Original Article
Clinical study on CTCs' value in evaluating chemotherapeutic effect of advanced NSCLC and prognosis

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Abstract: Objective: To explore the circulating tumor cells' (CTCs) value in evaluating chemotherapeutic effect of advanced non-small cell lung cancer (NSCLC) and prognosis. Methods: Eighty-six advanced NSCLC patients were divided into high expression group and low expression group. Using the median of CTCs expression values of all patients as the demarcation point, those higher than the median were deemed as high expression, and those lower than the median were deemed as low expression. The expression level of CTCs was determined by flow cytometry before and after 4 cycles of chemotherapy. The CTCs expression level was compared with chemotherapeutic effect, progression free survival (PFS) and overall survival (OS). Results: In the aspect of therapeutic effect, the low CTCs expression group had a response rate (RR) of 47.9% (23/48) and a disease control rate (DCR) of 72.9% (35/48). The high CTCs expression group had a RR of 31.5% (12/38) and a DCR of 57.8% (22/38). Patients with a low CTCs level before chemotherapy had better RR and clinical remission rate than those with a high CTCs level. Compared with patients without dramatic decline in the CTCs level after chemotherapy, those with dramatic decline in the CTCs level after chemotherapy had better chemotherapeutic effect. The difference between two groups was statistically significant (P < 0.05). In the aspect of prognosis, the median OS of low CTCs expression group and high CTCs expression group was 21.5 months (95% CI: 17.1-26.9 months) and 16.3 months (95% CI: 11.8-20.5 months), respectively. The median PFS was 10.5 months (95% CI: 6.9-13.8 months) and 5.1 months (95% CI: 3.3-7.2 months), respectively. The comparison of the two groups showed P < 0.05, indicating statistical significance. Patients with low CTCs expression had longer OS and PFS compared with those with high expression. Conclusion: The CTCs level can be used as an index predicting the chemotherapeutic effect of advanced NSCLC patients. The chemotherapeutic effect was negatively correlated with the CTCs level. Dynamic change of the CTCs level can be an effective index to evaluate chemotherapeutic effect. Meanwhile, the CTCs expression level was negatively correlated with the prognosis of advanced NSCLC patients. The change of CTCs expression levels can be used as an index evaluating the prognosis of advanced NSCLC patients.

Keywords: NSCLC, CTCs, chemotherapy, therapeutic effect, prognosis

Introduction

Presently, the morbidity of lung cancer is high. Lung cancer has become a tumor with the fastest growth rate and the largest harmfulness. 80% patients are suffered from non-small cell lung cancer (NSCLC), while 15-25% are suffered from small cell lung cancer (SCLC) [1]. The surgical treatment can obviously extend the survival time of early stage lung cancer patients. However, the early symptoms of lung cancer can hardly be noticed, without distinctive manifestation. Most patients are in locally advanced stage or have distant metastasis to brain, bone, adrenal gland and liver, etc. when they are diagnosed. Surgery cannot be conducted, and the prognosis is poor. The five-year survival rate of stage I-IIIA lung cancer patients after excision is only 30%-40%. The overall five-year survival rate is less than 15%. Most patients die of recurrence of lung cancer and distant metastasis [2].

With the development of molecular targeted therapy for lung cancer, epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI)

has become the first-line treatment choice for advanced NSCLC patients with EGFR mutation. But for advanced NSCLC patients with wild type EGFR, the two-drug chemotherapy regimen based on platinum still is a standard therapeutic regimen for advanced NSCLC patients with unresectable tumor. The RR of two-drug chemotherapy regimens based on platinum is 20-50%. Different patients have significantly different RR and survival rate, which is closely related to factors such as tumor heterogeneity, tumor microenvironment, formation of tumor vessels and multi-drug resistance [3]. Meanwhile, the discovery of tumor and the monitoring of therapeutic process depend on imageological examination and serum tumor marker examination. However, the tumor size indicated by imaging is not in conformity with the grade malignancy or invasion & metastasis ability [4]. Presently, a specific tumor marker with high sensitivity, which can dynamically evaluate the chemotherapeutic effect and timely find the recurrence and metastasis of tumor, is lacking. Therefore, a method, which can dynamically evaluate the chemotherapeutic effect and timely find the recurrence and metastasis of tumor, will improve chemotherapeutic effect, extend survival time, and avoid excessive or delayed treatment.

Circulating tumor cells (CTCs) refer to tumor cells which are released by the primary lesion or metastasis lesion due to internal or external causes and enter blood circulation [5]. Tumor cells can be frequently detected in peripheral circulating blood of patients with cancer. Although it does not mean the existence of metastasis, many clinical studies indicate that the change of CTCs expression level is closely related to therapeutic effect and prognosis of patients with cancer [6-8]. Presently, CTCs have been approved to be used in dynamic monitoring and therapeutic evaluation of advanced breast cancer and colorectal cancer by FDA [9, 10]. Meanwhile, many clinical trials also verify that CTC is also an effective dynamic evaluation and prognosis method for other malignant tumors including prostate cancer [11, 12]. However, in the field of lung cancer, no final conclusion has yet been reached on whether CTC is effective in chemotherapeutic effect evaluation and prognosis. Therefore, we used CTCs as the research object, which were from peripheral blood of advanced NSCLC patients receiving chemotherapy, and explored CTCs' value in evaluating chemotherapeutic effect of advanced NSCLC and prognosis.

Materials and method

Clinical data

Eighty-six advanced (stage IIIB, IV) NSCLC patients, who were admitted by Tumor Hospital of Yunnan Province between January 2012 and December 2014 and confirmed by histopathology, were selected. Inclusion criteria: Age > 18 years old; Those confirmed as NSCLC (adenocarcinoma or squamous carcinoma) by pathology or exfoliative cytology diagnosis; Patients who were detected as wild type EGFR or refused EGFR-TKI treatment; Stage IIIb-IV patients indicated by imaging examination; PS score was 0-2; Expected survival time was longer than 3 months; Those having not received systemic or local anti-cancer treatment (such as surgery, chemotherapy or radiotherapy) previously; Those without a history of other malignant tumors; Those having received two-drug chemotherapy regimen based on platinum for at least 4 cycles; The follow-up was at least 10 months (including patients died within 10 months). Exclusion criteria: Lacking pathological diagnosis of lung cancer; Having received systemic or local anti-cancer treatment; Poor compliance; Patients not having indication of chemotherapy; Patients with pneumonia, empyema or other signs of infection. Among all the cases, 46 were male and 40 were female (male to female ratio of 1.15:1), aged 34-76 years with an average of 61 years. There were 49 adenocarcinoma cases and 37 squamous carcinoma cases. In terms of TNM staging, there were 23 cases in stage IIIB and 63 cases in stage IV. In terms of PS score, 50 cases had a score of 0-1, and 36 cases had 2. Thoracic and abdominal CT, color Doppler ultrasound, brain MRI, bone scanning, ECG, blood routine examination, blood biochemistry and tumor marker examination were performed before and after treatment. Based on our experimental data, according to statistical method, 85.5-the median of CTCs expression values of all patients- was used as the demarcation point. Patients with CTCs expression values higher than the median were classified into high CTCs expression group, and those lower than the median were classified into low CTCs expression group.

Table 1. Comparison of positive rate in CTCs detection of lung cancer group and healthy group

Group	n	Positive CTCs cases (%)	χ^2	P
Lung cancer group	86	57 (66.2%)	17.68	< 0.001
Healthy group	20	0		

Therapeutic method

The TNM stage of patient was determined according to thoracic and abdominal CT, brain MRI and bone scanning. At least 4 cycles of two-drug chemotherapy based on platinum were given. Thoracic and abdominal CT and chemotherapeutic effect evaluation were conducted after every two cycles of chemotherapy. The chemotherapy regimen: Gemcitabine 1 g/m^2 d1, combined with cisplatin 75 mg/m^2 d1 or carboplatin 5AUC d1; Paclitaxel 175 mg/m^2 d1 combined with cisplatin 75 mg/m^2 d1 or carboplatin 5AUC d1. Peripheral blood was collected to conduct CTCs detection at one day before the first chemotherapy and after 4 cycles of chemotherapy. The survival data were obtained through the follow-up till December 31, 2014.

Experimental method

Specimen collection: The median cubital vein blood was collected by vacuum blood taking needle at one day before the first chemotherapy and after 4 cycles of chemotherapy. The first 2 ml blood after puncture was abandoned. 7.5 ml venous blood hereafter was collected. The heparin was added and mixed with blood for anticoagulation. Detection was conducted within 2 h after collection.

Antibody and reagent: CD45-PC5, CK18-FITC, EGFR-PE and NH4CL hemolysin were purchased from Beckmancoulter (USA). Flow cytometry was also from Beckmancoulter (USA). Mouse anti human cytokeratin 18 (CK18/PE) & mouse (Isotype Control/PE) kit, mouse anti human CD45 (Leuko-cyte Common Antigen)/ PE-Cy5 & mouse Isotype Control, mouse anti human EGFR/FIT & mouse Isotype Control/ FITC, FACSTM Permeabilizing Solution2 (Perm2) permeabilization medium (10×) and lymphocyte separation medium were purchased from Beckmancoulter (USA).

CTCs detection: 20 ul CD45-PC5, 20 ul EGFR-PE and 20 ul CK18-FITC were added into the test tube. Then 100 ul whole blood with heparin for anticoagulation was added. After shaking up, the test tube was kept in dark place at room temperature for 30 min. Then 2 ml NH_4Cl hemolysin was added for hemolysis. Detection was conducted 15 min later. The cells that expressed CD45-CK18+ were tumor cells (CTC) remained in peripheral blood of the patients. 5 ml heparin anticoagulation blood of the patients was taken and mixed. The blood was diluted by PBS solution or normal saline at a ratio of 1:1. 5 ml Ficoll lymphocyte separation medium was firstly added into a 15 ML centrifuge tube. 10 ml diluted blood was slowly added to the upper layer of Ficoll along the tube wall. Centrifugation was conducted at 2300 r/min for 30 min. Cells in the monocyte layer were collected. 2 ml PBS solution was added. After gently mixing, centrifugation was conducted at 1000 r/min for 5 min, with the supernatant abandoned. Two 100 ul flow reaction tubes were taken. 20 ul CD45/ PE-Cy5 and 20 ul CK18/PE were added into the experimental tube. The homotype IgG-PC5 and IgG-PE were added to the control tube. After 30 min standing in dark place at room temperature, 2 ml PBS solution was added. After gently mixing, centrifugation was conducted at 1000 r/min for 5 min. The supernatant was abandoned. 500 ul 1% paraformaldehyde was added to two tubes respectively. After 10 min reaction in dark place at room temperature, rinsing by PBS solution, centrifugation, and abandonment of supernatant were conducted. 500 ul permeabilization medium was added to two tubes respectively. After 10 min reaction in dark place at room temperature, rinsing by PBS solution, centrifugation, and abandonment of supernatant were carried out.

Therapeutic effect evaluation

In accordance with WHO solid tumor evaluation criteria, the therapeutic effect was divided into complete remission (CR), partial remission (PR), stable disease (SD) and progressive disease (PD). CR+PR meant effective. CR+PR+SD meant control. The progression free survival (PFS) referred to the first day of treatment to the progressive disease day. The overall survival (OS) referred to the first day of treatment to the date of death or lost follow-up. PFS and OS were calculated based on months.

Statistical methods

SPSS16.0 statistical package was used. Comparison of rates of enumeration data was

Table 2. Relationship between CTCs expression of advanced NSCLC and clinical factors

Clinical factors	n	CTCs expression value (X±S)	Z	P
Sex				
Male	46	139.9±219.3	2.161	0.131
Female	40	18.6±22.5		
Age				
≥ 60	30	97.8±176.1	1.683	0.163
< 60	56	83.5±154.6		
Pathological pattern				
Squamous carcinoma	37	103.8±184.6	0.938	0.378
Adenocarcinoma	49	125.6±184.5		
Differentiated degree				
Well & moderately differentiated	21	97.1±138.6		
Moderately differentiated	27	87.9±116.5	1.391	0.235
Poorly differentiated	38	113.1±139.1		
Clinical stage				
IIIB	23	78. 5±21.8	0.835	0.39
IV	63	138.6±168.8		
PS score				
0-1	50	106.5±159.1	1.681	0.271
2	36	83.5±163.5		
Chemotherapy regimens				
Gemcitabine + Cisplatin	25	68.6±103.5		
Paclitaxel + Cisplatin	23	87.1±136.3	2.38	0.159
Gemcitabine + Carboplatin	20	94.8±119.1		
Paclitaxel + Carboplatin	18	79.7±93.8		

performed by χ^2 test. Comparison of two groups of frequency under matched pair design was performed by χ^2 test for matched pair design. Correlation of CTCs and clinical factors and comparison of before and after chemotherapy did not conform to normal distribution after normality test. Therefore, they were expressed as median (range) and analyzed by matched pair rank-sum test. Kaplan-Meier method, Log rank test and Cox multi-factor regression analysis were adopted for survival analysis. P < 0.05 indicated significant difference.

Results

Comparison of positive rate in CTCs detection of lung cancer group and healthy group

The tumor cells were detected in peripheral blood of 57 cases among 86 patients in lung cancer group before chemotherapy. The positive rate of CTCs detection was 66.2%. The tumor cells were not detected in peripheral blood of all 20 cases of control group. The difference in CTCs detection result between the two groups was statistically significant (P < 0.001). See **Table 1**.

Relationship between CTCs and clinical factors

In 86 cases, the differences in CTCs expression among different sex, age, pathological pattern, differentiated degree, clinical stage, PS score and chemotherapy regimen groups were not statistically significant (P > 0.05). See **Table 2**.

Relationship between CTCs and chemotherapeutic effect

The CTCs expression value of this experiment was 92-382. 85.5- the median of CTCs expression values of all patients was used as the demarcation point. Patients with CTCs expression values higher than the median were classified into high CTCs expression group, and those with CTCs expression values lower than the median were classified into low CTCs expression group. Among 86 cases of this group, there were 2 cases of CR, 33 cases of PR, 22 cases of SD and 29 cases of PD. The overall response rate (CR+PR) was 40.6% (35/86). The disease control rate (CR+PR+SD) was 66.2% (57/86). The low CTCs expression group had an response rate (RR) of 47.9% (23/48) and a disease control rate (DCR) of 72.9% (35/48). The high CTCs expression group had a RR of 31.5% (12/38) and a DCR of 57.8% (22/38). The difference of the two groups was statistically significant (P < 0.05), see **Table 3**.

Change of CTCs expression level before and after chemotherapy

When we compared the change of CTCs expression levels before and after treatment for 57/86 cases having response, the difference was statistically significant (P < 0.05). When we com-

Table 3. Relationship between CTCs expression level and chemotherapeutic effect

Group	n	Mean CTCs (X±S)	CR	PR	SD	PD	RR (%)	X^2	P	DCR	X^2	P
Low expression group	48	58.5±31.3	2	21	12	13	47.9	11.47	0.007	72.9	8.665	0.003
High expression group	38	138.5±165.8	0	12	10	16	31.5			57.8		

Table 4. Change of CTCs expression levels before and after chemotherapy

Group	n	CTCs expression levels (X±S)		t	P
		Before treatment	After treatment		
Response group	57	21.8±19.1	13.3±11.6	2.398	0.013
No response group	29	168.3±269.1	156.6±237.1	1.385	0.076

pared the change of CTCs expression levels before and after treatment for 29 cases having no response, the difference was not statistically significant (P > 0.05). See **Table 4**.

Relationship between CTCs expression level and survival time

The median OS of cases in this group was 19.8 months (95% CI: 16.9-26.9 months). The one-year and three-year survival rate was 59.3% and 14.3%, respectively. The median OS of low CTCs expression group and high CTCs expression group was 21.5 months (95% CI: 17.1-26.9 months) and 16.3 months (95% CI: 11.8-20.5 months), respectively. The one-year survival rate of the two groups was 63.5% and 50.1%, respectively. The three-year survival rate of the two groups was 15.6% and 8.1%, respectively. Log rank test indicated that the difference of the two groups was statistically significant (P = 0.01). See **Figure 1**.

The median PFS of all cases was 7.8 months (95% CI: 5.1-9.6 months). The median PFS of low CTCs expression group and high CTCs expression group was 10.5 months (95% CI: 6.9-13.8 months) and 5.1 months (95% CI: 3.3-7.2 months), respectively. The comparison of the two groups showed P < 0.05, indicting significant difference. Log rank test indicated that the difference of the two groups was statistically significant (P = 0.005). See **Figure 2**.

Discussion

In 1869, Ashworth proposed the concept of CTCs because he found cells similar to tumor in the peripheral blood of patients who died of cancer [13]. In the proliferation and development process of malignant tumor, tumor cells detach continuously. Some detached tumor cells may have change in DNA, protein modification and cell phenotype through epithelial mesenchymal transition (EMT), capable of invading. These cells can enter peripheral blood circulation system and become CTCs with metastasis ability [14]. Therefore, CTCs detection is of clinical significance in malignant tumor diagnosis, therapeutic effect evaluation and prognosis. Our study indicated that the difference in CTCs levels of advanced NSCLC patients among different sex, age, pathological pattern, differentiated degree, clinical stage and PS score groups was not statistically significant (P > 0.05). This indicates that some patients have had occult metastasis that cannot be found by conventional pathological, cytological and imageological examination upon the first visit. Tumor cells found in peripheral blood are a direct evidence of micrometastasis of malignant tumor. Such micrometastasis is the main reason leading to treatment failure and death of patients.

There is only a small quantity of CTCs in peripheral blood of NSCLC patients. CTCs can hardly be detected even in peripheral blood of advanced NSCLC patients. For this reason, it is important to establish an accurate, reliable and repeatable method to detect CTCs in peripheral blood. Presently, common methods for CTCs detection mainly include flow cytometry, RT-PCR and Cell SearchTM system method [15]. These techniques can obviously improve the detection rate of CTCs, of which, flow cytometry is a high speed technique for cell analysis and sorting. It can execute the same cells. Mainly utilizing fluorescent antibody staining, it can conduct multi-parameter analysis for physicochemical properties of cells [16]. Flow cytometry has become a main technique used for CTCs detection due to simple, reliable and rapid properties. This study used flow cytometry to
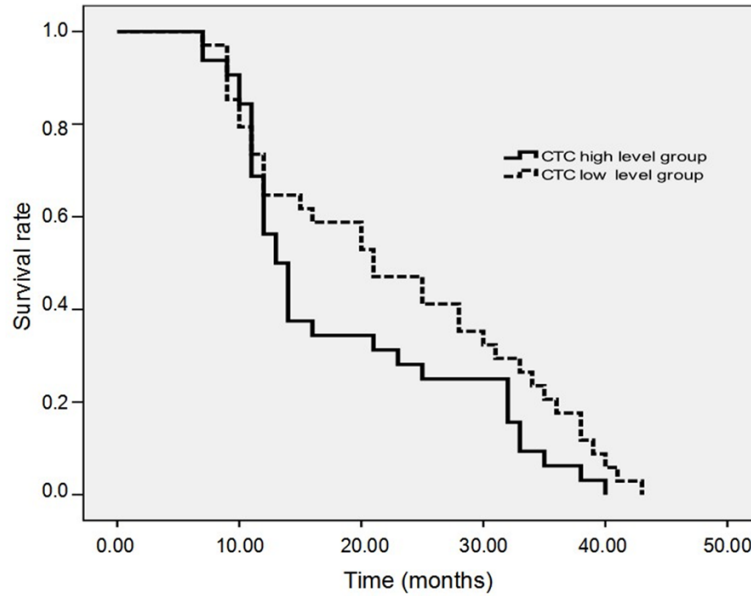
Figure 1. Relationship between CTCs expression of advanced NSCLC and survival time.

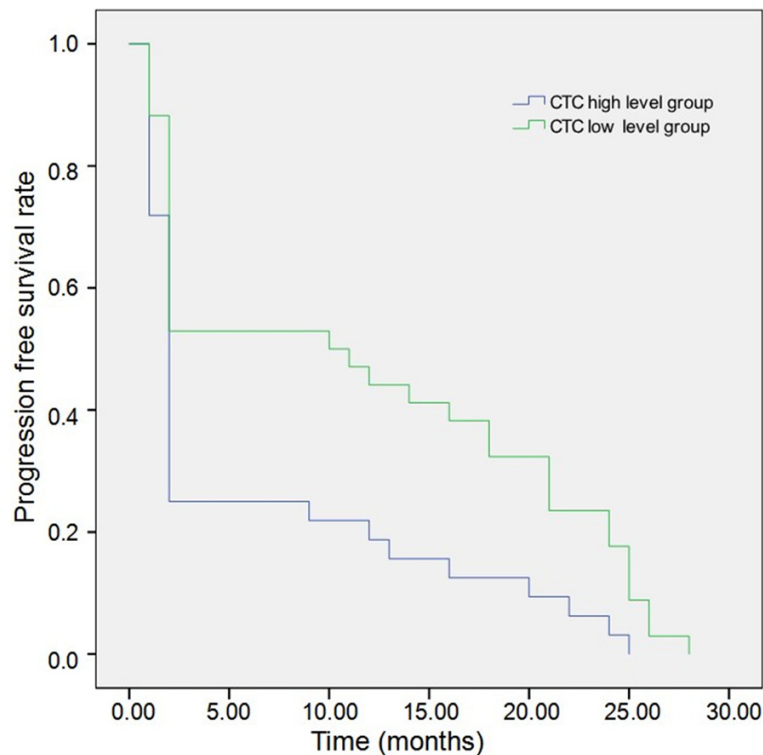**Figure 2.** Relationship between CTCs expression of advanced NSCLC and PFS.

peripheral blood of 57 cases among 86 patients in lung cancer group before chemotherapy. The positive rate was 66.2%. This is consistent with the results reported by Krebs MG et al. [17]. The tumor cells were not detected in peripheral blood of all 20 cases of control group. This indicates that the detection method of flow cytometry is highly specific.

Presently, the main therapy for advanced NSCLC is chemotherapy. The two-drug chemotherapy regimen based on platinum still is a standard therapeutic regimen for advanced NSCLC patients with unresectable tumor. However, RR of the two-drug chemotherapy regimen based on platinum is 20-50%. Different patients have significantly different RR and survival rate. The research results of Krebs MG et al. indicate that the change of CTCs expression levels is negatively correlated with chemotherapeutic effect of lung cancer [18]. Hou JM et al. report that CTCs can be used as an effective index to evaluate the chemotherapeutic effect of SCLC and prognosis [19]. Our experimental result indicated that after 4 cycles of chemotherapy for advanced NSCLC patients, the low CTCs expression group had a RR of 47.9% (23/48) and a DCR of 72.9% (35/48). The high CTCs expression group had a RR of 31.5% (12/38) and a DCR of 57.8% (22/38). Patients with a low CTCs level before chemotherapy had better RR and clinical remission rate than those with a high CTCs level. Compared with the patients without dramatic decline in CTCs levels after chemotherapy, those with dramatic decline in CTCs levels after

detect CTCs expression levels in peripheral blood of advanced NSCLC patients. The results indicated that the tumor cells were detected in

chemotherapy had better chemotherapeutic effect. This shows that the CTCs level can be used as an index to evaluate the chemotherapeutic effect of advanced NSCLC patients. Meanwhile, we can timely evaluate the chemotherapeutic effect and avoid insufficient or excessive chemotherapy through dynamic change of CTCs levels.

Presently, many studies indicate that the CTCs level can predict invasiveness of tumor. The existence of CTCs indicates poor prognosis of patients. Advanced NSCLC patients with a high CTCs level before chemotherapy have shorter PFS and OS compared with those with a low CTCs level [20-23]. Our research results also indicated that the median OS of low CTCs expression group and high CTCs expression group was 21.5 months (95% CI: 17.1-26.9 months) and 16.3 months (95% CI: 11.8-20.5 months), respectively. The median PFS was 10.5 months (95% CI: 6.9-13.8 months) and 5.1 months (95% CI: 3.3-7.2 months), respectively. The reason may be that compared with high CTCs expression group, low CTCs expression group has better chemotherapeutic effect and DCR, which further bring better OS and PFS to patients. This indicated that the CTCs expression level was negatively correlated with the prognosis of advanced NSCLC patients. Patients with a low CTCs expression had longer OS and PFS compared with those with high expression. The change of CTCs expression levels can be used as an index evaluating the prognosis of advanced NSCLC patients.

Our experimental results indicate that the CTCs level can be used as an index predicting the chemotherapeutic effect of advanced NSCLC patients. The chemotherapeutic effect is negatively correlated with the CTCs level. The dynamic change intensity of CTCs levels is closely related to chemotherapeutic effect, that is, the dynamic change of CTCs levels can be an effective index to evaluate chemotherapeutic effect. Meanwhile, the CTCs expression level is negatively correlated with the prognosis of advanced NSCLC patients. The change of CTCs expression levels can be used as an index evaluating the prognosis of advanced NSCLC patients. However, the sample size of this experimental study is small. A follow-up study with extended sample size is needed to further verify whether the CTCs level can be used as an

index evaluating the therapeutic effect of different chemotherapy regimens for advanced NSCLC and prognosis.

Disclosure of conflict of interest

None.

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