1%±4.8% respectively, which is slightly lower than the positive control group (anti-EpCAM to A549, 90.6%±4.9%) or similar (anti-EpCAM to NCI-H1975, 67.2%±3.0%). There is no statistical difference in the binding affinity of Pep and anti-EpCAM to A549 and NCI-H1975 ($P>0.05$). In the isotype controls group, the binding affinities of mouse IgG2bκ-FITC are 0.9%±0.3% and 1.1%±0.9% respectively. Meanwhile, in the negative group, the binding affinities of blank to A549 and NCI-H1975 is 0.3% and 0.6%, respectively. Typical images of A549 and NCI-H1975 flow fluorescence distribution were shown in *Figure 2B* and *Figure 2C*.

Moreover, A549 and NCI-H1975 cell lines were used to evaluate the capture efficiency of Pep@MNPs and anti-

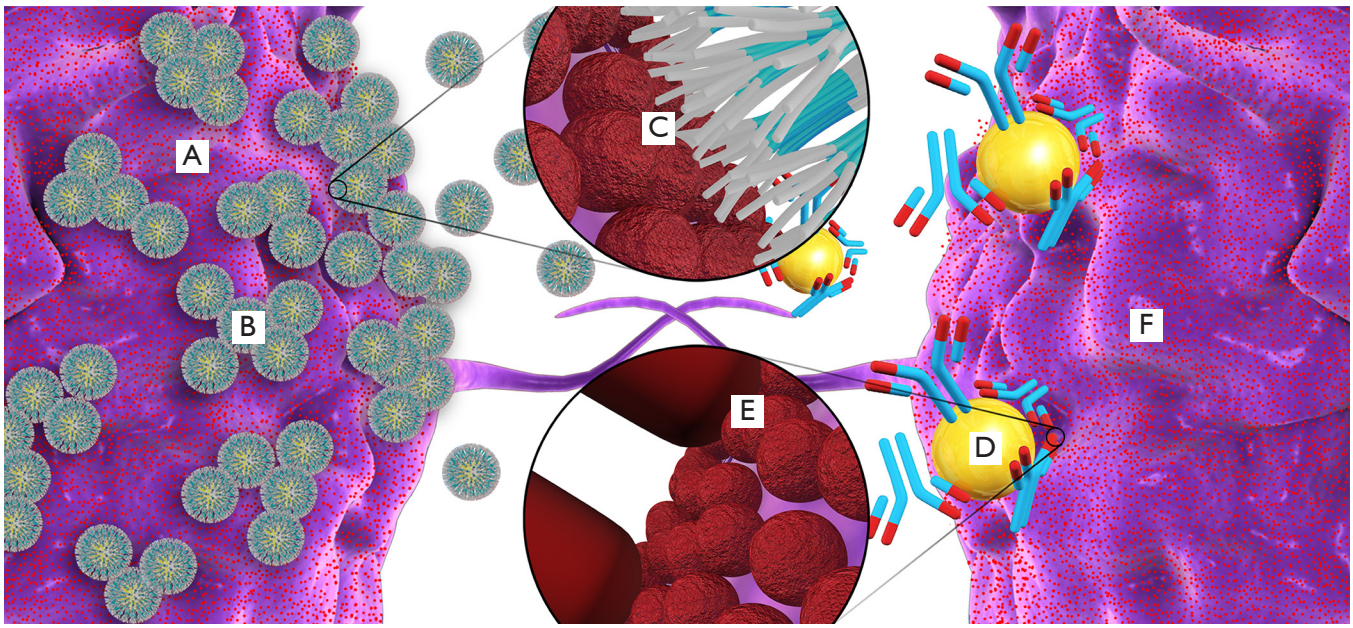

Figure 1 Schematic illustration of the TumorFisher and CellSearch method used to capture circulating tumor cells. A and F represent circulating tumor cells in purple color with rough surfaces, which are cells with epithelial origin. In the left half image, the small fuzzy spherical particles (B) spreading on the left CTC cell surface are iron oxide magnetic nanoparticles functionalized by EpCAM recognition peptides (yellow balls covered with grey strings). In the right half image, D denotes the magnetic beads functionalized with anti-EpCAM (D), yellow sphere with randomly oriented Y-shaped antibodies). In the middle zoom-in images, it is clearly observed that much more peptides (the white and light blue strings) interact with EpCAM molecules (brown rough spheres) in the upper circles, while much less anti-EpCAM molecules interact with EpCAM molecules.

EpCAM@MNPs in isolating CTCs from mimicking CTC samples. As shown in *Figure 2D*, the capture efficiencies of Pep@MNPs and anti-EpCAM@MNPs from A549 are 57.3%±7.0% and 56.3%±10.1%, respectively. And the capture efficiencies of Pep@MNPs and anti-EpCAM@MNPs from NCI-H1975 are 37.3%±6.1% and 30.3%±4.0%, respectively. The results indicated that the capture efficiency of Pep@MNPs and anti-EpCAM@MNPs to A549 and NCI-H1975 were comparable, and there is no statistical difference.

The conventional definition of CTCs was adopted in this study. Cells that $DAPI^+/CK^+/CD45^-$ and met the phenotypic morphological characteristics were designated as CTCs, and $DAPI^+/CK^-/CD45^+$ cells were designated as leukocytes. We imaged the cells on an inverted microscope. *Figure 2E* shows typical immune-fluorescence staining results for isolated A549 (top row) and NCI-H1975 (button row) by Pep@MNPs assay.

Comparation between TumorFisher and CellSearch systems in clinical applications

In order to compare the clinical effect of TumorFisher and CellSearch system, 7 patients with NSCLC were enrolled in this study. The patient characteristics are summarized in *Table 1*. Among them, 6 patients were in stage I, and 1 patient was in stage II. Patients were drawn blood in morning for blood samples from elbow veins, the blood samples were separated and analyzed with TumorFisher and CellSearch system. According to our research data, the most appropriate cut-off value of the TumorFisher system is 1 cell/2 mL blood, if the patient detected ≥1 CTCs in 2 mL of blood, we would define it positive, while the cut-off value of CellSearch is 5 cells/7.5 mL (40). Thus, the efficiency of TumorFisher is 5/7 (71.4%), which is much higher than CellSearch (0/7, 0%). It is worth mentioning that the false negative of TumorFisher (28.6%) is much lower than CellSearch

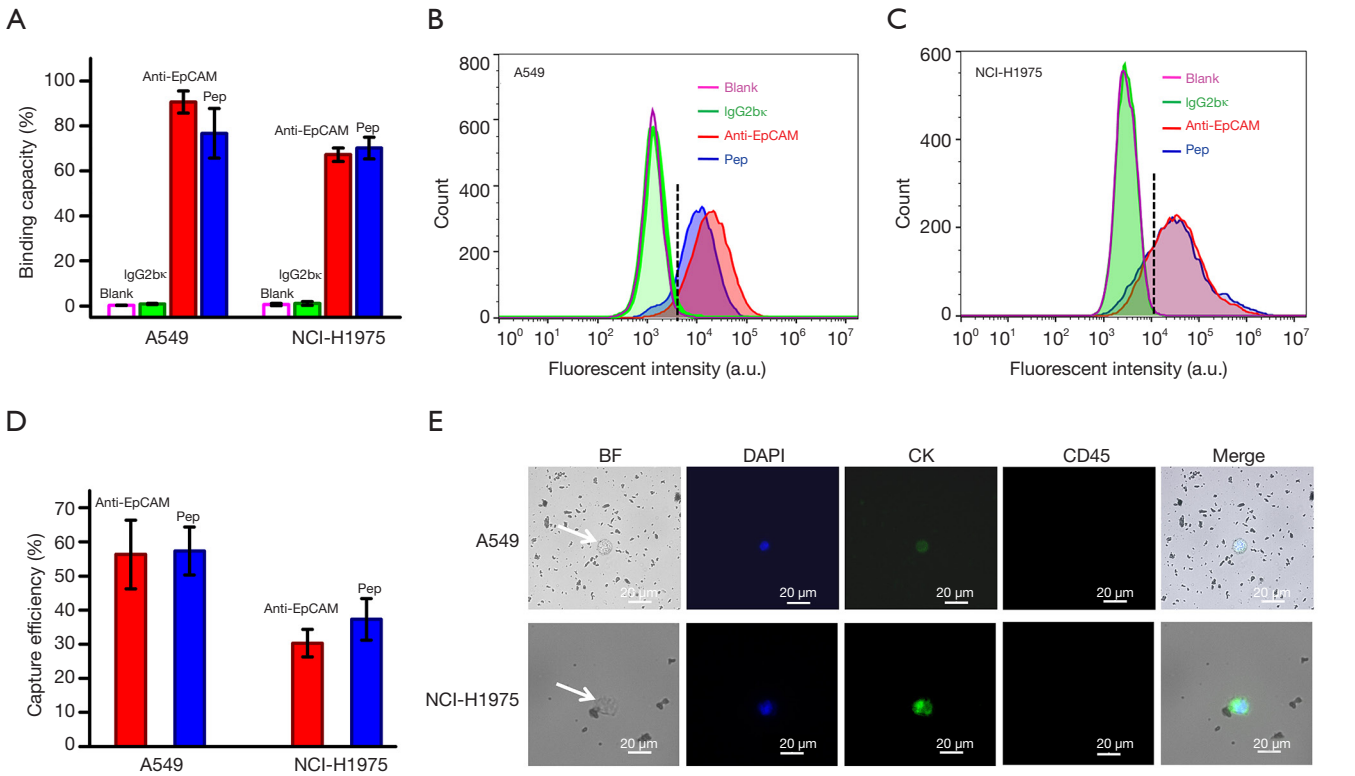

Figure 2 Comparison at the cell lines. Percentage of the binding affinity for IgG2bκ, anti-EpCAM and Pep on A549 and NCI-H1975 (A). Fluorescence distribution of A549 (B) and NCI-H1975 (C) incubated with IgG2bκ, anti-EpCAM and Pep by FCM measurements. The purple distribution corresponds to blank control, the green distribution corresponds to IgG2bκ, the red distribution corresponds to anti-EpCAM, and the blue distribution corresponds to Pep. The fluorescence threshold is marked by the black dotted line. Percentage refers to the percentage of the cell fluorescence by FITC binding to the total cells. (D) The capture efficiency of anti-EpCAM and Pep. (E) Immunofluorescent images of A549 (top panel) and NCI-H1975 (button panel) captured from blood. Three-color immunocytochemistry method was applied to identify CTCs from non-specifically trapped blood cells. Experiments were done in triplicates and the reported error is the standard deviations. The corresponding fluorescent channels are DAPI nuclear staining (blue color, Em =461 nm), AF488-labeled anti-CK (green color, Em =525 nm), PE-labeled anti-CD45 (red color, Em =578 nm). The top and button panels show fluorescent images of typical tumor cells of epithelial origin with $DAPI^+CK^+CD45^-$. EpCAM, epithelial cell adhesion molecules; Pep, peptide; CTCs, circulating tumor cell; PE, phycoerythrin; CK, cytokeratin.

(100%), as shown in *Figure 3A*. The representative immunofluorescent images of CTC and WBC found in NSCLC patients were shown as *Figure 3B*. The three-color immunocytochemistry method, based on DAPI nuclear staining (blue color, Em =461 nm), AF488-labeled anti-CK (green color, Em =525 nm), PE-labeled anti-CD45 (red color, Em =578 nm), was applied to identify CTCs from non-specifically trapped WBC cells. The larger cell size and nuclear size of CTCs than the WBCs are clearly shown in the images, and the $DAPI^+$/CK^+/$CD45^-$ cells with intact cell features are identified as CTCs and the $DAPI^+$/CK^-/$CD45^+$ ones as WBCs.

The applications of TumorFisher in clinic

To further explore the clinical value of CTC counting in different stages of disease, 81 patients with stage I–IV NSCLC were enrolled in this study from August 2016 to November 2017 in Department of Thoracic Surgery, PUMCH. The patient characteristics are summarized in *Table 2*. Of the patients enrolled, 29 were in stage I, 22 were in stage II, 21 were in stage III, and 9 were in stage IV. There were 67 patients with adenocarcinoma, accounting for 82.7% of the total number. After statistical analysis, we found that smoking and non-smoking patients have significant differences in detection rates (P=0.0231), the

Table 1 Clinical characteristics of the 7 enrolled Early Stage non-small cell lung cancer patients

Data of patients (N=7)	Age (years)	Gender (M/F)	Smoking history (Y/N)	Tumor size (cm)	pT stage	pN stage	pM stage	TNM stage	Histological subtypes[a]	TumorFisher CTC number (/2 mL)	CellSearch CTC number (/7.5 mL)
1	55	F	N	2.3	T1c	N0	M0	IA3	ADC (Acinar)	5	0
2	52	F	N	1.2	T1b	N0	M0	IA2	ADC (Lepidic+ Acinar)	0	0
3	64	F	N	2.4	T1c	N0	M0	IA3	ADC (Acinar)	8	0
4	61	F	N	1.0	T1a	N0	M0	IA1	ADC (Lepidic+ Acinar)	1	0
5	45	M	Y	2.0	T1c	N0	M0	IA3	ADC (Lepidic+ Acinar)	3	0
6	73	M	Y	5.0	T2b	N0	M0	IIA	ADC (Solid+ Acinar)	0	0
7	41	F	N	3.0	T1c	N0	M0	IA3	ASC	1	0

[a], adenocarcinoma subtypes by International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS) classification. N, non-smoker; Y, smoker; ADC, adenocarcinoma; ASC, adeno-squamous carcinoma; CTCs, circulating tumor cell.

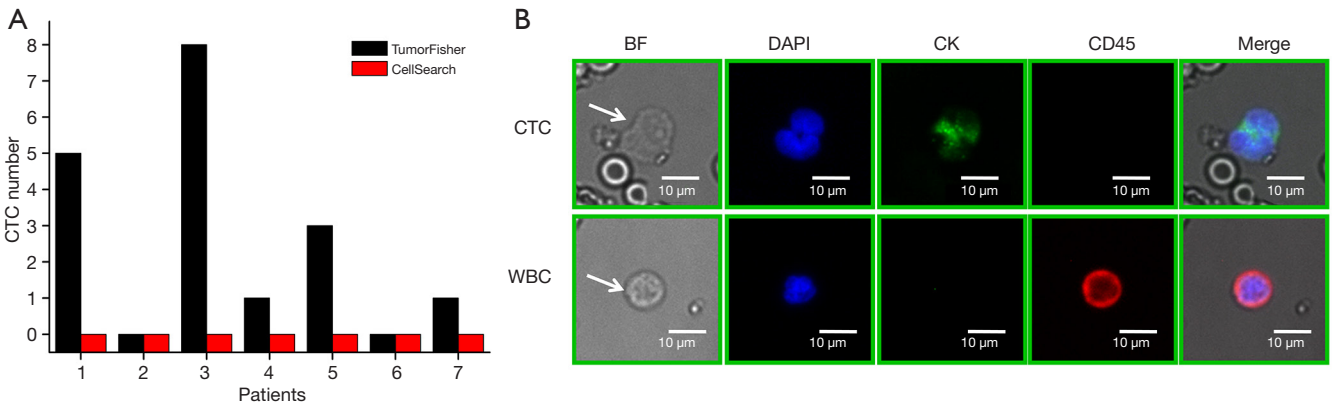

Figure 3 Comparison at the clinical. (A) The CTC numbers of each patient detected by TumorFisher and CellSearch. (B) The typical fluorescent micrographs of CTC (circulating tumor cell) and white blood cell (WBC) found in blood samples from NSCLC patients by TumorFisher technology. The corresponding fluorescent channels are the same as *Figure 2*. The top panels show fluorescent images of CTC with CK-positive features with green fluorescence, and the lower panels show typical WBC fluorescent images. CTC, circulating tumor cell; NSCLC, non-small cell lung cancer; CK, cytokeratin; BF, bright field.

detection rate of smoker and non-smoking patients are 37% and 63%, respectively. And there is no significant difference between other factors.

As shown in *Figure 4A*, the CTC numbers of NSCLC patients varied with different stages. Each point in the figure represents the CTC numbers detected by one patient, and different colors are used to distinguish patients at different disease stages. In this clinical cohort, the overall detection rate of TumorFisher was 59/81 (72.8%), the detection rates of adenocarcinoma and squamous carcinoma were 52/67 (77.6%) and (9/14, 64.3%), respectively. There was almost no difference in the detection rate among patients in stages I, II, and III, and they are maintained at high level (*Figure 4B*). The detection rates of TumorFisher in stage I–IV were 21/29 (72.4%), 17/22 (77.3%), 16/21 (76.2%), and 6/9 (66.7%) respectively. All P values >0.05, there are no statistical difference between different pathological types and stages in CTC detection rates.

Discussion

Lung cancer is the leading malignancy with the highest

Table 2 Characteristics of the 81 NSCLC patients cohort

Variable	Value
Total, n (%)	81 (100.0)
Age, mean ± SD (years)	59.3±9.3
Smoking situation, n (%)	
Smoker	30 (37.0)
Non-smoker	51 (63.0)
Sex, n (%)	
Female	48 (59.3)
Male	33 (40.7)
Histological subtype[a], n (%)	
ADC	67 (82.7)
SC	14 (17.3)
Stage, n (%)	
IA	27 (33.3)
IB	2 (2.5)
IIA	3 (3.7)
IIB	19 (23.5)
IIIA	17 (21.0)
IIIB	3 (3.7)
IIIC	1 (1.2)
IV	1 (1.2)
IVA	7 (8.6)
IVB	1 (1.2)
CTC number (/2 mL)	
≥1	59 (72.8)
=0	22 (27.2)

[a], adenocarcinoma subtypes by International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS) classification. ADC, adenocarcinoma; SC, squamous carcinoma; NSCLC, non-small cell lung cancer; CTCs, circulating tumor cell.

morbidity and mortality in the Chinese populations. Despite recent implementation of novel targeted agents, 5-year survival of lung cancer remains at a low rate of 19%. Screening of high risky subjects with LDCT (Low Dose Computed Tomography) can reduce lung cancer-related mortality by 20%, but the high false positive rate of lung nodules leads to morbidities from interventions, unnecessary imaging, and costs (41). Moreover, Rampinelli and coworkers reported that the radiation exposure from LDCT and positron emission tomography-computed tomography (PET-CT) scans for lung cancer is non-negligible (42).

An assay that accurately implements early screening for NSCLC would be greatly welcomed and could improve survival. Ideally, the assay could be safely performed in all patients (i.e., noninvasive), involve minimal or no discomfort. CTC assays may fulfill such roles. Recurrence and metastasis of lung cancer occur via CTCs and seriously affect the survival of patients even after surgery (43). Improving the rate of early screening has significant importance for improving the survival rate of patients, and the key to improving prognosis is to use better approaches for surveillance, continuous detection of CTCs can offer valuable insights.

CTC is extremely rare in peripheral blood, there are about 10 million WBCs and 5 billion red blood cells in 1 mL peripheral blood, but only several CTCs (9). Therefore, it is necessary to enrich the cells before detection. At present, there are two mainly methods to enrich and identify CTCs, based on the principle of morphology and the principle of immune adsorption. The immune adsorption method is the most widely used. It divided into two kinds, positive and negative cell sorting. For positive sorting, EpCAM was used as the marker, captured the CTCs by antigen-antibody specific binding. Representative techniques include CellSearch, CTC chip, etc. (44,45). CellSearch was the most widely used method for CTC detection in the past few years and has been approved by FDA in breast, colorectal and prostate cancer, but the recovery rate of CTCs in lung cancer is only about 20–40% (32,34). For negative sorting, after CD45 antibody specific adsorption, using cell markers identify the CTCs. In addition, isolation by size of epithelial tumour cells (ISET) and some non-commercial morphology-based methods have been employed in CTC detection in small samples of patients with NSCLC (46). The positive rates ranged from 13% to 87% (47,48). The main challenge of CTCs in early NSCLC screening is the lack of stable and highly sensitive methods.

The method used in this study has shown superiority in CTC detection. Peps play key roles in participating in ligand-receptor and protein-protein interactions, since the recognitions are mainly involving in the short Peps at the contact interface (49,50). Compared with the reported CTC analysis results in NSCLC patients, our detection rate (72.8%) is at the upstream level (21). In our study, the

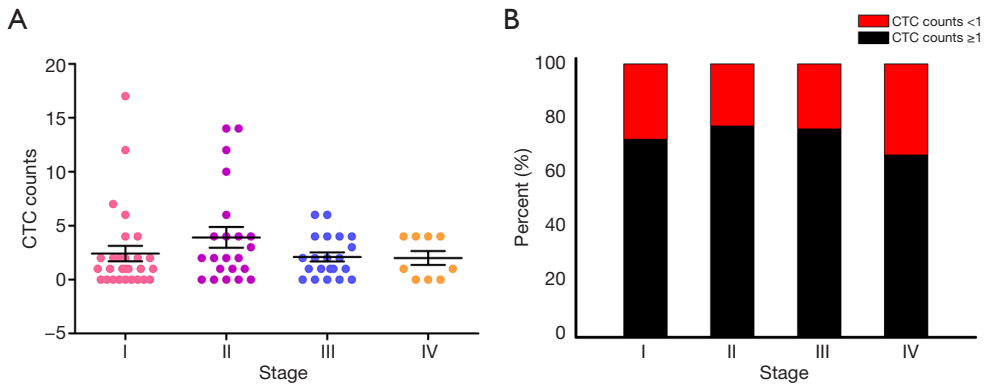

Figure 4 Clinical application of TumorFisher in NSCLC. (A) Number of CTCs in patients with different disease stages. The highest average number of CTCs was detected in stage II patients. One dot represents the CTC counts of detection by one patient, pink dots represent the CTC counts of patients in stage I, purple dots represents the CTC counts of patients in stage II, blue dots represents the CTC counts of patients in stage III, and yellow dots represents the CTC counts of patients in stage IV. (B) Percentage of patients with CTC numbers greater than 1 count/2 mL blood or not on different NSCLC stages. The detection rate of TumorFisher in NSCLC is not linearly related to the disease stage. The detection rates in stages I, II and III are similar, and the detection rate in stage IV is comparatively low. NSCLC, non-small cell lung cancer; CTCs, circulating tumor cell.

number of detected CTCs ranged from 0–8 cells/2 mL, while CellSearch was 0 cell/7.5 mL. The high efficiency result is ascribed to the high density and well-defined orientation of biotin-Pep modified on the magnetic nanoparticle surface with streptavidin functionalization, and also the multivalent interactions because of the Pep assembly structures via disulfide bond between cysteine residues.

For patients with different stages of disease, the CTC number differs. Tanaka and coworkers reported that the positive rates of CTC in lung cancer with stage I–III was only 0–20% (33). In stage I NSCLC, the detection of CTC is more challenging. The detection rates of ISET and a nano-enrichment technique are 49% and 17.4%, respectively (21,51). Our study used a highly sensitive method to detect CTCs of NSCLC patients at the early stage, which has a certain guiding role in clinical practice. The overall detection rate of this method was 72.8%. It is worth mentioning that in stage I, II, III patients, the detection rate reached 72.4%, 77.3%, 76.2% respectively. The results show that magnetic nanoparticles detection technology is expected to be an indicator of early NSCLC screening. Meanwhile, our data shows that there is no linear correlation between the CTC numbers and the clinical stage. Different research results differ, the number of CTCs enriched based on morphological methods is linearly correlated with the clinical stage (52). This result may be related to many reasons, such as epithelial-mesenchymal transition of tumor cells, and a small number of studies (10,53). This small-sample single-center prospective cohort study still has certain limitations. Before TumorFisher can be applied to NSCLC clinically, the next large-sample, multi-center study is still needed.

In summary, the highly efficient CTC detection by TumorFisher was demonstrated both in NSCLC cell line level and patient sample level. The comparison between CTC detection rate TumorFisher (5/7, 71.4%) and CellSearch system (all false negative) showed obviously superiority of our TumorFisher technique. The higher CTC detection rates (66.7–77.3%) were demonstrated for NSCLC patient blood samples ranging from stage I to stage IV, which manifested the possible application of TumorFisher in prognosis, dynamic monitoring of treatment of lung cancer. The distinguished high detection rate in early stage NSCLC patients indicated the feasibility of the TumorFisher technique in early screening of lung cancer. Large scale clinical cohort study could be performed in the future to further confirm the clinical utilities of this technique in not only NSCLC, but also in other lung cancers and even in other cancer types.

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Footnote

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