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Analysis of diagnostic value of CTC and CTDNA in early lung cancer

Hongwei Yao[1*], Lixin Wen[1], Ziyan Li[2], Chunwei Xia[3*]

[1] Department of Respiratory Medicine, Changzhou Cancer Hospital, Changzhou 213001, China
[2] Department of Pathology, Changzhou Cancer Hospital, Changzhou 213001, China
[3] Department of Respiratory Medicine, Affiliated Nanjing Brain Hospital, Nanjing Medical University, Nanjing 21000, China

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ABSTRACT

This paper is the exploration and analysis of the diagnostic value of circulating tumor cells (CTC) and circulating tumor deoxyribonucleic acid (ctDNA) in early non-small cell lung cancer (NSCLC).A total of 125 NSCLC patients that hospitalized from March 2019 to December 2020 were selected, of which 64 subjects with stage I-II were listed as the early-stage group, and 61 patients with stage III-IV were classified as the intermediate-advanced group; In addition, 47 patients with benign pulmonary nodules hospitalized within same period were selected as a group of benign pulmonary nodules, and 50 healthy subjects were enrolled as a control group. The levels of CTC and ctDNA, serum tumor markers carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), cytokeratin 21-1 (CYFRA21-1) and gastrin-releasing peptide precursor (pro-GRP) were detected. The diagnostic value of CTC and ctDNA in early NSCLC patients was analyzed by ROC curve. The area under the curve of CTC count for early-stage lung cancer was 0. 949 (P=0. 000, 95%CI: 0. 918-0. 980), and ctDNA was 0. 914 (P=0. 000, 95%CI: 0. 866~0. 963). There were significant differences in CTC count and ctDNA among subjects in each group (*P<0. 05*). CTC count and ctDNA of the early and intermediate-advanced group were remarkably higher than those of the control group and group of benign pulmonary nodules (*P<0. 05*), and CTC count and ctDNA of patients in the middle-late group were critically higher than those in the early-stage group (*P<0. 05*). There were significant differences in serum CEA, NSE, CYFRA21-1 and pro-GRP levels among all groups (*P<0. 05*). The results showed that CTC and ctDNA in NSCLC patients were significantly positively correlated with CEA, NSE, CYFRA21-1 and pro-GRP (*P<0. 05*). Peripheral blood CTC and ctDNA levels are significantly increased in NSCLC patients. They are positively correlated with serum tumor markers CEA, NSE, CYFRA21-1 and pro-GRP levels, and are related to tumor progression. CTC and ctDNA have high value in the clinical diagnosis of early NSCLC.

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Introduction

Lung cancer is the most common malignant tumor worldwide. There are nearly 1. 8 million newly increased every year, and 1. 4 million patients die every year, making it the leading cause of death of Chinese residents due to malignant tumors (1). Lung cancer falls into small cell lung cancer and non-small cell lung cancer (NSCLC) according to pathological classification, among which NSCLC takes the proportion of 85% of lung cancer (2). Due to the atypical early clinical symptoms, most sufferers were in the advanced stage when clinically diagnosed, and lost the chance of surgical resection, thus leading to a low 5-year overall survival rate (3,4). Circulating tumor cells (CTCs) refer to tumor cells that exist outside primary cancer and metastases and are mainly derived from the continuous proliferation of solid tumor cells (5). CTCs have certain invasiveness. After escaping from tumor tissue, the cells entered the circulation system and became circulating tumor cells with the ability to invasion and metastasis. Circulating tumor deoxyribonucleic acid (ctDNA) is a DNA fragment that carries the original tumor mutation in the circulatory system. It is repeatable, less invasive, real-time monitoring, and can reveal the genetic characteristics of tumors at the molecular level (6). It was found that the tumor cells and ctDNA could be released into the peripheral blood in the early stage of tumor formation. Qualitative and quantitative analysis of CTC and ctDNA in the circulatory system has been found to be associated with the diagnosis, progression, follow-up and prognosis of tumors, and can be used as an alternative to “liquid biopsy” of primary tumors. This study investigated and analyzed the diagnostic value of CTC and ctDNA in early NSCLC. The following reports are presented.

Materials and Methods

Clinical data

125 NSCLC subjects hospitalized from March 2019 to December 2020 were enrolled for TNM staging according to the 2017 UICC staging criteria. Among them, 64 patients with stage I-II were classified into the early group, and 61 with stage III-IV were selected as an intermediate-advanced group; In addition, 47 patients with benign pulmonary nodules hospitalized within the same period were listed as a group of benign pulmonary nodules, and 50

* Corresponding author. Email: klmyt77@163. com; xiachunwei@njmu. edu. cn

healthy subjects were enrolled as a control group. There were 33 males and 31 females in the early group. They were aged between 36 and 89 years, with an average of (56. 17±8. 39) years. There were 32 males and 29 females in the intermediate-advanced group, aged 36-78 years, with an average age of (56. 93±9. 56) years. In the group of benign pulmonary nodules, there were 25 males and 22 females aged 29-81, and the evaluation age was (57. 02 ± 11. 21) years. There were 27 males and 23 females in the control group, with an average age of (55. 97±8. 95) years (ranged 36-76 years). There was no significant difference in gender and age among the three groups (*P>0. 05*). The study was approved by the Ethics Committee of our hospital.

Inclusion and exclusion criteria

Inclusion criteria: NSCLC group: Subjects that were pathologically confirmed as NSCLS by bronchoscopic biopsy or skin-lung puncture biopsy, and had received surgery, radiotherapy or chemotherapy. Group of benign pulmonary nodules: The patient's chest CT showed lung nodule lesions, and pathologically confirmed no basis for nausea. Control group: The patient's chest CT showed no definite lung lesions, such as tuberculosis, pneumonia, diffuse lesions, and bronchiectasis. All subjects in the four groups signed the informed consent voluntarily.

Exclusion criteria: Patients accompanied by primary malignant tumors other than NSCLS. Those with severe heart, liver and/or renal insufficiency. Subjects that are unable to undergo tracheoscopy.

Methods

Acquisition of blood samples: 5ml of early morning peripheral fasting venous blood of the four groups was collected and stored in ACD anticoagulant vacuum blood collection vessels at 4℃ refrigerator for measurement, and CTC count detection was completed within 24h. The 5ml peripheral blood was placed in the procoagulant tube for 10min, centrifuged for 10min (3500r/min) to separate the serum, and subsequently placed in the refrigerator at -80℃ for a later test.

Detection of peripheral blood CTCs: Peripheral blood CTCs were combinative and detected by Lyle's Immunomagnetic Beads Negative Enrichment Method and Fluorescence in situ hybridization (FISH). The peripheral blood was washed with CS1 buffer and centrifuged for 5 min (650 × g); then added with CS2 erythrocyte lysate, centrifuged for 5 min (650×g), discarded the supernatant; CS1 buffer was added again to resuspended cells, magnetic beads were added to negative enrichment of white blood cells, and finally, CF1 fixative was added for fixation and drying. The slides were fixed with CF1 and left at 37℃ with 2×SSC for 10min. The slides were dehydrated with 75%, 85% and 100% ethanol solutions for 2-5 min in turn. 10 μl CEP8 probe was added to the sample area, denatured at 76℃ for 5min, hybridized for 1. 5h, soaked in formamide for 15min, and washed twice with 2×SSC for 5min each. The antibody-containing CD45-AF594 fluorescent antibody was also added to the specimen area and incubated at room temperature in the dark for 1h and counterstained with DAPI staining solution after washing. Finally, the images of the cellular areas were analyzed by fluorescence microscope. The criteria for a positive interpretation of CTCs: cells with DAPI+/CD45-/chromosome polyploidy are identified as CTC, the number of CTCS ≥2, then it was considered as positive CTC.

Detection of ctDNA in peripheral blood: A DNA sequencing library was constructed by QIAamp Circulating Acid Kit (Qiagen, Germany). The preliminary quantification was by Qubit 2. 0 fluorometer. The fragment length was inserted by Agilent 2100 bioanalyzer (Agilent, USA), and the effective concentration of the library was detected by real-time PCR. Finally, ctDNA was detected by next-generation sequencing (NGS).

Test of serum tumor markers: One serum sample was taken and the levels of carcinoembryonic antigen (CEA), neuron-specific enolase (NSE) and cytokeratin 21-1 (CYFRA21-1) in serum were detected by electrochemiluminescence (detection reagent was purchased from Roche reagent of Germany). Gastrin-releasing peptide precursor (pro-GRP) was gauged by enzyme-linked immunosorbent assay (the detection kit was purchased from Roche, Germany). The technical process was complied with kit instruction manual.

Statistical analysis

The statistical analysis was conducted by SPSS 25. 0. The measurement data were expressed as ($\bar{x}$±s). The comparison of measurement data among multiple groups was performed by variance analysis, and the post-hoc test was performed by LSD-t test. Enumeration data were expressed as a percentage. The comparison of multiple groups of counting data was conducted by the rank-sum test, and that of the count data of two groups was by the chi-square test. Correlation analysis was performed by Pearson correlation analysis, and the diagnostic value of CTC and ctDNA for early lung cancer was analyzed by ROC curve. *P<0. 05* was considered statistically significant.

Results

Comparison of CTC count and ctDNA

There were significant differences in CTC count and ctDNA among the subjects in each group (*P<0. 05*). The degree of CTC count and ctDNA of the early and intermediate-advanced group were remarkably higher than those of the control group and group of benign pulmonary nodules (*P<0. 05*), and CTC count and ctDNA in the intermediate-late group were higher than those in the early-stage group (*P<0. 05*)(Table 1).

Comparison of serum tumor markers

There were significant differences in serum CEA, NSE, CYFRA21-1 and pro-GRP among all groups (*P<0. 05*). CEA, NSE, Cyfra21-1 and pro-GRP in early and intermediate-advanced NSCLC patients were higher than those in the control group and benign pulmonary nodules group (*P<0. 05*), and the parameters in the intermediate-advanced group were higher than those in early-stage patients (*P<0. 05*)(Table 2).

The connection between CTC&ctDNA and clinical features of NSCLC

There was no connection between CTC&ctDNA and gender, age and pathological type of NSCLC (*P>0. 05*), while CTC&ctDNA was correlated with TNM stage (P<0. 05)(Tables 3 and 4).

Table 1. Comparison of CTC and ctDNA in each group ($\bar{x}$±s).

Group	Number of Cases	CTC count (FR/ml)	CtDNA (ng/ml)
Control group	50	0. 12±0. 33	27. 45±5. 26
Group of benign pulmonary nodules	47	0. 13±0. 34	29. 14±6. 21
Early-stage group	64	2. 84±0. 84*#	103. 25±15. 64*#
Intermediate-advanced group	61	7. 26±2. 14*#Δ	185. 25±33. 95*#Δ
F	-	419. 287	772. 427
P	-	0. 000	0. 000

Note: Compared with the control group, *$P<0. 05$; Compared with the benign pulmonary nodules group, #$P<0. 05$; Compared with the early-stage group, Δ$P<0. 05$.

Table 2. Comparison of tumor markers ($\bar{x}$±s).

Group	Number of Cases	CEA (ng/ml)	NSE (ng/ml)	Cyfra21-1 (ng/ml)	pro-GRP (pg/ml)
Control group	50	2. 11±0. 33	8. 34±1. 48	2. 72±0. 58	13. 54±3. 47
Benign pulmonary nodules group	47	2. 25±0. 46	8. 65±1. 67	2. 81±0. 69	14. 62±4. 10
Early-stage group	64	25. 54±3. 15*#	27. 67±5. 86*#	9. 15±1. 35*#	105. 47±20. 54*#
Intermediate-advanced group	61	37. 58±8. 75*#Δ	36. 35±7. 39*#Δ	13. 55±2. 14*#Δ	126. 52±30. 25*#Δ
F	-	710. 667	412. 607	781. 397	507. 393
P	-	0. 000	0. 000	0. 000	0. 000

Note: Compared with the control group, *$P<0. 05$; Compared with the benign pulmonary nodules group, #$P<0. 05$; Compared with the early-stage group, Δ$P<0. 05$.

Table 3. Connection between CTC and clinical features of NSCLC.

Clinical features	Number of cases	CTC count (FR/ml)	t	*P*
Gender				
Male	65	5. 12±2. 96	0. 521	0. 603
Female	60	4. 87±2. 49		
Age (years)				
<60	79	4. 87±2. 60	0. 676	0. 500
≥60	46	5. 22±2. 97		
The pathologic types				
Adenocarcinoma	88	5. 05±2. 72	0. 285	0. 776
Squamous cell carcinoma	37	4. 89±2. 81		
TNM-staging				
Stage I	23	2. 17±0. 58	169. 203	0. 000
Stage II	41	3. 22±0. 72		
Stage III	21	5. 29±1. 65		
Stage IV	40	8. 30±1. 57		

Table 4. Connection between ctDNA and clinical features of NSCLC.

Clinical features	Number of cases	ctDNA (ng/ml)	t/F	*P*
Gender				
Male	65	144. 75±52. 44	0. 351	0. 726
Female	60	141. 67±44. 77		
Age (years)				
<60	79	141. 00±50. 43	0. 680	0. 498
≥60	46	147. 16±45. 97		
The pathologic types				
Adenocarcinoma	88	144. 29±48. 68	0. 360	0. 720
Squamous cell carcinoma	37	140. 84±49. 47		
TNM-staging				
Stage I	23	95. 95±18. 34	109. 871	0. 000
Stage II	41	107. 35±12. 34		
Stage III	21	174. 71±34. 71		
Stage IV	40	190. 79±32. 63		

Table 5. Correlation between serum tumor markers and CTC&ctDNA in NSCLC.

Indicators	Statistics	CEA	NSE	Cyfra21-1	pro-GRP
CTC	R	0. 734	0. 718	0. 793	0. 419
	P	0. 000	0. 000	0. 000	0. 000
ctDNA	R	0. 634	0. 586	0. 728	0. 333
	P	0. 000	0. 000	0. 000	0. 000

Correlation Analysis

The correlation between CTC&ctDNA and serum tumor markers in NSCLC was analyzed. The results showed that CTC and ctDNA in NSCLC patients were significantly positively correlated with CEA, NSE, CYFRA21-1 and pro-GRP (*P<0. 05*)(Table 5 and FIG. 1).

ROC curve analysis of the diagnostic value of CTC&ctDNA in early lung cancer

The diagnostic value of CTC and ctDNA was analyzed in the early-stage group, the benign pulmonary nodules group and the control group. The results revealed that the under-curve area of CTC for early-stage lung cancer was 0. 949 (P=0. 000, 95%CI: 0. 918-0. 980), ctDNA was 0. 914 (P=0. 000, 95%CI: 0. 866~0. 963)(FIG. 2).

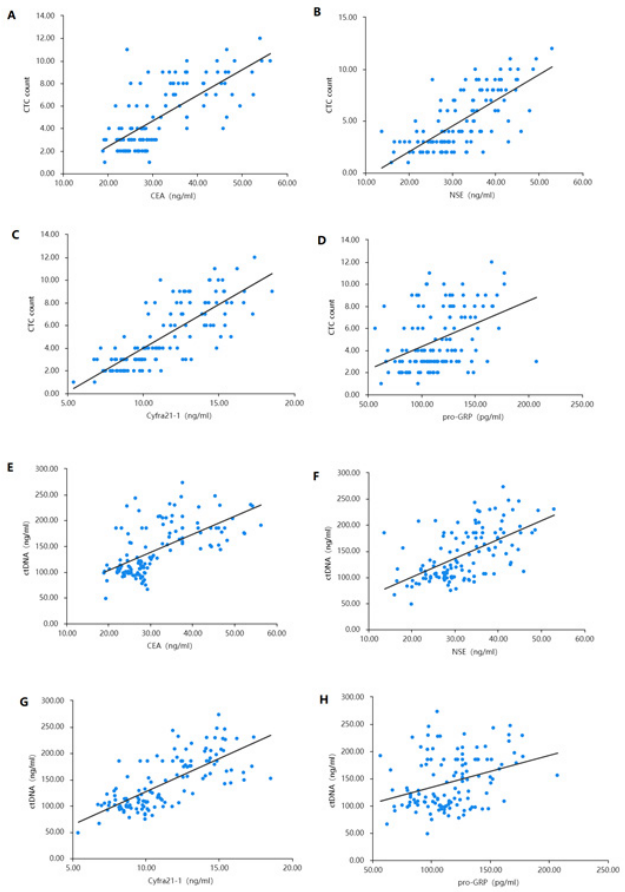

Figure 1. Correlation analysis. A: Correlation between CTC count and CEA, r=0. 734, P=0. 000. B: Correlation between CTC count and NSE, r=0. 718, P=0. 000. C: Correlation between CTC count and Cyfra21-1, r=0. 793, P =0. 000. D: Correlation between CTC counts and pro-GRP, r=0. 419, P=0. 000. E: Correlation between ctDNA and CEA, r=0. 634, P=0. 000. F: Correlation between ctDNA and NSE, r=0. 586, P=0. 000. G: Correlation between ctDNA and Cyfra21-1, r=0. 728, P=0. 000. H: Correlation between ctDNA and pro-GRP, r=0. 333, P=0. 000.

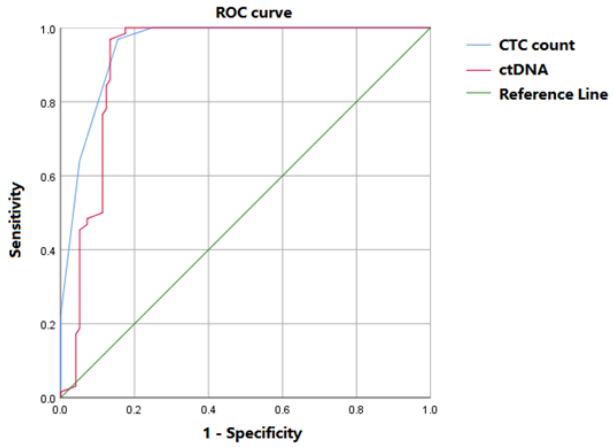

Figure 2. CThe diagnostic value of CTC&ctDNA in early lung cancer by ROC curve analysis.

Discussion

Epidemiological investigations show that the morbidity and mortality of lung cancer in China currently are the first among malignant tumors, and the number of cases is still increasing year by year (7). Lung cancer is also the malignant tumor with the highest mortality worldwide, and about 1. 6 million patients die each year due to it (8, 9). Lung cancer includes small cell lung cancer and non-small cell lung cancer (NSCLC), of which over 80% of patients belong to the latter. Attention has been paid to early screening and early diagnosis and treatment all over the world, aiming to reduce the mortality of patients. Although the abundant work has brought certain achievements, many early-stage lung cancer patients are still missed in clinical work, which results in a high mortality rate (10). The main reasons are as follows. Firstly, although the low-dose CT currently adopted for early screening has a certain effect on the diagnosis, it has a high false-positive rate and false-negative rate, which leads to a large number of missed or misdiagnosed patients (11). Some patients, therefore, miss the best time for treatment. Secondly, although the application of serum tumor markers (a serological test including CEA, NSE, Cyfra21-1, pro-GRP, etc.) can reflect the tumor situation of patients to a certain extent, it has the defects of low sensitivity, specificity and accuracy. Finally, the most important current diagnosis of NSCLC is to rely on the cytotype of tumor tissue biopsy (12,13). However, the acquisition of biopsy tissue requires invasive operations such as CT-guided biopsy, bronchoscopic biopsy, and surgical resection, and the incidence of complications after biopsy is high. At the same time, patients' willingness to biopsy is usually low. In addition, sometimes due to the poor location of the tumor, such as being close to the blood vessels and heart, the biopsy operation is difficult and the number of tissues taken is small, which can generate to false negative outcomes. The results of pathological detec-

tion are also easily affected by internal and external factors such as environment and tumor heterogeneity, which also limits the application of tissue biopsy in the diagnosis of early NSCLC.

With the development of medicine, the individualized medical model with precise diagnosis and precise treatment as the core has gradually attracted attention (14). Since tumor metabolites can be present in body fluids, liquid biopsies, especially peripheral blood, have emerged as a source of biomarker analysis for lung cancer. Among them, CTC and ctDNA are the most important markers (15,16). CTCs are various tumor cells existing in peripheral blood, which primarily come from the shed solid tumor lesions that enter the peripheral blood. Most of the CTCs will self-apoptosis or be phagocytosed by phagocytes, but a small part will gradually progress to metastases, proliferate, differentiate and fall off in the body, eventually leading to an increase in the cell content of peripheral blood NSCLC (17,18). ctDNA is DNA that is derived from tumor tissue in peripheral blood. It covers all the genetic information of tumor tissue and can be elevated in the early stage of the tumor (19). CTC and ctDNA are regarded as a new generation of tumor markers due to their non-invasive operation, convenient and repeatable sampling, and high sensitivity and specificity (20). This study investigated the diagnostic value of CTC and ctDNA in early lung cancer. According to the above studies, there were significant differences in CTC count and ctDNA among groups. The CTC count and ctDNA degree of the early and intermediate-advanced group were higher than those of the control group and group of benign pulmonary nodules, and patients in the intermediate-late group had notably higher parameters than the early-stage group. Similar to the results of scholars' studies, CTC counts and ctDNA levels can be significantly increased in the peripheral blood of NSCLC patients, and they can appear in early-stage patients (21,22). This provides a relevant basis for the application of clinical diagnosis of early NSCLC.

CEA is one of the acidic glycoproteins and is widely present on the surface of tumor cells. CEA level can reflect the number of tumor cells in the body to a certain extent[23]. NSE is an acidic protease that is unique to neuroendocrine cells and neurons. NSE can promote neuroendocrine and has a high expression level in the blood circulation of NSCLC patients. It has currently been widely adopted as one of the diagnostic markers of lung cancer (23,24). Cyfra21-1 is synthesized and secreted by tumor cells in the process of differentiation. It is a tumor marker of lung cancer widely used in clinical practice, and is mainly expressed in epithelial cells (25). pro-GRP is a gastrointestinal hormone that mediates smooth muscle cell contraction by stimulating gastric G cells to secrete gastrin, thereby affecting cell-cell interactions. It is indirectly involved in the process of tumor proliferation and differentiation (26). The relevance of CTC and ctDNA and the above four tumor markers in NSCLC were analyzed in this research. It showed that CTC and ctDNA in NSCLC patients were significantly positively correlated with CEA, NSE, Cyfra21-1 and pro-GRP. This may be because CEA, NSE, Cyfra21-1 and pro-GRP can increase with the occurrence, development and content of tumor cells in NSCLC. Meanwhile, the proliferation, differentiation and metastasis of tumor cells can lead to an increase in tumor shedding, which leads to the increase of CTC and ctDNA. Therefore, CTC&ctDNA is positively correlated with the number of tumor abscission (27-30).

This study conducted ROC curve analysis on subjects in the early-stage group, benign lung nodule group and control group in order to further analyze the value of CTC&ctDNA in early-stage NSCLC. It turned out that the under-curve area of CTC count in early lung cancer was 0. 949, and that of ctDNA was 0. 914, which proved the attribute of this diagnostic value.

In summary, peripheral blood CTC and ctDNA levels are significantly increased in NSCLC patients. They are positively correlated with CEA, NSE, CYFRA21-1 and pro-GRP levels, and are related to tumor progression. CTC and ctDNA have high value in the clinical diagnosis of early NSCLC.

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