Circulating tumor DNA detection in peripheral blood in postoperative efficacy evaluation and recurrence risk prediction of lung cancer

Kun Lv, Xiongtiao Yang, Zhihua Li, Chengfang Ma, Qiongchuan Hong[*]

Department of Thoracic Surgery, Shenzhen Longgang Central Hospital, Shenzhen, 518116, China

ARTICLE INFO	ABSTRACT
	This study mainly explores the application in the evaluation of postoperative efficacy and recurrence risk prediction. In this study, 50 lung cancer patients treated with thoracic surgery were selected. Collect the peripheral blood of these patients before the operation, separate the blood sample from the plasma by high-speed centrifugal separation, extract the DNA in the plasma by the magnetic bead method, and then extract the ctDNA. A liquid-phase chip capture system is used to highly concentrate target DNA. We use the IlluminaHiseq platform to sort with high throughput and high depth. As an indicator of ctDNA detection and analysis, the AF value of gene mutation frequency is used for calibration. The sensitivity of CEA detection in peripheral blood of tumor patients (TP) is 30% (15/50), and the sensitivity of ctDNA detection is significantly higher than that of CEA detection. Research results show that the number of CTCs in peripheral blood is closely related to the occurrence of tumors.

Introduction

Anti-angiogenesis factors are negative regulators of the growth of various malignant tumors. However, there is no uniform standard regarding methods for evaluating the therapeutic effects of anti-angiogenesis drugs (1). The detection of circulating tumor DNA in peripheral blood is very common in tumor detection (2). Wada *et al*. believe that TKI can treat NSCLC (3). The advent of next-generation sequencing (NGS) has made this possible (2).

The treatment of lung cancer is related to gene expression in cells (4, 5). Studies evaluated the efficacy of the dual endothelin receptor antagonist Macitentan (6, 7). In the cytotoxicity test, the effect of Macitentan on the chemical protection mediated by astrocytes and brain endothelial cells was determined (8, 9). The survival rates of mice with established MDA-MB-231 breast cancer or PC-14 non-small cell lung cancer (NSCLC) brain metastases were compared (10, 11).

In this study, the target sequence was used to detect the gene mutation driver of the tumor. TumorDNA (tumor DNA, tDNA) and plasma ctDNA were used as samples to determine the type and frequency of plasma ctDNA and tDNA mutations before and after sequence changes and before and after surgery, and the induction analysis method was implemented (12, 13). The frequency of ctDNA variation before and after surgery was analyzed, and the sensitivity of ctDNA and lung cancer tumor markers was compared (14, 15).

Materials and methods
Research Objects

The study included 50 lung cancer patients who were hospitalized from November 2011 to December 2012. Collected 4 ml of blood samples from each nerve terminal. Among them, 25-year-old men and 25-year-old women are 44 to 90 years old, with an average age of 62 years. The effectiveness is based on

the following criteria: the effectiveness evaluation criteria for solid tumors divided into PD, SD, PR and CR. PD and SD are classified as ineffective groups, and PR and CR are classified as effective groups.

Peripheral Blood Collection

(i) Collection tube: ordinary blood tube containing EDTA-K2 anticoagulant.

(ii) Collection time: lung cancer patients: 2 days before chemotherapy. Surgery patients: 2 days before surgery. Physical examination center: during physical examination. All personnel took blood in the morning on an empty stomach.

(iii) Collection volume: 4ml (2ml of cubital venous blood of the patient is collected and discarded, and 4ml is collected in the anticoagulant tube).

Screening for Markers with Both Tumor Stemness and Emt Characteristics

The A549 anti-alkaloid cell line (A549/GR) and EMT A549 (A549/TGF-1) cells were initially constructed, and the next two cell lines were stained by immunofluorescence. The appearance of CXCR4 was confirmed after staining. According to the CXCR4 labeling result, A549/GR cells flow. Extract RNA from cells (A549, A549/TGF-β1, circulating tumor cells + A549/GR), and perform transcription HumanEMTRT2 ProfiilerTMPCRArray experiment. Obtain higher mRNA markers, perform TCGA database and path analysis for verification.

Two-Site Elisa Method to Detect the Level of FS Protein In Serum

(i) Encapsulation: Using lung cancer two-site ELISA, the anti-lung cancer monoclonal antibody was diluted to 3.3 µg/mol with 0.04 mol (pH 9.6) carbonate buffer solution, and each specimen was coated with formaldehyde solution. Place it at 3°C overnight.

(ii) Sealing: wash the board with distilled water once the next day, and seal it with 0.8% BSA at 60°C for 2 hours. Tear off the sealed label and clean the board twice.

(iii) Add test samples: add 30 mol to each sample, and dilute standard protein (0.3-14µg/mol) or sample.

(iv) Increase the anti-lung cancer polyclonal antibody: incubate at 30 °C for 2 hours, wash the plate

twice, add 80 mol of anti-lung cancer polyclonal antibody (7:300) to each group of specimens.

(v) Add enzyme-labeled antibody: heat at 30°C for 2 hours, wash the plate twice and add 70 mol of protease-labeled IgG antibody (1:60) to the specimen.

(vii) Matrix color development: Bake at 50°C for 3 hours, clean the plate twice, add 80 molOPD matrix solution to each pore, and leave it at room temperature for 2-4 minutes. The sample well is colorless compared to the well. In order to terminate the reaction, 2 mol of LHSO4 was added to each gap.

(viii) Detection: Measured by ELISA under the light absorption value of 300nm (A550nm) to draw a calibration line for calculating the lung cancer content of the sample.

Detection of Circulating Tumor Cells in Peripheral Blood

(i) Check the quality of the sample and confirm that the sample meets the standard. Add 2ml of normal saline to the 10ml centrifuge tube with the label and add the fixative (7%PFA) within 19 days, mix well, and use a low-temperature sterilization straw to add 4ml Blood samples. Put it into the absorption tube, and perform two suction operations, and stir thoroughly. Set the room temperature to a constant temperature and keep it for 15 minutes. After fixing, open the upper plug of the filter, add the sample to the filter, and insert the upper plug. The machine test was followed.

(ii) Take out the filter after separation, open the upper plug, pour 200 mL of methanol into the filter, and fix it at room temperature (22-25 °C) for 3 minutes. Remove the filter, take out the color filter, and stick it on the center of the glass. Dry for 3 minutes at room temperature. Draw a circle slightly larger than the filter circle, and let it dry after three minutes. Add 50ml of A pigment solution and stir for 2 minutes, then add 300IPBS to dilute the A pigment solution, and then discard the diluted pigment solution. Add 50ml of B pigment solution and stir for 57 seconds, discard the B pigment solution. In order to clean the B dye residue, DI was added to the filter membrane, and it was confirmed that there was no obvious dye residue in the filter membrane. Set the oven at 60 °C for 6 minutes. Use a pipette to paste it on a clean sliding glass and apply it evenly to the size area of the filter film. Transfer the dyed color filter to

the sliding glass coated with glue, and place it in a drying oven at 50°C for 30 minutes. Drip 90% glycerin on the glass cover, seal it with rubber glue around the glass cover and place the temperature at 40°C. In order to confirm that the glass cover is fixed on the slide, the diagnosis of CTC needs to meet the following criteria: A.

Nuclear abnormalities, irregular nuclear morphology, nodules, lobes, etc., abnormal cells show nuclear sticks, and cell nuclei show horseshoe characteristics. B. The nucleus is relatively large. C. Single hole diameter of the core (>16μm). D. As the chromosomes of cancer cells increase, coarser particles appear and the staining is uneven. E. The thickness of the nuclear membrane is irregular. F. The cell undergoes abnormal mitosis. The image of the cell was divided into 4 independent parts and examined by an expert alone. In order to distinguish CTC from lymph node cells or single cells, immunocytochemical verification of CD45 is performed under special circumstances.

Monitoring Indicators and Efficacy Evaluation

The amount of CTC and CK (cytokeratin) in the peripheral blood is measured by the floating control method before and after treatment. All patients were evaluated at the end of the third treatment cycle, and the effectiveness was confirmed after 3 weeks. The effectiveness evaluation criteria for solid tumors are expressed by WHO. The clinical effective rate is the ratio of CR+PR to the total number of cases. Clinical benefit rate: the ratio of CR+PR+MR+SD to the total number of cases. Time to Tumor Progression (TTP): The recorded time of the patient's disease progression.

Statistical Methods

Using frequency law verification, the frequency of each group was compared. The identification range is all theoretical frequencies T≥5, and actual frequencies T<5. The correction formula of the correct probability method is used to verify the theoretical frequency T<1. When the P-value is greater than 0.05, the judgment is established. When the P-value is less than 0.05, it is judged as invalid.

Results and discussion

Stability of Peripheral Blood Circulating Lung Cancer Cells mRNA

Fluorescence quantitative RT-PCR method was used to detect the changes in peripheral blood of healthy people after 1, 2, 3, 4, 5, and 6 hours at room temperature. The changes in the mRNA of circulating lung cancer cells in the peripheral blood are shown in Figure 1. The expression level of circulating lung cancer mRNA in the peripheral blood of healthy people was relatively constant at room temperature for about 3 hours and decreased significantly after 6 hours. Therefore, the serum should be separated within 3 hours during sample testing. The healthy adults were divided into the young and middle-aged group (aged 20-40 years old) and the elderly group (aged> 60 years old), with 20 cases in each group, half male and half female.
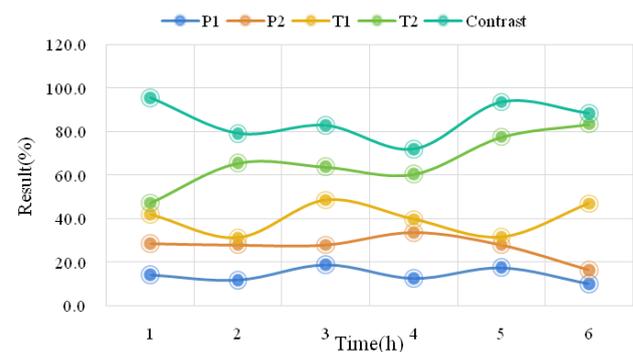

Figure 1. Changes in mRNA of peripheral lung cancer cells

The recovery status of lung cancer patients is shown in Table 1. The follow-up time was 5 months. There were 25 CTC-positive patients, with a positive rate of 71%. Among them, 15 cases had local recurrence and distant metastasis. The most common sites were brain, bone, mediastinal lymph nodes and multiple metastases in the lungs. There were 15 CTC-negative patients. Distant metastases occurred in 2 cases, and the metastasis sites were mediastinal lymph nodes and bones. CTM was only detected in CTC-positive patients. A total of 10 CTC-positive patients had CTM, with a positive rate of 0.58%. All patients with CTM-positive NSCLC had tumor recurrence and metastasis after surgery. The postoperative pathological test results were 32% (11/50). CEA

detection sensitivity is 30% (15/50),ctDNA detection sensitivity is significantly higher than CEA detection.

Circulating Muc1mrna Levels in Peripheral Blood of TP

Figure 2 shows the changes in circulating MUC1mRNA levels in the peripheral blood of TP. The level of circulating MUC1mRNA in peripheral blood of patients with benign tumors, kidney cancer and gastric cancer increased compared with healthy controls, but there was no statistically significant difference. In the study, peripheral blood CTCs of NSCLC patients were collected within 24 hours before surgery and 1 month after surgery. The rate of CTC-positive patients at baseline was 40%. The detection rate of patients with CTC decreased to 32% at 1 month after surgery, the difference was statistically significant. The presence of CTC after surgery was significantly related to early recurrence (p=0.018) and shorter DFS (p=0.006). There are reasons to believe that CTC counts in NSCLC patients one month after surgery can predict the possibility of their recurrence and metastasis.

Table1. Rehabilitation of lung cancer patients

Groups Parameter	Pulmonary Ca group	COPD group	Statistics	P
	(n=25)	(n=25)		
Average age	62.2±4.8	62.8±6.1	0.35	0.71
Dyspnea (cases, %)				
Class II	10 (50.00)	12 (50.00)	0.32	0.44
Class I	16 (50.00)	11 (50.00)	0.32	0.56
Cough (cases, %)				
Class II	10 (50.00)	6(50.00)	0.38	0.32
Class I	15 (50.00)	18 (50.00)	0.37	0.46

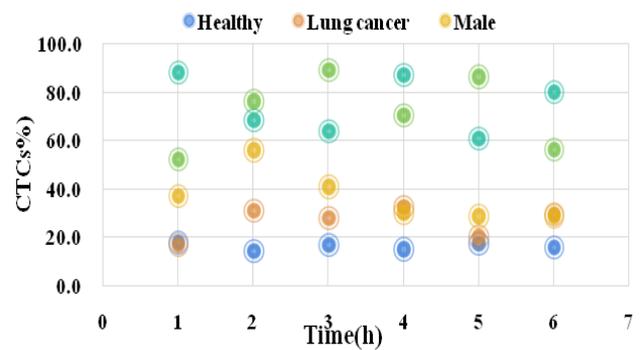## Detection of Circulating Tumor Cells in Patients with Lung Cancer

Figure 3 shows the results of circulating tumor cells in lung cancer patients. CTCs labeled with a single CD146 accounted for (0.7±0.43)% of all cells, mature CTCs labeled with three CD molecules accounted for

(0.4596±0.33)% of all cells, (P values were 0.02, 0.019)). Healthy subjects (n=18): CTCs labeled with a single CD146 accounted for (0.20±0.05)% of all cells, and mature CTCs labeled with three CD molecules accounted for (0.16±0.120)% of all cells. The average value of CTCs in lung cancer patients was 10.33 cells/mL, and the range was 5-40 cells/mL. The average value of CTCs in normal people's peripheral blood was 0.8 cells/mL, and the range was 1-8 cells/mL. The results showed that the reference value for distinguishing lung cancer patients from healthy controls was CTCs>8/mL. This result shows that CTCs can be used as predictive markers for liquid biopsy.
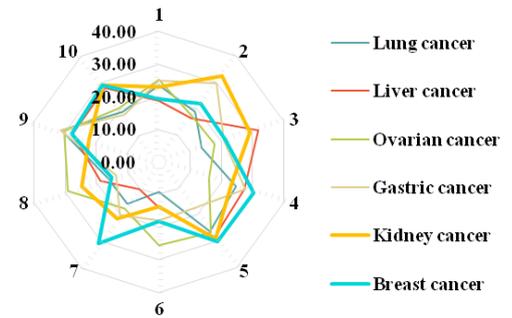
Figure 2. Changes in circulating MUC1mRNA levels in peripheral blood of TP
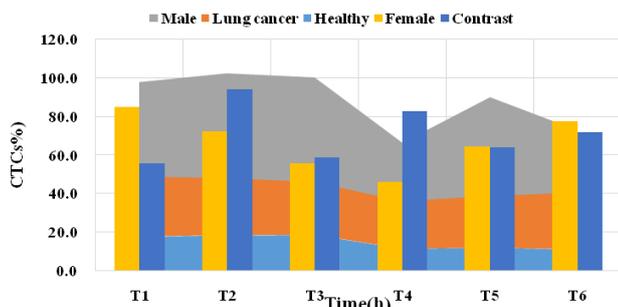
Figure 3. Detection results of circulating tumor cells in patients with lung cancer

Relationship between Pathological Staging and Different Types

The statistical results of TNM staging of lung cancer patients are shown in Figure 4. The detection rate of interstitial CTCs is statistically significant with the TNM (P-value: interstitial 0.02). With the later pathological staging, the positive rate of interstitial

CTCs tends to increase. The positive rates of qualitative CTCs during T1, T2, T3, and T4 are 7.5%, 41.0%, 42.0%, 67.7%, respectively. N0 The positive rates of qualitative CTCs during, N1, N2, and N3 were 28%, 36%, 5%, and 60%, respectively. The positive rates of qualitative CTCs during M0 and M1 were 33% and 72%, respectively. The later the clinical stage, the higher the detection rate of interstitial CTCs (the positive rates of interstitial CTCs in stages I, II, II, and IV are 21%, 22%, 54%, and 72%, respectively). The detection rate of CTCs is closely related to recurrence and distant metastasis, and the detection rate of qualitative CTC is still very high (M0 positive rate is 36.0%). Circulating tumor cells (CTCs) are more effective, timely, sensitive and reproducible than imaging examinations in monitoring early micrometastasis in patients.

Figure 4. Statistics of TNM staging of lung cancer patients

Conclusions

Peripheral blood ctDNA is a characteristic tumor biomarker. Compared with traditional tissue biopsy specimens, it has the characteristics of convenience, non-invasiveness, and patient compliance. In this paper, the stability of circulating tumor cells in the peripheral blood of patients with lung cancer, the relationship between pathological staging and different types of circulating tumor cells (CTC) were tested. The results showed that the number of CTCs in peripheral blood is closely related to the occurrence of tumors and can predict tumor The occurrence and development provide important reference information for the diagnosis and treatment of diseases. In monitoring early micrometastasis in patients, circulating tumor cells (CTC) are more effective, timely, sensitive and reproducible than imaging examinations. It is hoped that after more and deeper researches in the future, lung cancer factor diagnosis

and individualized treatment can be improved to a new level.

Interest conflict

The authors declare no conflict of interest.

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