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Detection of CTCs and CSCs in the staging and metastasis of non-small cell lung cancer based on microfluidic chip and the diagnostic significance

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Abstract. – OBJECTIVE: Dynamic monitoring of CTCs/CSCs can assist in the diagnosis and prognosis of tumors. This study explores the diagnostic significance of microfluidic chip technology in the detection of CTCs/CSCs in clinical staging and metastasis of patients with non-small cell lung cancer (NSCLC). That lays a solid foundation for the use of microfluidic chips to monitor CTCs/CSCs for the stage and metastasis of patients with non-small cell lung cancer.

PATIENTS AND METHODS: This study collected 80 patients with lung cancer from October 2017 to October 2018. Meanwhile, 30 healthy people and 30 patients with benign lung diseases were selected during the same period as the control group 1 and the control group 2, respectively. CellSearch (Huntington Valley, PA, USA) and microfluidic chip were used to detect CTCs, the sensitivities were recorded. ELISA methods were used to detect the concentrations of tumor markers VEGF-C, CEA, and CA125 in serum, and their association with CTCs and CSCs was analyzed. In addition, after 3 months, we followed up 40 patients with lung cancer, recorded their prognosis, and extracted peripheral blood to detect changes in their CTCs and CSCs. The CellSearch (Huntington Valley, PA, USA) system and the microfluidic chip system were used to detect the CTCs in patients with lung cancer, and the sensitivity and specificity of the patients were analyzed. The changes in CTCs and CSCs in the peripheral blood of the patient were recorded.

RESULTS: It can be seen that the positive rate of CTCs and CSCs is not significantly correlated with the patients' age, gender, pathological type (adenocarcinoma, squamous cell carcinoma), etc. They are significantly correlated with clinical stage (I + II and III + IV) and metastasis (metastasis and non-metastasis) ($p<0.01$). Then, we divided the patients into groups for testing, and analyzed the association between different groups of patients and CTCs and CSCs. Compared with control group 1 and control group 2, the positive rates of CTCs and CSCs in lung cancer metastasis group and non-metastasis group were significantly different ($p<0.05$). Compared with the control group 1 and control group 2, the positive rates of CTCs and CSCs in stage I + II and III + IV of lung cancer were significantly different ($p<0.05$). The positive rate was significantly higher in the cancer metastasis group ($p<0.05$). The concentrations of tumor markers VEGF-C, CEA, CA125 in the serum of patients were consistent with CTCs-negative and CTC-positive lung cancer, with significant differences ($p<0.05$). CSCs negative and CSCs positive patients have similar results. Subsequently, we analyzed the sensitivity and specificity of CSCs, CTCs, and tumor markers for the diagnosis of NSCLC. The results showed that the sensitivity of CSCs and CTCs to diagnose patients was significantly higher than that of tumor markers.

CONCLUSIONS: This study shows that our microfluidic chip device can exhibit relatively good performance and can better detect CTCs and CSCs. Monitoring CTCs and CSCs of patients can provide a basis for judging the stage and metastasis of patients.

Key Words:
Microfluidic chip, Lung cancer, CTCs, CSCs, Serum tumor markers, Prognosis.

Abbreviations

CTC, Circulating Tumor Cell; CSC, Cancer Stem-like Cells; TP, True Positive Rate; FPR, False Positive Rate; CR, complete response; PR, partial response; SD; stable disease; PD, progressive disease.

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Introduction

Lung cancer is the most common cancer. About 84% of new lung cancers were non-small cell lung cancer (NSCLC) and 15% were small cell lung cancer (SCLC). Most patients are diagnosed at advanced stage[1,2], therefore, early screening for NSCLC is essential. At present, it is more common to monitor circulating tumor cells (CTCs) in real-time through peripheral blood[3].

Circulating tumor cells (CTCs) in peripheral blood, which fall off from the primary tumor lesion and invade into the peripheral blood circulating tumor cells, are derived from tumor tissues. They are closely related to the occurrence and progression of cancer and generally regarded as a kind of metastasis, so it is of high research value[4,5]. As we all know, there are few CTCs in the blood. 1 mL of blood may contain less than ten CTCs, which may circulate in the blood disguised as a small mass of cells, so it is very difficult for its molecular analysis and enrichment[6,7]. It has been found in different cancers that the dynamic monitoring of CTCs can assist the diagnosis and prognosis of tumors[8-11].

Moreover, there have been some studies confirming[12,13] the existence of cancer stem-like cells (CSCs), which are also known as tumor initiating cells (TICs) and metastasis-initiating cells (MICs). These rare cells have been found in various solid tumors[14,15] and hematological diseases[16] for disease diagnosis. Therefore, dual monitoring of CTCs and CSCs is effective for treating cancers and prognosis. How to sort and enrich CTCs from whole blood has become the main problem in further analysis and identification of CTCs. Microfluidic chips has been used in sorting and enriching of CTCs. It has the advantages of rapid analysis, easy to carry, low reagent consumption and high flux.

Microfluidic techniques were used to detect CTCs and CSCs in a study on pancreatic cancer. It found that CTCs were labeled as EpCAM and CSCs were labeled as CD133. the results showed that the positive rate of CTCs was as high as 84.4%, and the positive rate of CSCs as high as 70.8%. So it is more effective to use microfluidic chip to detect CTCs and CSCs[17]. In another study[18], a novel multi-flow microfluidic device was designed to guarantee that the purity was more than 87% and the recovery was more than 93% without labels, and after testing, CTCs positive were detected in 6 of 8 patients with lung cancer.

There are two main methods for CTCs detection: one is based on CellSearch system (Huntington Valley, PA, USA) for prognostic monitoring[19,20], the other is based on physical properties. In view of Cellsearch system (Huntington Valley, PA, USA), epithelial-mesenchymal transition (EMT) may occur during tumor metastasis, which limits the efficacy of the system for detecting CTCs in clinical practice. Therefore, due to its disadvantages (high cost, manual operation, false positive/false negative), its widely clinical application was hindered[21]. The method based on physical properties is based on the different physical properties of tumor cells and normal blood cells, such as size, density, charge, etc. The system is easy to deform by the extrusion of the membrane during membrane filtration, which is not conducive to further detection.

Furthermore, it is possibly to miss CTCs in the current detection of microfluidic chip with the disadvantages of time-consuming sorting process and so on, which weakens the clinical application of microfluidic CTCs sorting based on antigen properties. It is self-evident important to design a cheap, easy-to-sample, easy-to-operate and repeatable microfluidic chip for CTCs/CSCs detection.

Aiming at the above problems, this study used a microfluidic chip based on DLD sorting system, magnetic field negative sorting system and immune affinity sorting system, which can effectively sort, enrich and detect tumor cells, so as to assist doctors in clinical staging and diagnosis of tumor metastasis in patients with NSCLC.

Patients and Methods

General Information

This study collected 80 patients with NSCLC from October 2017 to October 2018. Meanwhile, 30 healthy people and 30 patients with benign diseases in lung were selected as control group 1 and control group 2. There were three groups in this study (lung cancer group, control group 1 and control group 2). Among the 80 patients, there were 49 men and 31 women. 41 patients were at the age of 60 or less and 39 people at the age of more than 60. There were 44 patients with adenocarcinoma and 36 patients with squamous cell carcinoma, 22 patients in stage I+II and 58 patients in stage IIII+IIIV, 56 patients without metastasis and 24 patients with metastasis. For the convenience of analysis, the patients in the lung cancer group were divided into I+II group, III+IV group, metastatic group, non-metastatic group.

There was no significant difference in the ratio of men and women.

Information collection of patients: 1. demographic information: age, sex, nationality, place of origin, etc. 2. lifestyle information: diet, smoking, drinking and exercise; 3. physical examination information: height, weight, abdominal circumference, blood pressure, heart rate, etc.; 4. medical history information: current medical history, past medical history, family history, etc.; 5. imaging information: chest radiographs, chest CT and/or MRI examination. According to the principle of informed consent, 5 ml venous blood was collected on an empty stomach with BD anticoagulant tube in the morning and stored at room temperature.

Inclusion criteria: patients: 1. all patients were diagnosed with lung cancer by pathological biopsy; 2. patients without chemoradiotherapy before receiving treatment; 3. patients' health status KPS score was at least 60 and the estimated survival time was more than 3 months; 4. patients with compliance.

Control group 1: 1. patients showed normal status in routine physical examination; 2. patients without nodules in chest radiographs; 3. patients without lung diseases; 4. patients without other tumor-like diseases.

Control group 2: 1. patients with benign nodules in chest radiographs; 2. patients without other tumor-like diseases.

Exclusion criteria:

Patients: 1. patients whose pathological biopsy results did not meet the criteria of this study; 2. patients treated; 3. patients with a history of psychiatric diseases; 4. patients with severe infections; 5. patients with severe diseases in other important organs, etc.

Control group 1: 1. patients with nodules in chest radiographs; 2. patients with abnormal blood test results; 3. patients with chest tightness, shortness of breath and other symptoms.

Control group 2: 1. patients with other tumor diseases; 2. patients with incomplete clinical information.

Microfluidic Chip Device for Detecting CTCs/CSCs

This study used a device with microfluidic chip for detecting CTCs/CSCs. The device mainly consisted of three parts: DLD sorting system, magnetic field negative sorting system and immune affinity sorting system. Peripheral blood samples from patients with lung cancer entered the DLD chip, and tumor cells and large white blood cells were enriched through the middle collecting outlet. Next, the tumor cells and white blood cells were incubated with magnetic beads coated with CD45 antibodies for 15 min, and then, injected from the negative magnetic separation chip feed. Since the target cells expressed CD45, it could form a complex with CD45 antibody-magnetic beads, and the motion trajectory changed under the action of the chip magnetic field, separating to the first sample outlet. Next, the tumor cells flew out into the immune affinity sorting chip consisted of three "S"- shaped fishbone chips, which could change how fluid flow through a chip, to improve the binding ability of cells to specific antibodies encapsulated in the underlying chip. SOX2 and OCT4 antibodies coated on the bottom chip could specifically capture CSCs. EpCAM could specifically capture CTCs, so that the CTCs and CSCs in tumor cells can be specifically detected and enriched.

Sorting and Enrichment of CTCs and CSCs

The peripheral blood of subjects in lung cancer group, control group 1 and control group 2 were all collected, and sorted and enriched in the above sorting device within 24 h. The operations are as follows: triangular microcolumn DLC array (microposts starter kit, MechProfiler, Swiss Microduits) was used for sorting and enriching CTCs for studying. Injection pump was used for injecting into tumor cells at a flow rate of 30 μL/min, 50 μL/min and 100 μL/min through the chip. Fluorescence microscopy with high-speed camera function (MDX1-T, Mingmei Optoelectronic Technology Co., Ltd., Guangzhou, China) was used for observing and shooting. The tumor cell suspension out from the outlets of the three chips was collected. Then, the negative purification platform of magnetic field and immune affinity system was used to detect CTCs and CSCs, and the recovery and positive rate were calculated respectively. The criteria of positive was: CTCs>0/2 mL.

CellSearch System and Microfluidic Chip for Detecting CTCs

We collected 20 patients with NSCLC with similar pathological information in each group. CellSearch system (Huntington Valley, PA, USA) and microfluidic chip were used to detect the blood of the patients, respectively, in order to compare the sensitivity and specificity between the two methods. The detection scheme of micro-

fluidic chip has been shown above, the detection scheme of CellSearch system (Huntington Valley, PA, USA) referred to the study by Lowes et al[22].

Detection of Serum Tumor Markers

5 mL of peripheral blood was taken from all the subjects, and the serum was separated at 3000 rpm/min for 10-minute centrifugation, then, the concentration of tumor markers, including VEGF-C (ELISA kit, UNOCI Biotechnology Co., Ltd., Hangzhou, China; Cargo No.: 70- EK1154-96), CEA (ELISA kit, Biovision, Milpitas, CA, USA; Cargo No.: K4805-100), CA125 (ELISA kit, Biovision, Milpitas, CA, USA; Cargo No.: K4803-100), NSE (ELISA kit, Biovision, Milpitas, CA, USA; Cargo No.: 351672), etc., was detected By Enzyme-Linked Immunosorbent Assay (ELISA) kit at the wavelength 450 nm.

Follow-up of Patients with Lung Cancer

After 3 months, we followed up 40 patients with lung cancer. CTCs in all the patients were detected before and after treatment. The treatments included surgery, chemotherapy, targeted therapy. The patients' conditions were recorded, including 4 main types: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). The correlation between prognosis of patients and CTCs and CSCs were analyzed.

Statistical Analysis

SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. Measurement information was expressed by mean ± standard deviation ($\bar{x}\pm s$). Independent sample *t*-test or ANOVA was used for statistical analysis of the normal distribution or information transformed from normal distribution. Patients' overall survival (OS) was recorded, Kaplan-Meier survival curve was used to describe the difference in survival time of the patient, and the log-rank test was used to compare the difference in efficacy between the experimental group and the control group. Enumeration information was expressed in ratio (%). Chi-square test was used for its statistical analysis. There was statistically significance when $p<0.05$. MedCalc 11.4.2.0 analysis software was used for drawing the receiver operating characteristic curve (ROC) of patients, CellSearch system (Huntington Valley, PA, USA) and microfluidic chip were used for evaluating the diagnostic value of CTCs and the sensitivity and specificity of different detection methods for lung cancer, the optimal cutoff value was determined according to the Youden indexes.

Results

The Sensitivity of CTCs Detected by Microfluidic Chip was Higher than CellSearch System

We used CellSearch system (Huntington Valley, PA, USA) and microfluidic chip to detect CTCs in patients with lung cancer. The sensitivity and specificity were analyzed. It was found that the sensitivity detected by microfluidic chip was higher than that of CellSearch system (Huntington Valley, PA, USA; 95.1% *vs* 92.1%), suggesting that the sensitivity of CTCs detected by microfluidic chip was higher with better detecting efficiency, and the results are shown in Figure 1.

Correlation Analysis Between CTCs and CSCs in Peripheral Blood and Clinical Information in Patients with NSCLC

According to the detection of CTCs and CSCs in the peripheral blood and the comparison with the clinical information of patients, we can know that the positive rate of CTCs and CSCs was not significantly correlated with the patient's age, sex, pathological type (adenocarcinoma and squamous cell carcinoma), but significantly correlated with clinical stage (I+II and IIII+IIIV), metastasis (metastasis and non-metastasis) ($p<0.01$), as detailed in Figure 2.

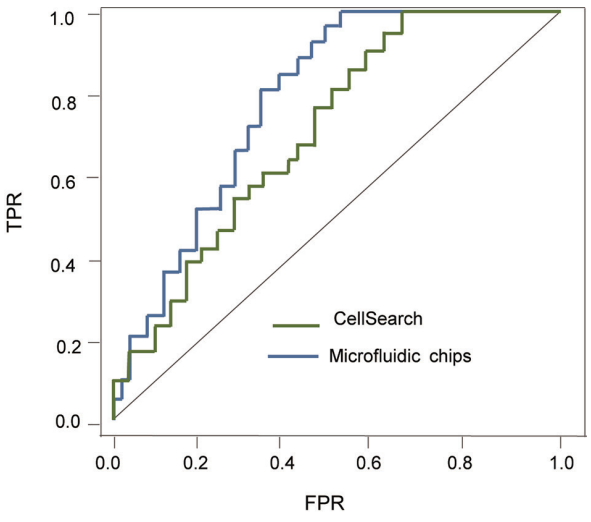

Figure 1. Comparison of sensitivity and specificity of CTCs detection by CellSearch System and microfluidic chip.

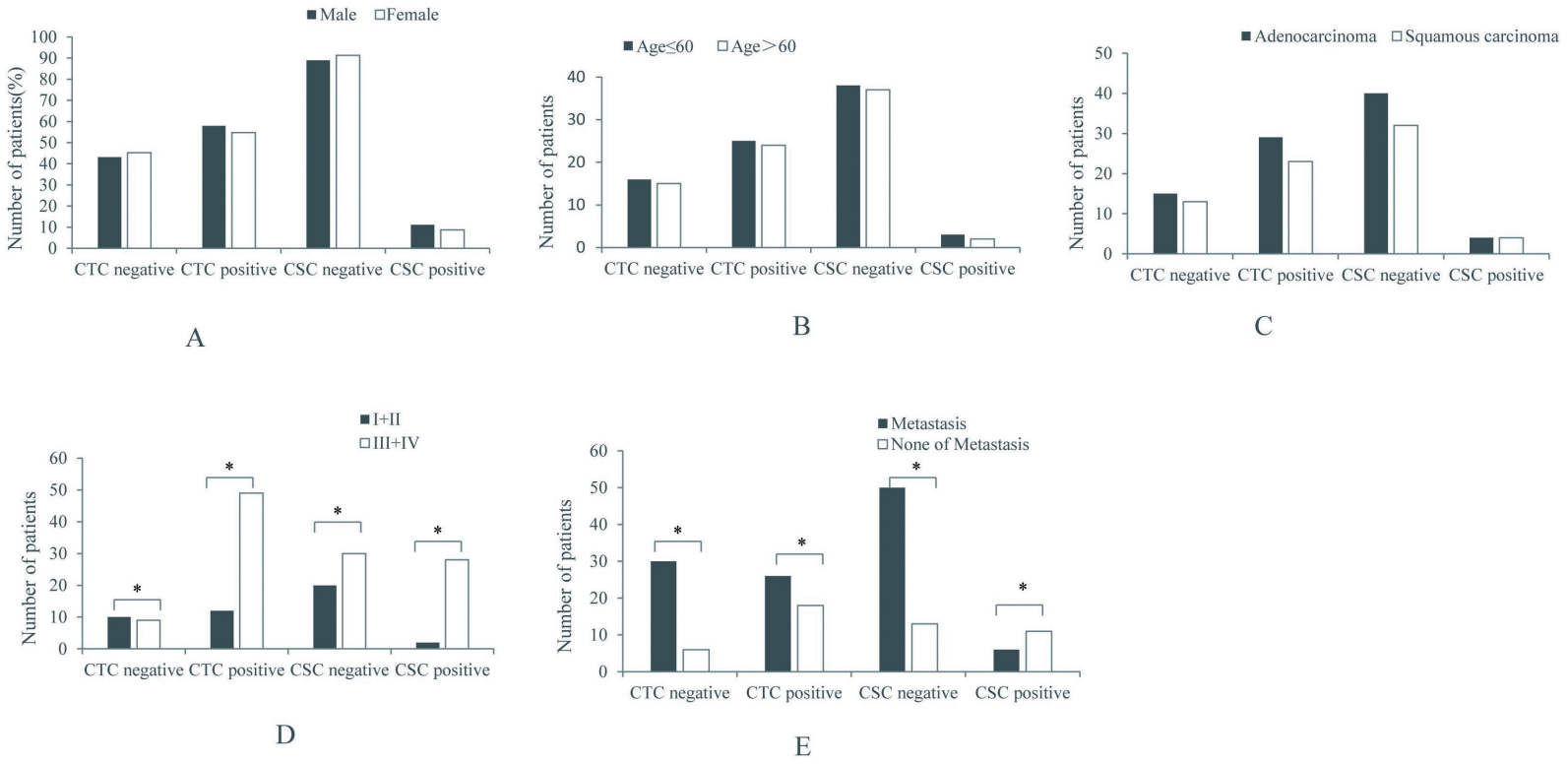

Figure 2. Comparison between clinical status and the results of CTCs and CSCs in patients with NSCLC. **A**, Comparison between patient's gender and the results of CTCs and CSCs. **B**, Comparison between patient's age and the results of CTCs and CSCs. **C,** Comparison between patient's pathological classification and the results of CTCs and CSCs. **D,** Comparison between patient's staging and the results of CTCs and CSCs. **E,** Comparison between patient's metastasis and the results of CTCs and CSCs.

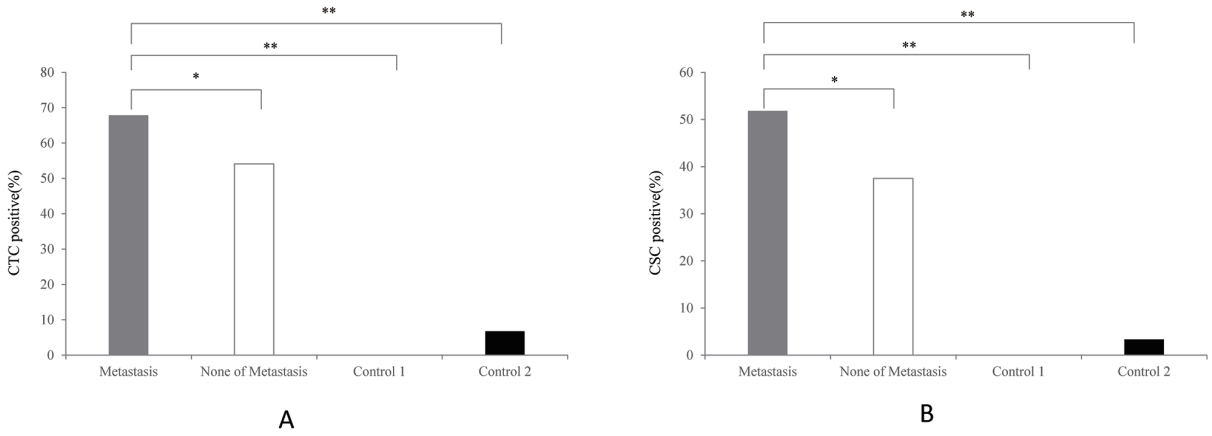

Figure 3. Difference between the metastasis and CTCs and CSCs positive rate detected by microfluidic chip in patients. **A**, Comparison of CTCs positive rate detected by microfluidic chip in different groups (stage I+II, stage III+IV, control group 1 and control group 2). **B**, Comparison of CSCs positive rate detected by microfluidic chip in different groups (stage I+II, stage III+IV, control group 1 and control group 2).

Comparison of CTCs and CSCs Positive Rate in Lung Cancer Metastasis Group, Lung Cancer Non-Metastasis Group, Control Group 1 and Control Group 2

According to the metastasis, 80 patients were divided into two groups, metastasis group and non-metastasis group, to compare CTCs and CSCs positive rates of different patients. According to the eighth edition of lung cancer staging, patients with lung cancer stage III-IV were divided into metastasis group, and patients with lung cancer stage I-II were divided into non-metastasis group. There was no significant statistical difference in the general information of the four groups. According to the results, compared with control group 1 and control group 2, there was significant difference in CTCs and CSCs positive rate between lung cancer metastasis group and lung cancer non-metastasis group ($p<0.05$). Compared with lung cancer non-metastasis group, the positive rate of lung cancer metastasis group was significantly higher ($p<0.05$). As shown in the Figure 3, it was suggested that the early detection of patients with microfluidic chip can be used to judge the metastasis of patients.

Relationship Between CTCs and CSCs Detection and Clinical Staging in Patients with NSCLC

According to the staging, 80 patients were divided into two groups of stage I+II group and stage IIII+IIIV group, respectively, to compare

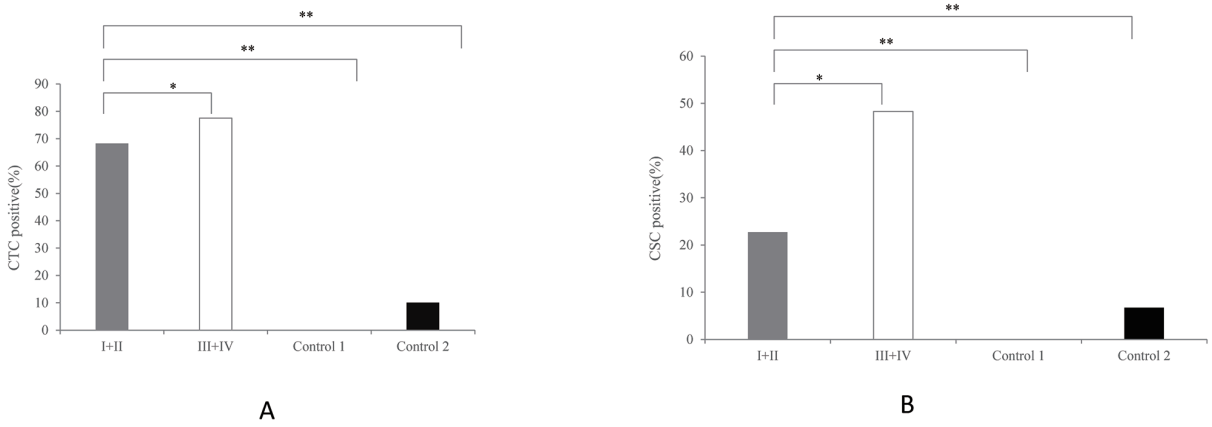

Figure 4. Difference between clinical staging and CTCs and CSCs positive rate detected by microfluidic chip. **A**, Comparison of CTCs positive rate detected by microfluidic chip in different groups (stage I+II, stage III+IV, control group 1 and control group 2). **B**, Comparison of CSCs positive rate detected by microfluidic chip in different groups (stage I+II, stage III+IV, control group 1 and control group 2).

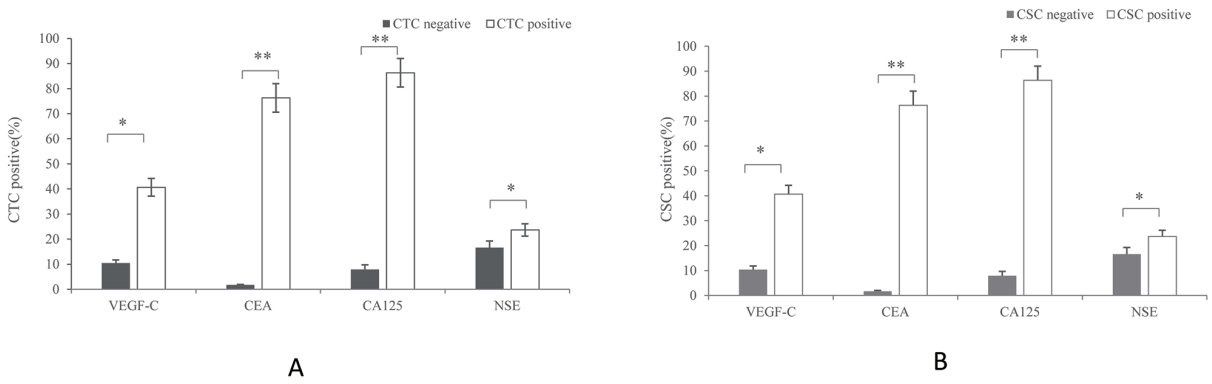

Figure 5. Relationship of serological tumor markers and the findings of CTCs and CSCs detection in patients. **A**, Relationship of serological tumor markers and CTCs positive rate. **B**, Relationship of serological tumor markers and CSCs positive rate. Of which, all VEGF-C, CEA, CA125 and NSE are serological tumor markers.

the positive rate of CTCs and CSCs in different patients. There was no significant statistical difference in the general information of the four groups. The results show that, compared with control group 1 and control group 2, there was significant difference in positive rate of CTCs and CSCs between lung cancer stage I+II group and lung cancer stage IIII+IIIV group ($p<0.05$). Compared with the patients in lung cancer stage I+II group, lung cancer stage III+IV group had a higher positive rate, and the difference was statistically significant ($p<0.05$), as shown in Figure 4, suggesting that the CTCs and CSCs detection with microfluidic chip technology can assist in the clinical staging of patients with NSCLC.

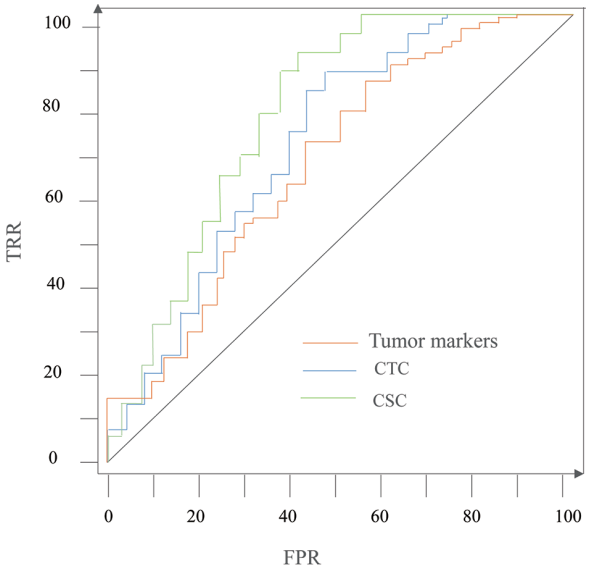

Figure 6. Comparison between ROC curves of CTCs and CSCs and serological tumor markers detected by microfluidic chip.

Differences in Sensitivity between Serological Tumor Markers and Microfluidic Chip Detection of CTCs and CSCs in Patients with Lung Cancer

We tested the concentration of serological tumor markers such as VEGF-C, CEA and CA125, and analyzed whether it had certain correlation with CTCs and CSCs. The results show that, the concentration of CTCs negative and CTCs positive of patients with lung cancer were consistent with the serological tumor markers such as VEGF-C, CEA and CA125. And there were significant differences, the results are shown in Figure 4. Subsequently, we compared the ROC curves of CTCs and CSCs with tumor markers. The sensitivity of CSCs was found to be 94.5% in the diagnosis of patients with lung cancer with the specificity of 71.0%, the sensitivity of CTCs was 93.6% with the specificity of 78.9%, the sensitivity of tumor markers was 89.1%, with the specificity of 86.3%, which is shown in Figure 5 and Figure 6.

Differences in the Findings of CTCs and CSCs Before and After Treatment for Patients with Tumors

We followed up 40 of these lung cancer patients. CTCs before and after treatment of all the patients with lung cancer were detected, the treatment included surgery, chemotherapy, targeted therapy. The patients' condition was recorded, which mainly included 4 main types: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). The find-

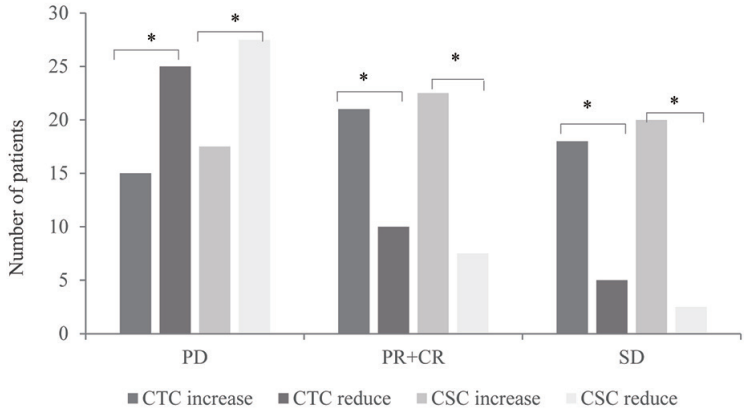

Figure 7. Differences in the findings of CTCs and CSCs before and after treatment for patients with lung cancer. Note: * represents the difference between the two groups, $p<0.05$.

ings showed that after all the treatments, CTCs and CSCs positive rate of patients with complete response and stable disease decreased significantly compared with those in patients with progressive disease, $p<0.05$. The details of the results are shown in Figure 7.

Discussion

The morbidity and mortality of lung cancer are in the first place in China. The incidence is relatively not evident, which is not easy to find. The patients are usually diagnosed at the late stage, with brain, bone and other distal metastasis, and poor prognosis, seriously threatening people's health [23,24]. At present, there are some limitations in the examination methods of lung cancer, such as imaging, serological tumor markers, pathology and cytological examination, which cannot monitor the dynamic changes of tumor progression in real time. It is very important to monitor the occurrence of tumor in the early stage and make early prevention[11]. There is need to detect circulating tumor cells (CTCs) and cancer stem cells (CSCs)[25,26].

We collected 80 patients with lung cancer from October 2017 to October 2018. Meanwhile, 30 healthy people and 30 patients with benign lung diseases were collected as control group 1 and control group 2. CellSearch system (Huntington Valley, PA, USA) and microfluidic chip were used to detect the CTCs of patients with NSCLC. The sensitivity and specificity of patients were recorded. We found that the sensitivity of detection with microfluidic chip was higher than that of CellSearch system (Huntington Valley, PA, USA; 95.1% *vs.* 92.1%), suggesting that the sensitivity of CTCs detected by microfluidic chip can be higher with better detection efficiency, the results are shown in Figure 1.

According to the detection of CTCs and CSCs in the peripheral blood and the comparison with the clinical information of patients, there was no significant correlation between the positive rate of CTCs and CSCs and the patient's age, sex, pathological type (adenocarcinoma, squamous cell carcinoma), there was significant correlation with the clinical stage (I+II and IIII+IIIV), metastasis (metastasis and non-metastasis) ($p<0.01$), see Figure 3 for details.

And then, according to the conditions, the 80 patients were divided into two groups, namely metastasis group and non-metastasis group, to compare CTCs and CSCs positive rate of different patients. The results showed that compared with healthy control group and benign control group, CTCs and CSCs positive rate of lung cancer metastasis group and lung cancer non-metastasis group was significantly different ($p<0.05$), and compared with the non-metastasis group, the positive rate of lung cancer metastasis group was significantly higher than lung cancer non-metastasis group ($p<0.05$). The results were shown in Figure 4.

According to the staging, we divided 80 patients into two groups, lung cancer stage I+II group and lung cancer stage IIII+IIIV group, to compare CTCs and CSCs positive rate of different patients. There was no significant statistical

difference in the general information of the four groups. The results showed that compared with control group 1 and control group 2, there was a significant difference in CTCs and CSCs positive rate between lung cancer stage I+II group and lung cancer stage IIII+IIIV group ($p<0.05$), and compared with the patients in lung cancer stage I+II group, the positive rate of lung cancer stage IIII+IIIV group was significantly higher and the difference was statistically significant ($p<0.05$). The results are shown in Figure 5.

Next, we detected the concentrations of serological tumor markers, such as VEGF-C, CEA, CA125 in patients, and analyzed whether it had a certain correlation with CTCs and CSCs. The results showed that the positive rate of CTCs and CSCs in patients with lung cancer was significantly different from tumor markers of VEGF-C, CEA, CA125, and the results are shown in Figure 6.

Finally, we followed up 40 of patients with lung cancer and tested CTCs of all patients with lung cancer before and after treatment. The treatments involved surgery, chemotherapy, targeted therapy. The patients' conditions were recorded. The results showed that the positive rate of CTCs and CSCs in patients with complete response and stable disease decreased significantly compared with patients with progressive disease after treatment, $p<0.05$. Details of the results are shown in Figure 7.

This study is the first to use microfluidic chip technology to detect the CTCs/CSCs of lung cancer patients, and divide the patients into Stage I+II group and Stage III+IV group for research. It is found that the positive rate of CTCs/CSCs in patients with stage III+IV is significantly higher than patients with stage I+II, and the concentration results of tumor markers are consistent with the results of CTCs/CSCs. The results of the study show that the use of microfluidic chips to detect CTCs/CSCs in patients has the potential value of diagnosing the stage and metastasis of non-small cell lung cancer, and is worthy of further study.

Conclusions

Summarily, the sensitivity and specificity of CTCs/CSCs detected by modified microfluidic chip are high, which also has great reference significance in the clinical staging and judging of metastasis, providing reference for early clinical monitoring. They were positively correlated with the concentration of tumor markers in serum, which was beneficial to the clinical treatment and prognosis of patients. According to the follow-up of the patients, we found that CTCs/CSCs positive rate was directly related to the patients with progressive disease. Microfluidic chip technology has great application prospect in the treatment of patients with lung cancer. However, at present, CTCs/CSCs detection with microfluidic chip technology is still under investigation.

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Availability of Data and Materials

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

Authors' Contributions

SW and ZS conceived and designed this study. XZ offered administrative support. SW, ZS, XZ, HD and XY prepared materials and carried out experiments. TW, TL and QW helped with data collection, analysis and interpretation. SW and ZS wrote the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Qiqihar Medical University. Signed written informed consents were obtained from the patients and/or guardians.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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