

Evaluation of sensitivity and specificity of CanPatrol™ technology for detection of circulating tumor cells in patients with non-small cell lung cancer

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Abstract

Background The early diagnosis of non-small cell lung cancer is of great significance to the prognosis of patients. However, traditional histopathology and imaging screening have certain limitations. Therefore, new methods are urgently needed to make up for the current diagnostic defects.

Objectives To evaluate of sensitivity and specificity of CanPatrol™ technology for detection of circulating tumor cells in patients with non-small cell lung cancer (NSCLC).

Methods Non-interventional clinical research approach was used in this study. CTCs in the peripheral blood of 98 patients with NSCLC (including 48 patients in stage I, 13 in stage II, 29 in stage III and 8 in stage IV) and 38 patients with benign pulmonary diseases were collected by the latest typing of CanPatrol™ detection technology and nanomembrane filtration technology. CTCs were divided into epithelial, epithelial-mesenchymal, and mesenchymal types by multiple mRNA in situ analysis. We followed a 3-year follow-up of the 98 NSCLC patients to observe the recurrence and metastasis of the tumor. Next, Kruskal-Wallis test was used to compare multiple groups of data, Mann-Whitney U test was used to compare data between the two groups, and ROC curve analysis was used to obtain the critical value. Then, the COX risk regression and Kaplan-Meier survival analysis were performed in 63 NSCLC patients who were effectively followed up with recurrence and metastasis as the outcome.

Results The epithelial, epithelial-mesenchymal and total CTCs were significantly higher in patients with NSCLC than in patients with benign lung disease ($P < 0.001$). The mesenchymal CTCs of NSCLC patients were slightly higher than those of benign lung diseases ($P = 0.013$). Both differences were statistically significant. The AUC of the ROC curve of the total CTCs was 0.837 (95% CI: 0.76–0.914), and the cut-off value corresponding to the most approximate index was 0.5 CTCs/5 ml, at which point the sensitivity was 81.6% and the specificity was 86.8%. COX regression analysis revealed that clinical stage was the factor affecting patient survival ($P = 0.006$), while gender, age and smoking were not statistically significant ($P > 0.05$). After excluding the confounders of staging, surgery, and chemotherapy, Kaplan-Meier survival analysis showed that patients in stage IIIA with CTCs ≥ 1 had significantly lower DFS than those with CTCs < 1 ($P = 0.022$).

Conclusion CanPatrol™ technology has good sensitivity and specificity in detecting CTCs in peripheral blood of NSCLC patients, and has certain value for clinical prognosis evaluation.

Introduction

The incidence of lung cancer (11.6% of total cancer cases) and mortality (18.4% of total cancer deaths) rank first in all malignancies.[1]According to histological classification, lung cancer can be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for about 85% of lung cancer and the main subtypes are lung adenocarcinoma and lung squamous cell carcinoma[2, 3]. Although screening, early diagnosis and treatment can improve the survival rate of lung cancer patients, the low sensitivity of the currently approved low-dose CT scan screening leads to a false positive rate of over 90%[4]. There are currently no additional biomarkers to improve the sensitivity of low-dose CT screening, especially for patients with uncertain lung nodules. In addition, histopathology and imaging which are the main methods to diagnose and evaluate treatment efficacy also have limitations. For example, there are certain restrictions in the actual operation of obtaining a tissue specimen for pathological examination with risking of bleeding, pneumothorax, and planting. Also, tissue biopsy is difficult to fully reflect the heterogeneity of the tumor, and cannot ideally predict the onset of resistance to targeted therapy[5]. As for imaging examination, it is difficult to find small metastatic lesions, which is lagging behind in monitoring the efficacy of chemotherapy and the resistance of targeted drugs[6]. Therefore, new methods are urgently needed to remedy the current shortcomings in order to improve the

screening, diagnoses and prognostic evaluation in lung cancer, and to achieve early prediction of treatment efficacy and dynamic monitoring of the condition.

Circulating tumor cells (CTCs) are tumor cells that enter the peripheral blood circulation spontaneously or by medical treatment caused. CTCs originate from the primary or metastatic tumor and can reflect the genetic information of tumor in real time[7]. Studies have shown that the detection of CTCs contributes to the early diagnosis of NSCLC, monitoring postoperative tumor recurrence and metastasis, and selecting individualized treatment strategies[8–10]. During the process of tumor cells detaching from the primary lesion into the blood circulation, most cells undergo epithelial-mesenchymal transition (EMT). Therefore, CTCs can be divided into epithelial CTCs, mesenchymal CTCs and epithelial-mesenchymal CTCs[11]. During EMT, the expression of epithelial genes such as epithelial cell adhesion molecule (EpCAM) and cytokeratins (CK) is down-regulated, while the expression of mesenchymal genes such as vimentin and twist is up-regulated[12]. Studies have also shown that a high proportion of mesenchymal CTCs predicts a worse prognosis for cancer patients, as well as a greater risk of metastasis, recurrence and drug resistance[13, 14]. Therefore, it is particularly important to further analyze the classification of CTCs based on the number of CTCs. By comparing both their changes, we can more comprehensively and accurately evaluate the tumor status, and achieve the accurate prognosis evaluation of NSCLC which will provide an important reference for the clinical treatment of NSCLC.

However, due to the scarcity of CTCs in the peripheral blood circulation and high individual heterogeneity, the sensitivity, specificity and efficiency of CTCs detection technology are highly challenged. Most of the methods currently available on the market can only detect epithelial CTCs and epithelial-mesenchymal CTCs with epithelial markers. Even CellSearch®, a CTCs testing organization approved by the US FDA, also miss out on the more migratory and infiltrating mesenchymal CTCs[8]. In a previous study, the optimized CanPatrol CTC enrichment technique was used to classify CTCs by using EMT markers in different types of cancers[15]. Therefore, here, we provide a more comprehensive and systematic data to explore the sensitivity and specificity of the latest CanPatrol™ technology for detection of CTCs in peripheral blood of NSCLC patients.

Materials And Methods

Study subjects. A total of 136 patients who admitted to the department of thoracic surgery of the first affiliated hospital of the Army Medical University from August 2015 to December 2015 were selected as the study subjects. The subjects were patients who had been diagnosed with NSCLC or pulmonary benign diseases through clinical manifestations, medical history and pathology. All the enrolled patients had no history of other malignancies and related anti-tumor treatments prior to participation in our study. Before surgical treatment, the peripheral blood samples of this study were performed within 2 weeks before and after the imaging examination.

Blood sampling and enrichment. 5 mL peripheral blood was collected using a blood collection needle No. 8 (WEGO, Shangdong, China) and an EDTA-containing anticoagulation blood collection tube (WEGO, Shangdong, China). The following pretreatments were performed within 4 h after blood sample collection. 15 ml of erythrocyte lysis was firstly added into the sample and mixed well. Then, placed at room temperature for 30 min to allow the erythrocytes were fully lysed. After centrifugation for 5 min the supernatant was discarded, 4 ml of PBS and 1 ml of RI fixative were added to fix the remain cells. The fixed cells were transferred to a filter tube (containing filter membrane: 8 μM pore size), and filtered up using a vacuum pump. The filtered cell samples were further fixed at room temperature for 1 hour by 4% formaldehyde.

Multiple mRNA in situ analysis. The fixed cell samples were treated with 0.1 mg/mL proteinase K to increase the cell membrane permeability. Next, specific capture probes (epithelial biomarker probe: EpCAM and CK8/18/19; mesenchymal biomarker probe: vimentin and twist; leukocyte marker: CD45) were added for hybridization. The sequence of these probes was listed in **supplementary table 1**. After incubating, the unbound probes were washed with 0.1 × SSC eluent

(Sigma, St. Louis, USA). Then incubated with the pre-amplification and the amplification solution to amplify the probe signal, and following incubated with three fluorescence-labeled probes at 40 °C. Namely, Alexa Fluor 594 (for epithelial biomarker probes EpCAM and ck8/18/19), Alexa Fluor 488 (for mesenchymal biomarker probes vimentin and twist) and Alexa Fluor 750 (for leukocyte marker CD45), and the sequence were listed in **supplementary table 2**. Finally, after staining nuclear with DAPI, the samples were observed using an automated fluorescence scanning microscope under a 100-fold field of view.

Positive criterion. Cell which has the number of fluorescence signal spot greater than or equal to 7 to be considered a valid count. Red fluorescence spot represents the epithelial marker expression, green fluorescence spot represents the mesenchymal marker expression. Both red and green fluorescence were observed represent the epithelial-mesenchymal type of CTCs (Table 1, Fig. 1).

Table 1
CTCs classification criteria.

| | Type | Red spot | Green spot | Gray spot | DAPI |
|---|------|----------|------------|-----------|------|
| CTCs | ⊠ | + | - | - | + |
| | ⊡ | + | + | - | + |
| | ⊢ | - | + | - | + |
| Type ⊠: epithelial CTCs, red fluorescence; Type ⊡: epithelial-mesenchymal CTCs, red and green fluorescence; Type ⊢: mesenchymal CTCs, green fluorescence. | | | | | |

Follow-up. A total of 98 NSCLC patients who underwent radical surgery were followed up by telephone or clinic. The follow-up contents were chest CT, abdominal color Doppler ultrasound, skull MRI, whole body bone scan, and PET-CT examination if necessary. The criteria for defining postoperative recurrence and metastasis in patients with lung cancer are imaging examinations suggesting that space-occupying lesions occur both inside and/or outside the lung. The follow-up period was 3 years and ended on December 31, 2018.

Statistical analysis. Data analysis and charting were performed using SPSS 25.0 (IBM, USA). Because of the CTCs levels were significantly skewed, the Kruskal-Wallis test was used for comparison between multigroup while the Mann-Whitney U test was used for comparison between the two groups. The inspection level was $\alpha = 0.05$. COX proportional hazard regression analysis was used to analyze the factors (staging, gender, age and smoking) affecting patients' survival, and the survival curve was plotted by Kaplan-Meier method. The cut-off value was determined by ROC curve.

Results

Patient characteristics. A total of 98 NSCLC patients were enrolled, including 65 males and 33 females, and the age distribution was between 18 and 82 years old (average age was 52 ± 9.3). There were 60 cases of lung adenocarcinoma, 33 cases of lung squamous cell carcinoma, and 5 cases of other NSCLCs. According to IASLC2009 (TNM staging standard for lung cancer, 2009, 7th edition), TNM staging was performed on the enrolled patients. Among them, 48 patients were stage I, 13 patients were stage II, 29 patients were stage III, and 8 patients were stage IV. There were 38 patients with benign lung diseases including 18 males and 20 females with the age distribution from 18 to 70 years (average age was 46 ± 11.7) (Table 2).

Table 2
Patients Characteristics and prevalence of circulating tumor cells.

| Characteristics | No. | CTCs(CTC Units/5ml) | | | | | | | | | | | |
|----------------------|-----|---------------------|---------|--------|------------|---------|--------|------------------|---------|-------|------------|------------|--------|
| | | Epithelial CTCs | | | Mixed CTCs | | | Mesenchymal CTCs | | | Total CTCs | | |
| | | M | P25-P75 | P | M | P25-P75 | P | M | P25-P75 | P | M | P25-P75 | P |
| Benign lung diseases | 38 | 0 | 0-0 | < 0.01 | 0 | 0-0 | < 0.01 | 0 | 0-0 | 0.013 | 0 | 0-0 | < 0.01 |
| NSCLC | 98 | 1 | 0-2 | | 1 | 0-3 | | 0 | 0-1 | | 3 | 1-6 | |
| Pathological type | | | | 0.845 | | | 0.528 | | | 0.904 | | | 0.579 |
| AC | 60 | 1 | 0-2.75 | | 1.5 | 0-3 | | 0 | 0-1 | | 3 | 1-6 | |
| SC | 33 | 1 | 0-2 | | 1 | 0-2 | | 2 | 0-0.5 | | 2 | 1-5 | |
| Others | 5 | 1 | 0.5-3 | | 2 | 0-3 | | 1 | 0.5-1 | | 2 | 1-7 | |
| TNM stage | | | | 0.850 | | | 0.954 | | | 0.505 | | | 0.926 |
| I | 48 | 1 | 0-2 | | 1 | 0-3 | | 0 | 0-1 | | 3 | 1-6 | |
| II | 13 | 1 | 0.5-3 | | 1 | 0-4 | | 0 | 0-1 | | 3 | 1-11 | |
| III | 29 | 1 | 0-2.5 | | 1 | 0-3 | | 0 | 0-0.5 | | 3 | 1-6 | |
| IV | 8 | 1.5 | 0-4.5 | | 1 | 0.25-5 | | 0.5 | 0-1.75 | | 3 | 0.25-12.75 | |
| Age | | | | | | | | | | | | | |
| ≤ 60y | 74 | 1 | 0-2 | 0.446 | 1 | 0-3 | 0.470 | 0 | 0-1 | 0.353 | 3 | 1-6 | 1 |
| > 60y | 24 | 1 | 0-2 | | 0 | 0-3.75 | | 0 | 0-0 | | 2.5 | 0-6 | |

Abbreviations: NSCLC, non-small cell lung cancer; AC, Adenocarcinoma; SC, Squamous carcinoma; CTCs, circulating tumor cells.

Comparison of the number of CTCs between groups. The number of all subtypes of CTCs and the total number of CTCs in NSCLC were higher than those in the benign lung disease group (Mann-Whitney U test: The U value of epithelial CTCs group was 822.5, $P < 0.01$; the U value of epithelial-mesenchymal CTCs group was 859, $P < 0.01$; the U value of mesenchymal CTCs group was 1487, $P = 0.013$; and the U value of total CTCs was 605.5, $P < 0.01$). There was no statistically significant difference in the number of CTCs between lung adenocarcinoma, lung squamous cell carcinoma and other NSCLC. According to the Kruskal-Wallis test, there was no statistically significant difference in the number of CTCs between TNM stages. Also, there was no significant difference in the number of CTCs between NSCLC patients at different ages (≤ 60 years or > 60 years) (Table 2).

ROC curve analysis to determine the cut-off value and assess the diagnostic performance. Taking the pathological results as standard, the ROC curve of the total number of CTCs in the NSCLC group was plotted comparing with those in the benign lung disease group (Fig. 2). The area under the curve (AUC) was 0.837, 95% CI was 0.76–0.914. The critical value

corresponding to the maximum value of Youden index was 0.5 CTC/5 mL. That was, when the number of CTCs ≥ 0.5 was considered as positive, the sensitivity was 81.6% and the specificity was 86.8%. Since the number of CTCs is an integer, CTCs ≥ 1 was considered as positive.

COX proportional hazard regression analysis. A total of 63 of the 98 NSCLC patients were effectively followed up for a period of three years. COX proportional hazard regression analysis revealed that tumor stage was a risk factor for recurrence and metastasis in NSCLC patients ($P = 0.006$), while gender, age and smoking were not risk factors for recurrence and metastasis ($P > 0.05$) (Table 3). The Exp(B) of tumor staging was 1.813, and the 95.0% CI was 1.186–2.772, indicating that for each upgrade of tumor stage, the risk of recurrence and metastasis was increased by 1.813times.

Table 3
COX proportional hazard regression analysis of follow-up information for 63 NSCLC patients.

| | <i>P</i> | Exp(B) | 95.0% CI for Exp(B) | |
|---------|----------|--------|---------------------|-------|
| | | | Lower | Upper |
| Stage | 0.006 | 1.813 | 1.186 | 2.772 |
| Smoking | 0.843 | 0.895 | 0.299 | 2.680 |
| Gender | 0.745 | 0.820 | 0.248 | 2.709 |
| Age | 0.517 | 0.985 | 0.941 | 1.031 |

Kaplan-Meier survival analysis.

Among the 63 followed up NSCLC patients, 14 patients with stage IIIA who underwent radical surgery and subsequent four rounds of adjuvant chemotherapy were divided into two groups according to the total number of CTCs (CTCs ≥ 1 , 10 cases and CTCs < 1 , 4 cases). Kaplan-Meier survival analysis results showed that the DFS (progression-free survival) of patients with total number of CTCs ≥ 1 was significantly lower than that of patients with total number of CTCs < 1 ($P = 0.022$) (Fig. 3).

Discussion

CTCs refer to tumor cells released into the peripheral blood by primary tumors and/or metastatic lesions. They are an important cause of postoperative recurrence and metastasis in patients with malignant solid tumors, as well as an important cause of tumor-related death. Therefore, detection of CTCs in peripheral blood is important for early diagnosis, efficacy and prognosis evaluation[8–10, 16]. however, due to the very limited number of CTCs in peripheral blood circulation, the heterogeneity of CTCs subtypes, and the easily aggregation into micro-plugs etc., the sensitivity, specificity and efficiency of CTCs detection technology are extremely challenged[17].

The key technology for CTCs detection is enrichment and identification. Currently, CTCs are sorted from other cells in the blood mainly through physical characteristics (such as the size, density, chargeability and deformability of CTCs, etc.) and biological characteristics (such as the cell surface antigen) [18]. Sorting CTCs according to physics characteristics is simple in operation and relatively low in cost, but cannot avoid the interference of individual heterogeneity, while sorting CTCs according to biological characteristics ensures the accuracy, but is limited by the types of cell surface expressed antigen. CTCs identification techniques include cell counting which is based on flow cytometry, and nucleic acid detection which is based on reverse transcriptase polymerase chain reaction. Cell counting method can quantitatively detect the number of CTCs and analyze various parameters of the CTCs (such as the size, morphology, intracellular and extracellular

biomarkers, as well as the genomic mutations), but the detection sensitivity is low and requiring a large volume of blood sample; The advantages of nucleic acid detection method are time saving, highly specific and requiring less blood samples, but this process inevitably destroy cell morphology and function, making further analysis impossible. In addition, due to the easy degradation of mRNA and the influence of non-specific amplification, the false positive rate increases[18–21]. The CellSearch system is currently widely recognized and used in the detection of lung cancer CTCs, which consists mainly of automated immunomagnetic separation systems and immunofluorescence analysis systems. The CTCs are isolated and enriched based on the EpCAM expression, but mesenchymal CTCs that had undergone epithelial-mesenchymal transformation could not be detected[8]. Therefore, currently, there is no ideal method for detecting CTCs in the peripheral blood of NSCLC patients.

The CanPatrol™ technology used in this study combined nanomembrane filtration technology and multiple RNA in situ analysis techniques to sort and identify CTCs. We used nanomembrane with a self-optimized pore size of 8µm to filter peripheral blood, so that the tumor cells in the peripheral blood were highly enriched. Previous studies have shown that the enrichment rate was as high as 89%, and the leukocyte removal rate was as high as 99.98% [22]. The advantage of this method is that it can completely sort all types of CTCs (epithelial, epithelial-mesenchymal and mesenchymal CTCs) without relying on specific biomarkers, and could be applied to enrich most of solid tumors' CTCs[15]. In addition, Canpatrol™ adopts a novel multiple mRNA in situ analysis method to hybridize the specific probes to the target gene, and further enhances the sensitivity and specificity of the detection through the fluorescence signal cascade amplification system. In this study, we compared CTCs in peripheral blood of patients with NSCLC and benign lung diseases. Statistical analysis showed that there were differences in the number of three subtypes of CTCs and total CTCs between the two groups. ROC curve analysis showed that the sensitivity and the specificity of CanPatrol™ technology for detection of peripheral blood CTCs in NSCLC was 81.6% and 86.8%, respectively. It can be concluded that this method has better diagnostic accuracy for NSCLC and has obvious diagnostic advantages compared with other methods. Additionally, as a non-specific physical enrichment technology, Canpatrol™ reduces the damage of tumor cells in peripheral blood preserving the original cellular information, such as morphology, cell function, molecular biology information, etc. Therefore, Canpatrol™ technology is beneficial for subsequent immunofluorescence, fluorescence in situ hybridization (FISH), gene expression, gene mutation detection, and microdissection based single cell sequencing analysis of CTCs. Moreover, this technology can also be used for cell culture and animal models to develop new drug and conduct the drug susceptibility testing, which would comprehensively and dynamically reveal tumor molecular information and guide the individualized treatment for cancer patients.

In this study, there was no statistically significant difference in the number of CTCs between lung adenocarcinoma, lung squamous cell carcinoma, and other NSCLCs which is consistent with previous studies[23, 24]. Theoretically, the higher stage of the tumor the greater number of CTCs[25]. Here, in our study, the number of all three subtypes CTCs and total CTCs increased with the increase of stage, but there was no statistical significance, which may be caused by too few cases (especially only 8 cases of stage IV). There was no statistical difference in the number of subtype CTCs and total CTCs between different ages (≤ 60 years or > 60 years), indicating that age is not a factor influencing CTCs, and our result is consistent with previous studies [23–25]. Through COX proportional hazard regression analysis of the follow-up data, we found that pathological stage is a risk factor for recurrence and metastasis which indicating that it is more scientific to plot the survival curve after risk screening and stratification. According to the ROC curve analysis and the cut-off value, the number of CTCs ≥ 1 was judged as positive. After a survival analysis of 14 patients with stage IIIA, we concluded that patients with NSCLC with total number of CTCs ≥ 1 have significantly lower DFS than patients with number < 1 , which is consistent with previous reports [23, 26]. Our data suggests that the number of total CTCs ≥ 1 in peripheral blood (5 ml) of NSCLC patients could predict the prognosis. However, it is necessary to expand the number of cases and extend the follow-up time to verify this conclusion.

In summary, CanPatrol™ has high sensitivity and specificity in detecting peripheral blood CTCs in NSCLC patients, which is of certain value in clinical diagnosis and prognosis.

Declarations

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Statement of Ethics

The study protocol has been approved by the Ethics committee of the First Affiliated Hospital of Third Military Medical University, PLA (2015).

Consent for publication

All patients signed an informed consent form and volunteered to participate in this study.

Disclosure Statement

The authors of this article declared they have no conflict of interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Author Contributions

Jingyao Li performed the Follow-up and analysis of the data. Yi Liao prepared the first draft of the manuscript. Yaling Ran and Guiyu Wang assisted in CTCs' enrichment and identification. Wei Wu, Yang Qiu, Jie Liu and Ningyu Wen help collected the blood samples. Tao Jing finalized the manuscript. Haidong Wang and Shixin Zhang instructed the study, as well as acquired funding to support the research.

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Figures

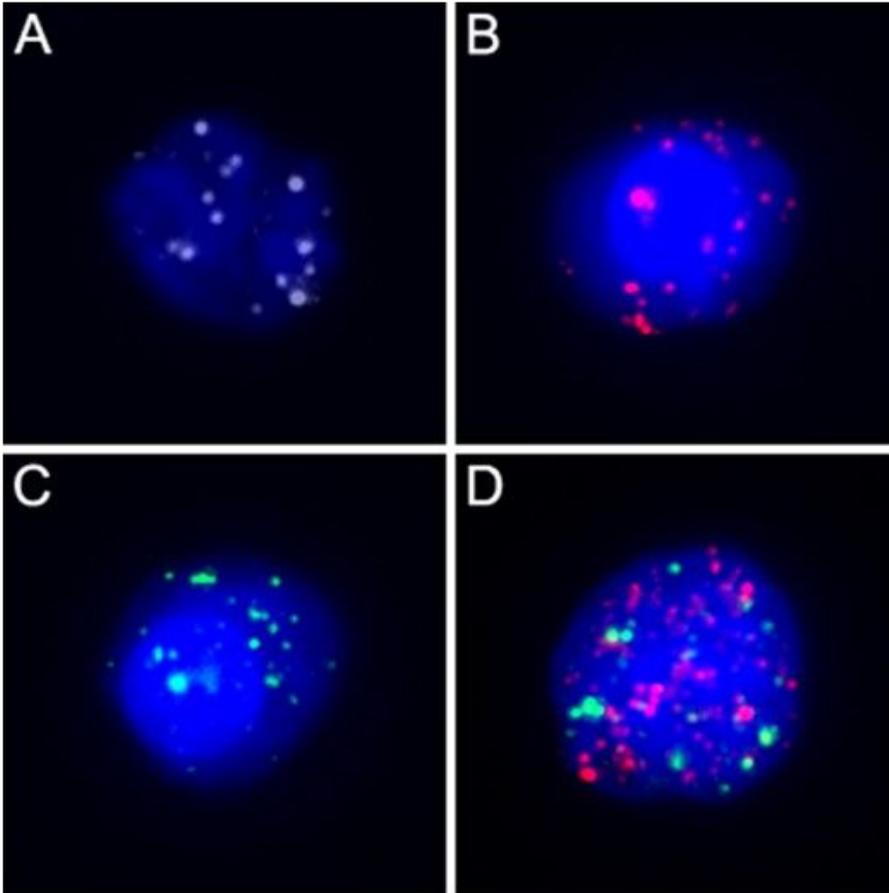


Figure 1

Fluorescence of CTCs. A. leukocyte. B. Type I CTCs (epithelial marker labeled, red fluorescence); C Type II CTCs (mesenchymal marker labeled, green fluorescence); D. Type III CTCs (epithelial and mesenchymal marker labeled, red and green fluorescence).

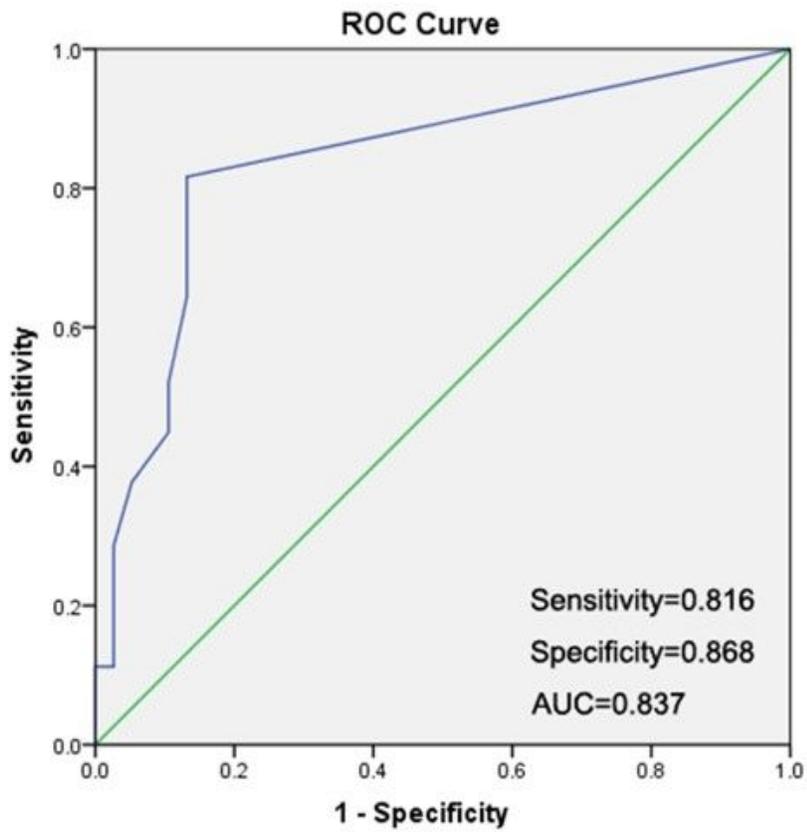


Figure 2

The ROC curve of CanPatrol™ technology-based CTCs of NSCLC.

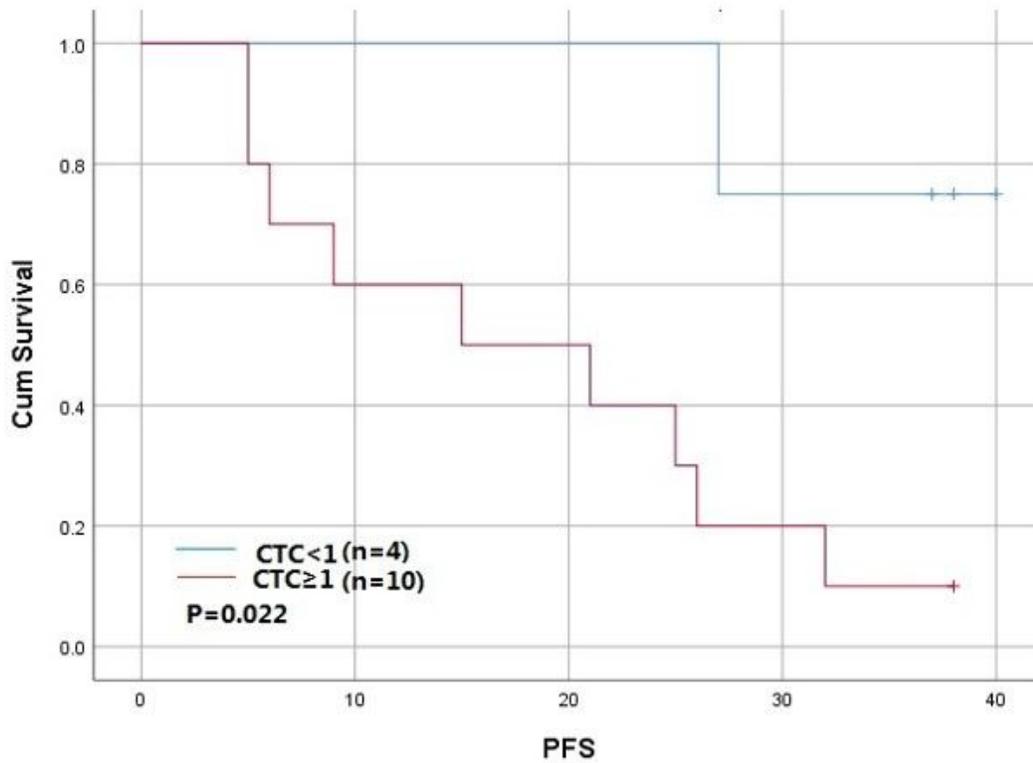


Figure 3

Survival curve of the stage IIIA NSCLC patients.

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