Simultaneous in Situ Detection of Protein Expression of Multiple Tumor Markers of CTC and Heteroploid of Chromosome 8 in Primary Lung Cancer

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Research

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Abstract

Background

Past studies have shown that circulating tumor cells (CTCs) play an important role in the clinical staging, efficacy monitoring and prognosis evaluation of lung cancer.

Methods

In this study we investigated the expression of CK18 and Vimentin on the surface of CTC and the aneuploidy of chromosome 8 in peripheral blood of 24 patients with metastatic primary lung cancer which were detected by subtraction enrichment and immunofluorescence in situ hybridization (SE-iFISH), and their correlation with clinicopathological features, curative effect and prognosis was analyzed.

Results

The positive rate of CTC was 95.83% (23/24). There was a certain correlation between the positive rate of CTC and smoking and a correlation between the number of CTC and the histopathological type of patients. The number of monoploid, diploid, triploid and polyploid CTC had no statistical correlation with progression-free survival (PFS) or overall survival (OS). However, a tetraploid CTC count of two or more was an unfavorable predictor of response and a tetraploid CTC count ≥ 1 is an unfavorable prognostic predictor of poor OS. The positive rate of CK18 + CTC in all 24 patients was 4.35% (1/23). Meanwhile, 7 out of these 24 patients were also tested for Vimentin, and the positive rate of Vimentin + CTC was 85.71% (6/7). Small-cell (≤ 5μm) CTC was found in 6 of these 7 patients and it accounted for 11.83% (11/93) of the total CTC. The tumor marker phenotype of small-cell CTC was CK18 - Vimentin +. In addition, circulating tumor microthrombi (CTM) were found in 2 of these 7 patients (28.57%).

Conclusion

SE-iFISH has a high detection rate of CTC in peripheral blood of patients with metastatic primary lung cancer, and it can identify small-cell CTC. Tetraploid CTC count ≥ 2 can predict poor PFS in patients with advanced lung cancer. Tetraploid CTC count ≥ 1 may predict poor OS in patients with advanced lung cancer. CTM can predict poor prognosis in patients with lung cancer.

Background

Lung cancer (LC) is a serious threat to human health, and its morbidity and mortality rank first in malignant tumors. The main treatment of lung cancer includes surgery, chemotherapy, radiotherapy, molecular targeted therapy, anti-angiogenesis therapy and immunotherapy. Sensitive and effective biomarkers in the course of treatment can evaluate the efficacy, monitor the progress of disease and

predict the prognosis of disease in real time, and then provide a basis for individualized and precise treatment of lung cancer.

At present, the evaluation of curative effect of lung cancer is mainly based on imaging and serum tumor markers, but imaging has a certain lag, and the sensitivity and specificity of serum tumor markers are poor. For example, the sensitivity of carcinoembryonic antigen (CEA) in the diagnosis of lung cancer is only about 40% [1, 2], and there are some false negative and false positive. CEA in the evaluation of efficacy and prognosis of lung cancer is still controversial.

With the emergence of "liquid biopsy" technology, circulating tumor cells (CTC) have become an important means of lung cancer evaluation. CTCs are malignant tumor cells which enter the circulating blood after shedding from primary tumor or metastatic tumor and have the characteristics of tumor genes or tumor antigens [3]. CTCs are mainly found in the blood of patients with malignant tumors. It is rare in the blood of patients with benign diseases and healthy people [4]. More and more studies show that CTCs play an important role in the metastasis of lung cancer[5, 6]. Researchers found that CTCs can form circulating tumor microthrombi (CTM ≥ 2 CTCs) have stronger viability and potential to form micrometastasis than single CTCs, so CTMs are risk factors for tumor metastasis [7].

The detection method of CTC is mainly composed of two main links: separation and identification. The sensitivity and specificity of CTC detection are determined by the effectiveness of the separation and identification methods. The biggest difference between subtraction enrichment technology (SE) and negative enrichment method is that SE uses special non-blood-derived cell separation medium to remove red blood cells instead of lysing red blood cells by hypotonicity. In the process of leukocyte elimination of 6 to 8.5 mL blood sample, a combinatorial population of multiple anti-leukocyte antibodies, rather than a single anti-CD45 antibody, is coupled to specially coated magnetic beads, thereby ensuring maximal specific removal of leukocytes and minimal non-specific adhesion to tumor cells. The CTC enriched by SE has no hypotonic damage and is not combined with the antibody, so it keeps good biological activity. The SE method does not depend on the expression of tumor cell surface markers, therefore, the tumor cells enriched by it are not limited by cell size and highly heterogeneous expression of tumor markers [8, 9]. In 2009, it was reported for the first time that the application of SE technology could effectively detect CTC in 86% of recurrent lung cancer patients [10].

Chromosomal instability (CIN) exists in tumor cells, and aneuploid cells can exist in both primary and metastatic lesions [11]. According to reports, chromosome 8 aneuploidy in CTCs is associated with progression-free survival (PFS) and overall survival (OS) in lung, breast, ovarian and oral cancers [12–14]. It has been extensively reported that chromosome 8 centromere probes (CEP 8) have been applied to the diagnosis of lung cancer, esophageal cancer, pancreatic cancer, gastric cancer, colorectal cancer, bladder cancer and liver cancer [6, 15–20].

Immunofluorescence in situ hybridization (iFISH) is a novel detection method that combines immunofluorescence staining with FISH, which can effectively detect non-hematogenous aneuploid CTC [21]. The targeted non-hematogenous cells (CD45 negative) were detected for chromosomal aneuploidy

by FISH and various tumor markers by immunofluorescence staining. Different from the conventional cytokeratin (CK) single-tumor-labeled fluorescence staining for identifying CTC, iFISH can be used on the basis of FISH and furthermore provide a dual-color double-tumor-labeled protein expression detection which can be freely selected (such as CK, EpCAM, HER-2, Vimentin, CD133, CA19-9 and the like) and combined (E.g. CK + EpCAM, HER-2 + EpCAM, Vimentin + EpCAM etc.).

It has been demonstrated that EpCAM-independent iFISH can identify CTC with high sensitivity through surface marker expression and ploidy analysis [22], a sensitivity of 92% in lung cancer [23]. On this basis, combining with SE technology can find more CTCs, especially CK negative CTCs with epithelial-mesenchymal transition (EMT) [24]. Benefiting from the advantages of SE combined with iFISH, we can identify and count the enriched tumor cells to explore its potential clinical value, especially in the evaluation of curative effect and prognosis of lung cancer.

CK18 is a structural protein which highly expresses in epithelial cells. Vimentin is the most commonly used molecular marker of epithelial-mesenchymal transition (EMT), and its expression reflects the degree of EMT. Its number is positively correlated with the degree of malignancy and poor prognosis of tumors [25].

This study integrated phenotypic analysis of CTC and karyotypic analysis of chromosome 8, and analyzed the chromosome ploidy of CTC in peripheral blood of patients with lung cancer based on different copy numbers of chromosome 8. The expression of cytokeratin 18 (CK18) and Vimentin on the surface of tumor cells was detected independent of epithelial cell adhesion molecule (EpCAM). We wantde to investigate the significance of simultaneous in situ detection of multiple tumor marker protein expression and chromosome 8 aneuploid of CTC in the diagnosis and treatment of primary lung cancer.

Methods

Clinical Data and Sample Collection

From July 1, 2017 to August 18, 2017, 24 patients with locally advanced or metastatic primary lung cancer (stage IIIB-IVB) were diagnosed by pathology in the Department of Respiratory and Critical Care Medicine of Northern Jiangsu People's Hospital. There were 16 males and 8 females. The age ranged from 42 to 77 years with a median age of 65 years. Histopathological classification: 17 adenocarcinoma cases, 4 squamous cell carcinoma cases and 3 small cell lung cancer cases. This study was approved by the Ethics Committee of Northern Jiangsu People's Hospital. In accordance with the principles of the Helsinki Declaration. All patients signed a written informed consent.

This study collected the following clinical data: gender, age, smoking history, histopathological type, EGFR gene mutation, ECOG score, serum CEA, imaging data, first-line treatment, progression-free survival (PFS), overall survival (OS).The clinical staging of non-small cell lung cancer (NSCLC) was performed according to the UICC/AJCC 8th edition TNM staging system. Staging criteria for small cell lung cancer

(SCLC) are established by the International Association for the Study of Lung Cancer (IASLC). It is divided into limited stage and extensive stage.

According to the histological type and EGFR gene mutation, the first-line treatment was determined. Among 17 patients with adenocarcinoma, 4 patients with EGFR mutation (+) were treated with EGFR-TKI. The rest 13 cases were detected EGFR mutation (-), 11 patients were treated with platinum-based chemotherapy (AP or TP), and 2 patients were not treated. All 4 squamous cell carcinoma patients with EGFR mutation (-) were treated with platinum-based chemotherapy. EGFR was not detected in 3 patients with SCLC. 2 cases were treated with EP regimen and 1 case was not treated. Peripheral blood samples were taken to detect serum CEA before treatment, and patients were followed up regularly to calculate PFS and OS(Table 1). RECIST Version 1.1 was used to evaluate the efficacy in solid tumors for objective assessment of clinical response.

Peripheral venous blood (6 ml) from each subject was collected into an EDTA anticoagulated tube and all samples were processed within 5 hours of collection.

Table 1
Summary of serum CEA, first-line treatment plan and survival data of 24 selected patients

Case No.	Pathological type	Clinical stage	EGFR mutation	ECOG score	Serum CEA	First-line treatment	PFS (m)	OS (m)
H1	adenocarc.	T2N0M1a	(-)	1	17.49	TP	8	12
H2	adenocarc.	T3N3M1a	(-)	0–1	60.04	AP	10	26
H3	adenocarc.	T2N2M1a	19(+)	0	6.36	TKI	10	25
H4	squa.carc.	T3N0M1a	(-)	0	8.77	GP	9	29
H5	SCLC	ES	No detection	3	21.21	no treatment	1	1.5
H6	adenocarc.	T1N0M1c	(-)	0	> 60	AP	1	8.5
H7	SCLC	ES	No detection	1	32.5	EP	9	15
H8	SCLC	ES	No detection	1	normal	EP	3	5
H9	squa.carc.	T2N2M1a	(-)	2	30.27	TP	5	5
H10	adenocarc.	T4N2M1a	(-)	3	normal	no treatment	2	2
H11	squa.carc.	T3N2M1a	(-)	0	normal	GP	4	8
H12	adenocarc.	T4N3M1c	(-)	3	16.32	no treatment	1	11
H13	adenocarc.	T4N1M1a	(-)	0	81.83	AP	4	14
H14	adenocarc.	T1N0M1a	(-)	0	normal	AP	3	6
H15	adenocarc.	T2N2M1a	(-)	0	16.97	AP	16.5	18
H16	adenocarc.	T4N3M1c	19(+)	3	normal	TKI	5	5
H17	adenocarc.	T1N3M1a	(-)	1	162.9	AP	10	28
B1	adenocarc.	T3N3M1c	(-)	1	normal	AP	8	11
B2	adenocarc.	T4N2M1a	21(+)	1	normal	TKI	11	19
B3	squa.carc.	T4N3M1c	(-)	2	normal	TP	6	10
B4	adenocarc.	T4N3M1c	19(+)	2	33.8	TKI	1	2
B5	adenocarc.	T2N3M0	(-)	0	6.52	AP	16	18

EGFR: human epidermal growth factor receptor; PFS: progression-free survival; OS: overall survival; AP: pemetrexed + platinum; TP: paclitaxel + platinum; EP: etoposide + platinum; GP: gemcitabine + platinum; TKI: tyrosine kinase inhibitor.

Case No.	Pathological type	Clinical stage	EGFR mutation	ECOG score	Serum CEA	First-line treatment	PFS (m)	OS (m)
B6	adenocarc.	T4N2M1c	(-)	2	8.32	AP	3	3
B7	adenocarc.	T2N2M1c	(-)	2	57.54	AP	3	4

EGFR: human epidermal growth factor receptor; PFS: progression-free survival; OS: overall survival; AP: pemetrexed + platinum; TP: paclitaxel + platinum; EP: etoposide + platinum; GP: gemcitabine + platinum; TKI: tyrosine kinase inhibitor.

Separation of CTC by SE

6 ml of peripheral anticoagulant blood is centrifuged at 600 G and separated, that supernatant fluid is discard, 3 ml of Cytelligen cell separation solution is added, Centrifuged at 350 G for 6 min. After centrifugation, all the liquid on the bottom layer of deposited red blood cells was transferred to a 50 ml centrifuge tube and slowly added to the magnetic beads buffer while shaking the centrifuge tube. Immunomagnetic beads of a mixture of anti-leukocyte antibodies (CK18, Vimentin) were added and incubated for 15 min at room temperature, after which they were placed on magnetic racks for magnetic separation. Aft 4 min that liquid in the centrifuge tube was carefully transfer to a new 15 ml centrifuge tube. Add cleaning solution 1×CRC twice, centrifuge, discard supernatant, and add tissue fixing solution. The final pellet cells were then counted. Apply the specimen solution to the specimen frame of iFISH slide (Cytelligen Co., USA) (1 person/slide). Specimens were dried and stored for tumor-labeled iFISH.

iFISH detection

Add a mixed solution prepared by the tissue fixing solution and the sample diluent into a CTC glass slide sample frame to cover the sample frame, and let it stand for 10 min at room temperature in the dark. The slide was inserted into the ethanol jar and allowed to stand for 2 min, and then taken out and dried. Add 10 ul of CEP 8 probe into that center of the specimen frame and cover with a cover glass to make the liquid spread around to the whole specimen frame. Seal that four edge of the cover glass with rubber cement, and directly put into a hybridization instrument for hybridization. Denaturation at 76 ℃ for 10 min, hybridization at 37 ℃ for 3 h. The cover glass was washed off, and 10 ul of staining solution DAPI for blood cell analysis was added dropwise to the glass specimen frame treated with the washing solution after drying. Placing cover glass, sealing, and storing at 2–8℃ in dark.

The images were observed under an Axio Imager. Z2 fluorescence microscope, and the CTC images were scanned and analyzed automatically by a Metafer 5 system.

Identification of CTC

CK18, Vimentin, CD45, DAPI and CEP 8 staining data observed with fluorescence microscopy were combined to identify CTCs. Immunostaining for CD45 was used to exclude hematogenous leukocytes, DAPI staining was used to observe the nucleus, CK18 and Vimentin are two tumor markers of CTC. For identifying CTC subtypes, CEP 8 is used to count that copy number of chromosome 8. CTCs were

classified into monosomic CTC, triploid CTC, tetraploid CTC and polyploid CTC (≥ 5 copies) based on the copy number of chromosome 8. CTC: DAPI + / CD45- / CK +- / Vimentin +- / CEP 8 + (monosomic, triploid, tetraploid, polyploid or diploid).

Measurement of CEA

Peripheral venous blood (3 ml) was collected into nonanticoagulant blood collecting tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifugated (1,500 G) at room temperature for 10 min. CEA was measured from the supernatant by an automatic immunoassay analyzer Cobas e601 (Roche, Pleasanton, CA, USA). The reference range of CEA was less than 4.7 U/ml.

Efficacy Evaluation Criteria

All patients received chemotherapy for 2 cycles (6–8 weeks) before the start of the third cycle of chemotherapy according to the "solid tumor efficacy evaluation criteria RECIST version 1.1" to evaluate the change of tumor size and clinical response objectively. Evaluation criteria were as follows: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD).

Survival analysis: Patients were followed up to December 31, 2019. 24 patients had relatively complete follow-up data, no progress or survival at the time of follow-up were truncated data, all variables were based on the medical records at the time of diagnosis.

Statistical method

Statistical analysis was performed using IBM SPSS 26.0 software. Chi-square test was used to analyze the correlation between the positive rate of CTC and the clinicopathological features. Mann-Whitney U and Kruskal-Wallis were used to analyze the correlation between the number of CTCs and clinicopathological features. Mann-Whitney U was used to analyze the correlation between CTC threshold of different ploidy of chromosome 8 and PFS or OS. $p < 0.05$ is statistically significant, and the p value is bilateral.

Results

Correlation between positive rate of CTC in peripheral blood and clinical characteristic

In this study, peripheral blood tumor cells of 24 patients with primary lung cancer were detected by SE-iFISH. The patients with NO. B1-B7 used double-tumor labeled CK18 and Vimentin five-color channels. CTCs were detected in 23 of the 24 patients, bringing the total number of detected CTCs to 285. The average number of CTC per case was 12.4 (285/23), and the positive rate of CTC was 95.83% (23/24). There was no significant correlation between the positive rate of CTC and the clinical characteristics of patients (gender, age, ECOG score, histological type, serum CEA), but there was a certain correlation between the positive rate of CTC and whether the patients smoked ($p < 0.05$) (Table 2). There was no

significant correlation between the number of CTC and the clinical characteristics (gender, age, ECOG score, serum CEA) ($p > 0.05$), but there was correlation between the number of CTC and the histological type ($p < 0.05$) (Table 3). There was a difference in the number of CTCs between adenocarcinoma and squamous cell carcinoma (Z = -2.334, $p = 0.02$), but no difference between adenocarcinoma and small cell carcinoma (Z = -1.435, $p = 0.151$) and between squamous cell carcinoma and small cell carcinoma (Z = -1.635, $p = 0.102$).

Table 2 Relationship between positive rate of CTC detection and clinical characteristics of patient

Clinical characteristics	N = 24		
	n (CTC cases)	χ^2 value	*p* value
Gender			
male	16(15)	0.522	0.470
female	8(8)		
Age			
≥60	16(16)	2.087	0.149
≤ 60	8(7)		
History of smoking			
yes	12(11)	6.020	0.014
no	12(12)		
ECOG score			
0–1	15(14)	0.626	0.429
≥ 2	9(9)		
Pathological type			
adenocarc.	17(16)	0.430	0.807
squa. carc.	4(4)		
SCLC	3(3)		
Serum CEA			
normal	8(8)	0.522	0.470
increased	16(15)		

Table 3 Relationship between numbers of CTC and clinical characteristics of patient

Clinical characteristics	N = 285		
	CTC number	Z value	*p* value
Gender			
male	7(2.25 ~ 11.5)	-1.137	0.255
female	8.5(6.25 ~ 11.75)		
Age			
⩾60	7.5(3 ~ 11.5)	-0.031	0.975
≤ 60	7(3.25 ~ 11.75)		
History of smoking			
yes	8(6.25 ~ 10.75)	-0.985	0.325
no	6(2 ~ 13.5)		
ECOG score			
0–1	7(3 ~ 12)	-0.15	0.881
≥ 2	7(3 ~ 10.5)		
Pathological type			
adenocarc.	9(6.5 ~ 13)		0.029
squa. carc.	2.5(2 ~ 4.5)		
SCLC	7(3~.)		
Serum CEA			
normal	7(3.5 ~ 10.25)	-0.369	0.712
increased	7.5(3.25 ~ 12)		

The relationship between CTC threshold of different ploidy of chromosome 8 and PFS or OS

We investigated the correlation of CTC counts at different ploidy levels of chromosome 8 with first-line response (PFS) and prognosis (OS) of patients (Table 4). As a result, there were 0 (19 cases), 1 (3 cases), 2 (1 case) and 6 (1 case) monomeric CTCs in peripheral blood of 24 patients before treatment. To determine the threshold of aneuploid CTCs for poor outcome, we compared the PFS and OS data of patients with monomeric CTC count ⩾ 1 with those of patients with monomeric CTC count < 1, and found no statistically significant difference between the two (Z = -0.572, $p = 0.567$; Z = -1.459, $p = 0.144$). Before treatment, there were 0 (19 cases), 1 (4 cases) and 2 (1 case) diploid CTCs in peripheral blood, respectively. There was no significant difference in PFS and OS between patients with diploid CTC count ⩾ 1 and those with diploid CTC count < 1 (Z = 0.000, $p = 1.000$; Z = -0.854, $p = 0.393$). There were 0 (10

cases), 1 (6 cases), 2 (4 cases), 3 (2 cases) and 4 (2 cases) triploid CTCs in peripheral blood before treatment, respectively. The PFS and OS data of patients with triploid CTC count ≥ 1 were compared with those of patients with triploid CTC count < 1. The results showed that there was no significant difference in PFS and OS between them （Z = -0.913, $p = 0.361$; Z= -0.938, $p = 0.348$）. The PFS and OS data were compared between patients with triploid CTC count ≥ 2 and patients with triploid CTC count < 2. There was no significant difference in PFS and OS between the two groups (Z = -0.585, $p = 0.558$; Z = -1.043, $p = 0.297$). There Were 0 (12 cases), 1 (7 cases), 2 (1 case), 3 (2 cases), 4 (1 cases) and 5 (1 case) tetraploid CTCs in peripheral blood before treatment, respectively. Comparison of PFS and OS data between patients with tetraploid CTC count ≥ 1 and those with tetraploid CTC count < 1 (Fig. 1a and 1b) showed no statistically significant difference in PFS （Z = -1.568, $p = 0.117$), but there was significant difference in OS (Z = -2.284, $p = 0.022$). Comparing the PFS and OS data of patients with tetraploid CTC count ≥ 2 and patients with tetraploid CTC count < 2 (Fig. 2a and 2b), the results showed that the PFS and OS of the two patients were significantly different （Z = -2.366, $p = 0.014$; Z = -2.366, $p = 0.018$）. Before treatment, there were 0 (4 cases), 1–4 (6 cases), 5–9 (10 cases) and ≥ 10 (4 cases) polyploid CTCs in peripheral blood, respectively. The PFS and OS data were compared between patients with polyploid CTC count ≥ 1 and those with polyploid CTC count < 1. The results showed that there was no significant difference in PFS and OS between the two groups （Z = -0.585, $p = 0.559$; Z = -1.319, $p = 0.187$）. The PFS and OS data of patients with polyploid CTC count ≥ 5 and patients with polyploid CTC count < 5 were compared. The results showed that there was no significant difference in PFS and OS between the two groups (Z = 0. 000, $p = 1.000$; Z = -0. 440, $p = 0.660$). As can be seen, there was no statistical correlation between CTC count of monosomic, diploid, triploid and polyploid in PFS and OS ($p > 0.05$). A tetraploid CTC count of 2 or more was an unfavorable predictor of response, it can predict poor PFS in patients with advanced lung cancer. A tetraploid CTC count ≥ 1 is an unfavorable prognostic predictor of poor OS in patients with advanced lung cancer.

Table 4 Relationship between CTC threshold of different ploidy of chromosome 8 and PFS or OS

Threshold grouping of different ploidy	PFS			OS		
	Median(25%~75%)	Z value	*p* value	Median(25%~75%)	Z value	*p* value
Monomeric CTC□1	5(3 ~ 10)	-0.572	0.567	11(5 ~ 19)	-1.459	0.144
Monomeric CTC ≥ 1	3(2 ~ 11)			4(2.5 ~ 14)		
Diploid CTC□1	5(3 ~ 10)	0.000	1.000	11(5 ~ 19)	-0.854	0.393
Diploid CTC ≥ 1	6(2 ~ 12)			10(2.5 ~ 14.5)		
Triploid CTC□1	4(2.5 ~ 7)	-0.913	0.361	8.25(4.5 ~ 15.25)	-0.938	0.348
Triploid CTC ≥ 1	8(2.75 ~ 10)			11.5(4.75 ~ 20)		
Triploid CTC□2	4.5(3 ~ 9.5)	-0.585	0.558	8.25(5 ~ 17)	-1.043	0.297
Triploid CTC ≥ 2	8.5(2.25 ~ 9.75)			13(5.75 ~ 25.5)		
Tetraploid CTC□1	4(3 ~ 5.75)	-1.568	0.117	5(3.25 ~ 10.75)	-2.284	0.022
Tetraploid CTC ≥ 1	9.5(1.5 ~ 10.75)			16.5(9.125 ~ 23.5)		
Tetraploid CTC□2	4(2.25 ~ 8)	-2.366	0.014	8.25(4.25 ~ 13.5)	-2.366	0.018
Tetraploid CTC ≥ 2	10(9.25 ~ 14.875)			22(15.75 ~ 27.5)		
Polyploid CTC□1	7(4.5 ~ 8.75)	-0.585	0.559	13(10.5 ~ 25.25)	-1.319	0.187
Polyploid CTC ≥ 1	4.5(2.25 ~ 10)			8.25(4.25 ~ 18)		
Polyploid CTC□5	5.5(3 ~ 9)	0.000	1.000	11(5 ~ 18.25)	-0.440	0.660
Polyploid CTC ≥ 5	4.5(1.75 ~ 10.25)			9.75(3.75 ~ 18.25)		

Relationship between the expression of tumor markers and the ploidy of chromosome 8

Two tumor markers, CK18 and Vimentin, were detected in this study. All patients were tested for CK18, only 1 case had both CK18 + CTC (1 triploid) and CK18 - CTC (1 tetraploid). The remaining 22 patients were CK18 - CTC, CK18 + CTC detection rate was 4.35%(1/23). Seven patients (NO. B1-B7) were tested for both CK18 and Vimentin, and all of them were negative for CK18, 5 cases □71.43%) had both Vimentin + CTC and Vimentin – CTC, 1 case (14.29%) had only Vimentin + CTC, and the other 1 case (14.29%) had only Vimentin - CTC. The positive rate of Vimentin + CTC was 85.71% (6/7). In 6 cases of Vimentin + CTC, there were 4 cases of monosomy, 5 cases of diploidy and 1 case of triploidy in chromosome 8. In 6 Cases of Vimentin - CTC, there were 3 cases of monosomy, 4 cases of triploidy and 2 cases of tetraploidy in chromosome 8. There were 6 cases of polyploidy, of which 5 cases had ≥ 7 polyploidy. Therefore, chromosome 8 of Vimentin + CTC was mostly diploid and monosomic, and absent of polysome. Vimentin

- CTC showed more heteroploid diversity of chromosome 8, including monosomy, triploid, tetraploid and polyploid, especially the probability of polyploid was higher (Fig. 3).

Tumor Marker Expression of Small Cell CTC and Chromosome 8 Aneuploid

In this study, 7 patients (NO. B1-B7) were detected for double tumor markers and chromosome 8 aneuploidy. Six patients were found to have small cell (≤ 5μm) CTC, the total number of small cell CTC was 11, the incidence rate was 85.71% (6/7), and the proportion of small cell CTC in total CTC was 11. The tumor marker phenotype of small cell CTC was CK18 - Vimentin +, including 4 monomers (36.36%), 6 diploids (54.55%) and one trisomy(9.09%). No tetraploid and polyploid were found.

Circulating Tumor Microthrombi (CTM)

Circulating tumor microthrombi (CTM ≥ 2 CTCs) were found in 2 of 7 patients (NO. B1-B7), with an incidence of 28.57% (2/7). The total number of CTM was 2, and the tumor marker phenotypes were CK18 - Vimentin + and CK18 – Vimentin -, respectively. The two cases were IVB adenocarcinoma patients, and the serum CEA was significantly increased. They were 33.80 ng/ml and 57.54 ng/ml, respectively, while the CEA of the other 5 patients was normal or slightly elevated. Patient B4 was treated with first-generation EGFR-TKI because of EGFR del 19 (+). But PFS is only 1 month and OS is 2 months. Patient B6 was treated with whole brain radiotherapy because of brain metastasis. PFS was 3 months and OS was 4 months.

Discussion

At the end of the 19th century, PAGET put forward the "seed and soil" theory of tumor metastasis. It is undeniable that the number of CTC in peripheral blood is very small, and some patients are even difficult to obtain in clinic. In the process of continuous adaptation to the microenvironment of peripheral blood, some CTCs undergo epithelial-mesenchymal transition (EMT). Some show apoptosis or reduced antigen expression [23]. In order to overcome the difficulty of CTC detection caused by the above problems, in 2018, Zhenlong Ye et al [6] studied the clinical significance of aneuploidy of CTC in patients with various malignant tumors. 594 blood samples from 479 cases of 19 different cancers and 30 healthy samples were collected for CTC detection and analysis by SE-iFISH. The results of this study show that no positive CTC was found in all 30 healthy samples. In the diagnosis of malignant tumors, the overall positive rate of CTC was 89. 0% (75. 0% -100. 0%). The results were basically consistent with the positive rate of CTC detection in this study (95. 83%).

Other studies [6, 26–29] have shown that an average of 11, 5, 8, and 4 CTCs per 7.5 mL were observed in lung, liver, kidney, and colorectal cancers, respectively. In this study, the average number of CTC detected per 6 mL blood of each case was 12. 4 (285/23), which was similar to the results of previous studies, but slightly better. In addition, more than 95% of the positive rate is also closely related to the clinical stage and pathological type of the patients enrolled in this study. All patients were in stage IV except one in

stage IIIB, and CTC was more easily detected in patients with metastasis. On the contrary, if the patient cannot complete the clinical staging evaluation, CTC measurement can also effectively predict whether the patient has metastasis, which has a certain value for the prognosis of patients. At the same time, patients with pathological type distribution: adenocarcinoma 70.83% (17 cases), small cell carcinoma 12.50% (3 cases), squamous cell carcinoma 16.67% (4 cases). It is well known that adenocarcinoma and small cell lung cancer are more likely to metastasize, and the proportion of adenocarcinoma and small cell lung cancer patients in this study is high (83.33%) and all of them were advanced lung cancer. Due to the high sensitivity of SE-iFISH, we got a high positive rate of 95.83% CTC detection results.

There was no significant correlation between the positive rate of CTC and the clinical characteristics of lung cancer patients, including gender, age, ECOG score, histopathological type and serum CEA. The results are basically consistent with those of other clinical studies (gender, age, histopathological type). However, some other studies [30–32] have pointed out that the detection rate of CTC in patients with lung cancer in clinical stage III ~ IV is higher than that in patients with lung cancer in clinical stage I ~ II, but this study did not find that there is a significant correlation between the detection rate of CTC and the clinical stage of lung cancer. The possible reason of this result is that the number of patients enrolled in this study is small, and all of them are stage IV metastatic lung cancer (only 1 case of stage IIIB), and there is a lack of early stage (stage I, stage II) and stage IIIA patients. But the results of this study showed that there was a significant difference between the detection rate of CTC and whether the patients smoked ($p = 0.014$), that is, the detection rate of CTC in smoking patients was lower than that in non-smoking patients. Only one patient in the whole sample was negative for CTC, and this patient had a history of smoking, so this conclusion needs to be further confirmed.

We also found that there was no significant correlation between the number of CTCs and the clinical characteristics of patients (including gender, age, ECOG score, serum CEA), but there was a certain correlation between the number of CTCs and the histopathological type of patients. There was a difference in the number of CTCs between adenocarcinoma and squamous cell carcinoma ($p = 0.02$), but there was no difference between adenocarcinoma and small cell carcinoma ($p = 0.151$) or between squamous cell carcinoma and small cell cancer ($p = 0.102$). To sum up, we can conclude that the three main pathological types of lung cancer (adenocarcinoma, squamous cell carcinoma, small cell carcinoma) have no significant difference in the detection rate of CTC in peripheral blood, but there are some differences in the number of CTC. The difference between adenocarcinoma and squamous cell carcinoma was statistically significant. In NSCLC, it has been found that CTC count is correlated with tumor metastasis [33, 34], while adenocarcinoma is a pathological type prone to hematogenous metastasis, squamous cell carcinoma is relatively inert, ana its growth and development is slow, not easy to early invasion and metastasis. So it is not difficult to understand, compared with squamous cell carcinoma, peripheral blood CTC count of adenocarcinoma patients is more. In theory, small cell lung cancer has the highest degree of malignancy and is most likely to undergo hematogenous metastasis and dissemination in the early stage. However, there was no significant difference in CTC count between small cell carcinoma and adenocarcinoma, small cell carcinoma and squamous cell carcinoma, which may be due to the small number of cases of small cell cancer and squamous cell carcinoma selected in

this project. There were only 3 cases of small cell carcinoma and 4 cases of squamous cell carcinoma. The median CTC counts (25% -75%) in lung cancer, 9 (6.5–13) of adenocarcinoma, 2.5 (2 ~ 4.5) of squamous cell carcinoma, 7 (3 ~.) of small cell carcinoma. If the sample size is expanded, it is expected that there may be some statistical difference in CTC count between small cell carcinoma and squamous cell carcinoma.

In this study, SE-iFISH was used to detect the expression of CK18 and Vimentin on the surface of lung cancer cells in peripheral blood, and combined with the ploidy of CEP 8 to determine CTC. Among the 23 patients in whom CTCs were detected, only one CK18 + triploid CTC on chromosome 8 was detected in one patient, and in the remaining 22 patients, all CTCs were CK18 -. The positive rate of CK18 + CTC was 4.35% (1/23). 5 of 7 patients had both Vimentin + and Vimentin -, only one had Vimentin + CTC, the others only had Vimentin - CTC, the detection rate of Vimentin + CTC was 85. 71% (6/7). It is noteworthy that chromosome 8 of Vimentin + CTC is mostly diploid and monosomic. Vimentin - CTC showed more heteroploid diversity of chromosome 8, including monosomy, triploid, tetraploid and polyploid, especially polyploid was more common. These results showed the complexity and complementarity of the expression of CTC surface protein markers and chromosome 8 aneuploidy in peripheral blood of patients with advanced lung cancer, and the detection sensitivity of chromosome 8 aneuploidy was the highest, reaching 100%. Vimentin + was the second with 85.71%, while CK18 + was the worst with 4.35%.

In Addition, small cell (≤ 5 um) CTC was detected in 6 of 7 patients (85.71%). Another method for enriching CTCs is based on the tumor cell size separation method (ISET), which can only capture CTCs with larger diameter than the filter pore (> 8 um) from the blood sample and is independent of the surface antigen of CTCs. But there are also a small number of CTCs with smaller diameter in peripheral blood. Similar to blood cells or even smaller, this method is easy to lose this part of the small size of CTCs. In this study, Vimentin expression and CEP 8 aneuploid were used to detect 85.71% (6/7) of patients with primary lung cancer who had small cell CTC in peripheral blood, which avoided the missed detection of small cell CTC ≤ 5 um.

Therefore, SE-iFISH platform improves the sensitivity of CTC detection, avoids the limitations of tumor heterogeneity, CK downregulation, deletion and small size easy to miss detection.

Chromosome aneuploidy exists in various tumor cells. Many studies have shown that aneuploid tumor cells are associated with poor prognosis. Polyploid tumor cells are genetically unstable and not sensitive to radiotherapy and chemotherapy. It is generally believed that the prognosis of patients with polyploid subcloned tumor cells is worse [35].

In this study, 23 of 24 patients were positive for CTC in peripheral blood, and all of them were detected with chromosome 8 heteroploid CTC, and the ploidy of chromosome 8 was obviously heterogeneous. The number of polyploid CTC was the largest, followed by triploid and tetraploid CTC. In this study, we compared the correlation between the cutoff value of different ploidy number of chromosome 8 and PFS of the first-line treatment or OS in order to find a reference method to predict the efficacy and poor prognosis. The results showed that a tetraploid CTC count ≥ 2 was an unfavorable predictor of response,

it can predict poor PFS in patients with advanced lung cancer. A tetraploid CTC count ≥ 1 is an unfavorable prognostic predictor of poor OS in patients with advanced lung cancer. It is suggested that quantitative analysis of chromosome 8 tetraploid in CTCs by SE-iFISH may be a useful tool for evaluating the therapeutic effect and judging the prognosis.

Studies have found that lung cancer metastasis is likely to have occurred in the early stage of lung cancer, and there is dissemination of lung cancer cells. A large number of CTCs aggregate and form CTM with an high metastatic potential, this stage is called micrometastasis, which is also known as occult metastasis. Therefore, CTM has stronger ability of invasion and metastasis than CTC. In This study, CTM was found in 2 of 7 lung cancer patients (28.57%, 2/7). Both of the two cases were stage IVB adenocarcinoma patients, and their serum CEA levels were 33.80ng/ml and 57.54ng/ml, respectively. After the first-line treatment, the PFS was only 1 month and 3 months respectively, and the OS was 2 months and 4 months respectively. It is suggested that the presence of CTM in peripheral blood may predict the later clinical stage, the worse therapeutic effect and the poor prognosis of lung cancer patients.

In summary, this study further confirms that SE-iFISH can effectively enrich CTC in the peripheral blood of patients with primary lung cancer. According to the specific needs of clinic or research, the combination of immunofluorescence staining with freely selected tumor biomarkers and chromosome FISH further improves the sensitivity and specificity of CTC detection. CTCs effectively detected by SE-iFISH can be used as a preliminary predictor of immediate efficacy and prognosis of patients with metastatic primary lung cancer. Quantification of chromosome 8 aneuploidy also shows potential as a predictive biomarker for disease progression and prognosis. Due to the small sample size, there are still some limitations in this study, which needs to be verified by a larger sample.

Conclusions

SE-iFISH has a high detection rate of CTC in peripheral blood of patients with metastatic primary lung cancer, and it can identify small-cell CTC. Tetraploid CTC count ≥ 2 can predict poor PFS in patients with advanced lung cancer. Tetraploid CTC count ≥ 1 may predict poor OS in patients with advanced lung cancer. CTM can predict poor prognosis in patients with lung cancer.

Abbreviations

CTC
circulating tumor cell; CEA:carcinoembryonic antigen; CTM:circulating tumor microthrombi; CEP8:centromere of chromosome 8; CK:cytokeratin; DAPI:4',6-diamidino-2-phenylindole; EpCAM:epithelial cellular adhesion molecule; SE-iFISH:subtraction enrichment and immunofluorescence in situ hybridization; EMT:epithelial-mesenchymal transition; NSCLC:non-small cell lung cancer; SCLC:small cell lung cancer; UICC/AJCC:Union for International Cancer Control/American Joint Committee on Cancer; IASLC:International Association for the Study of Lung Cancer. WBC:white blood cell; HER-2:human

epidermal growth factor receptor-2; CA19-9:carbohydrate antigen 19 – 9; ECOG:Eastern Cooperative Oncology Group.

Declarations

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Authors' contributions

XXX, SY and YHQ contributed in study design and manuscript revision; YHQ wrote the manuscript; YHQ and WZD collected samples and statistical analysis; YJJ and YHQ were in charge of the CTC detection procedure. All authors read and approved the final manuscript.

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Availability of data and materials

All data analysed during this study are included in this manuscript.

Ethics approval and consent to participate

All the patients were informed of sample collection and usage. The samples were collected and used in accordance with approval by the Institutional Ethical Committee Board (Northern Jiangsu People's Hospital, Yangzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

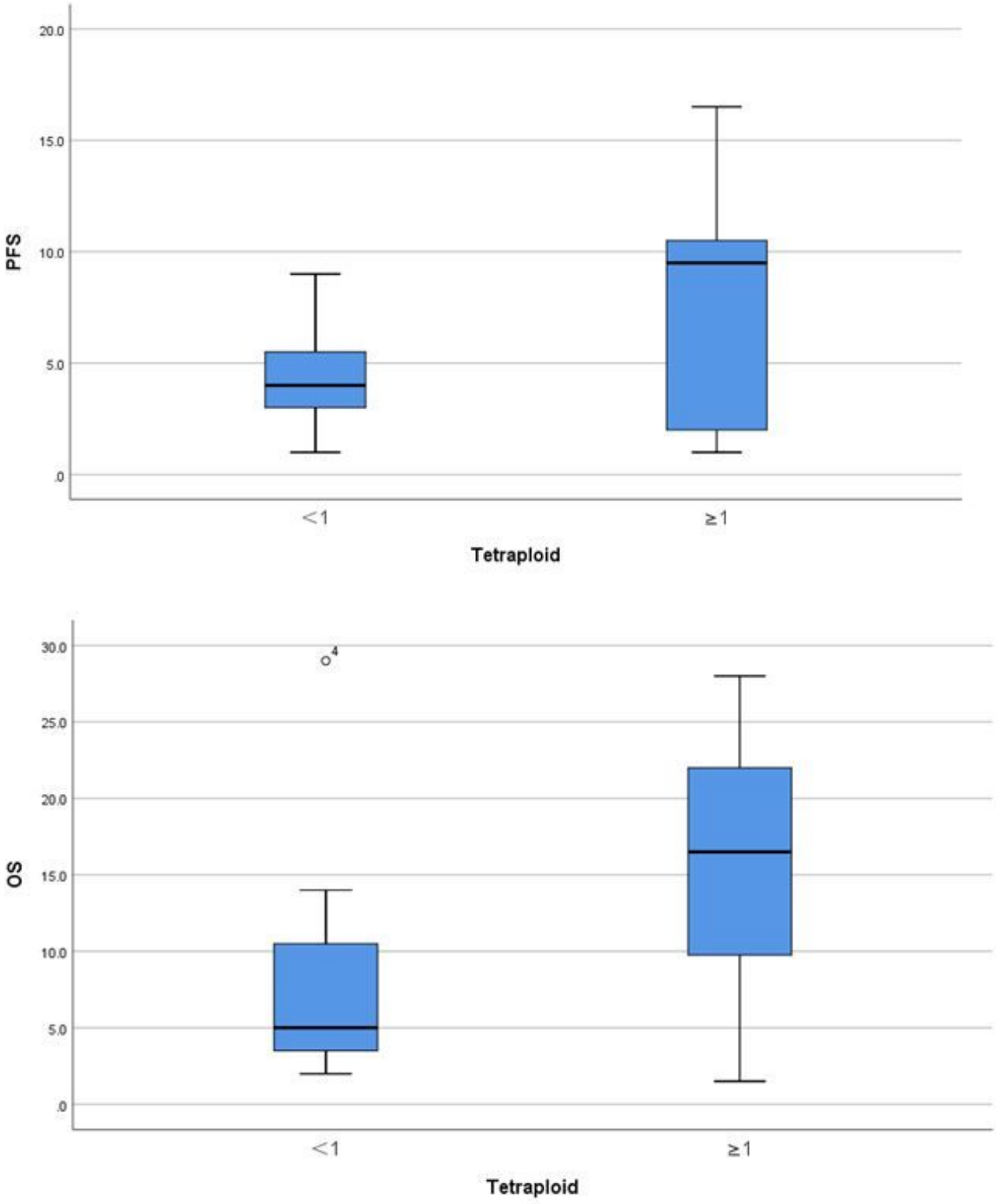
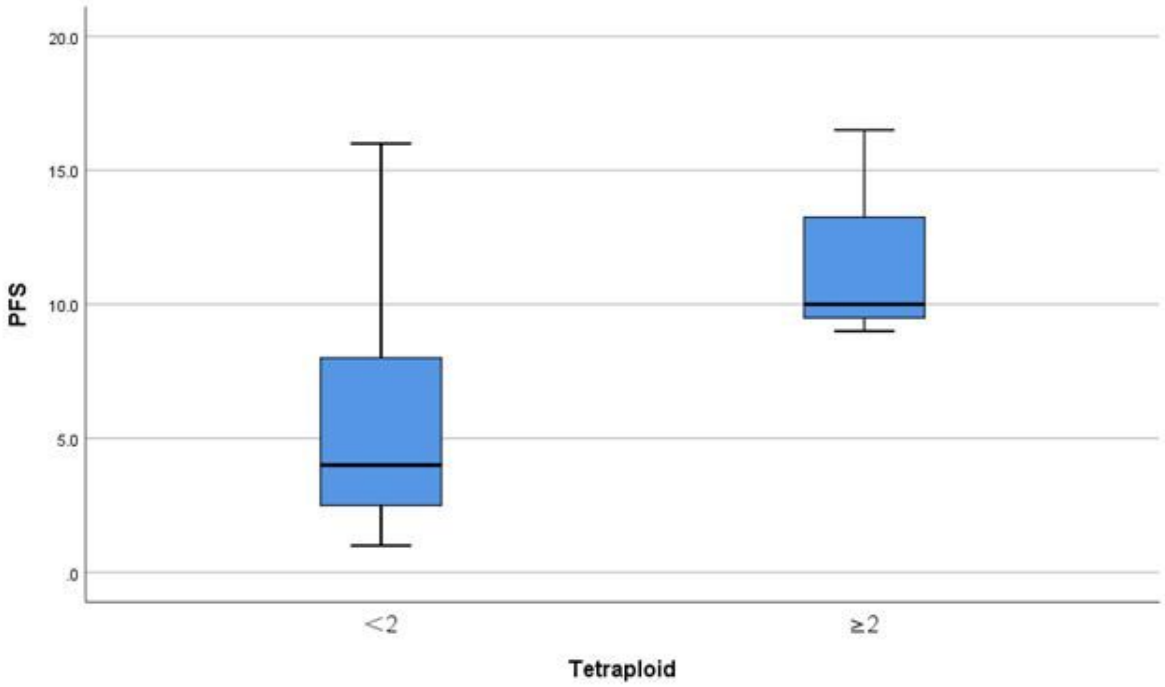


Figure 1

1a The relationship between the number of tetraploid CTC ≥ 1 or ＜ 1 and the patient's PFS 1b The relationship between the number of tetraploid CTC ≥ 1 or ＜ 1 and the patient's OS



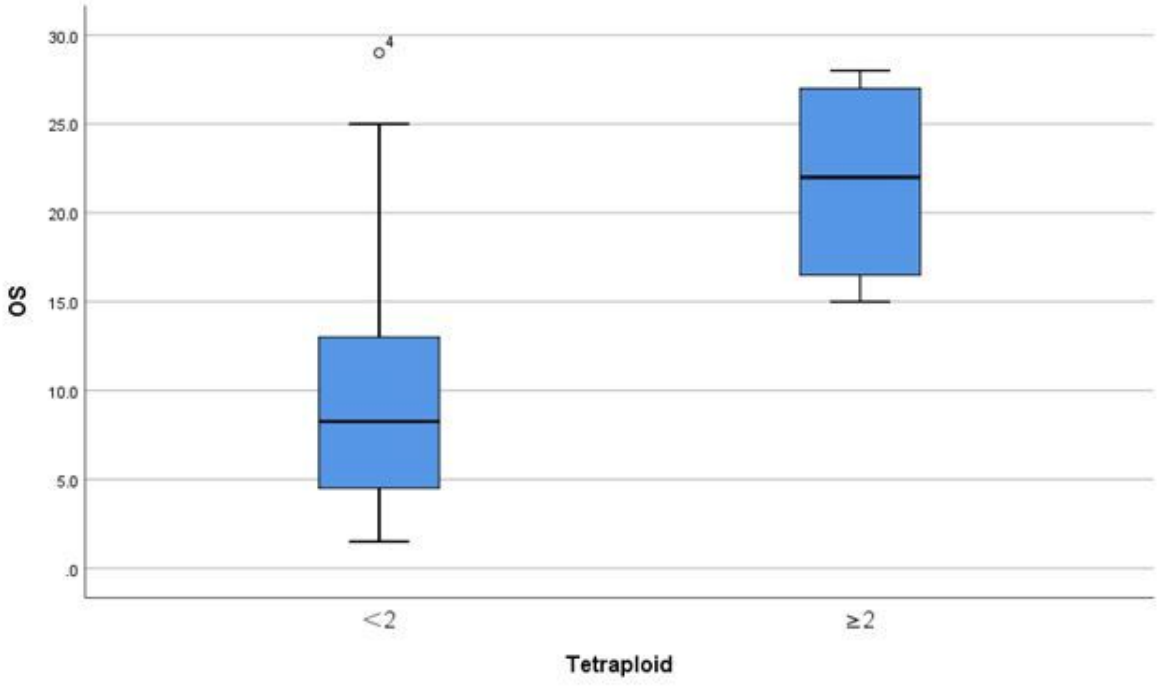
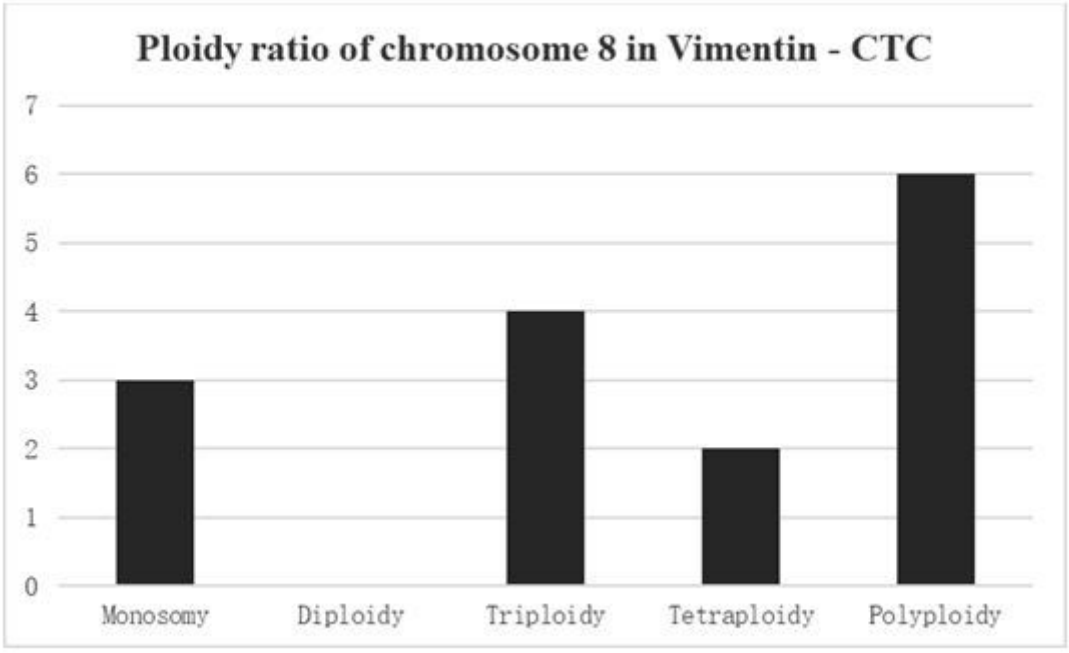


Figure 2

2a The relationship between the number of tetraploid CTC ≥ 2 or 2 and the patient's PFS 2b The relationship between the number of tetraploid CTC ≥ 2 or 2 and the patient's 0S



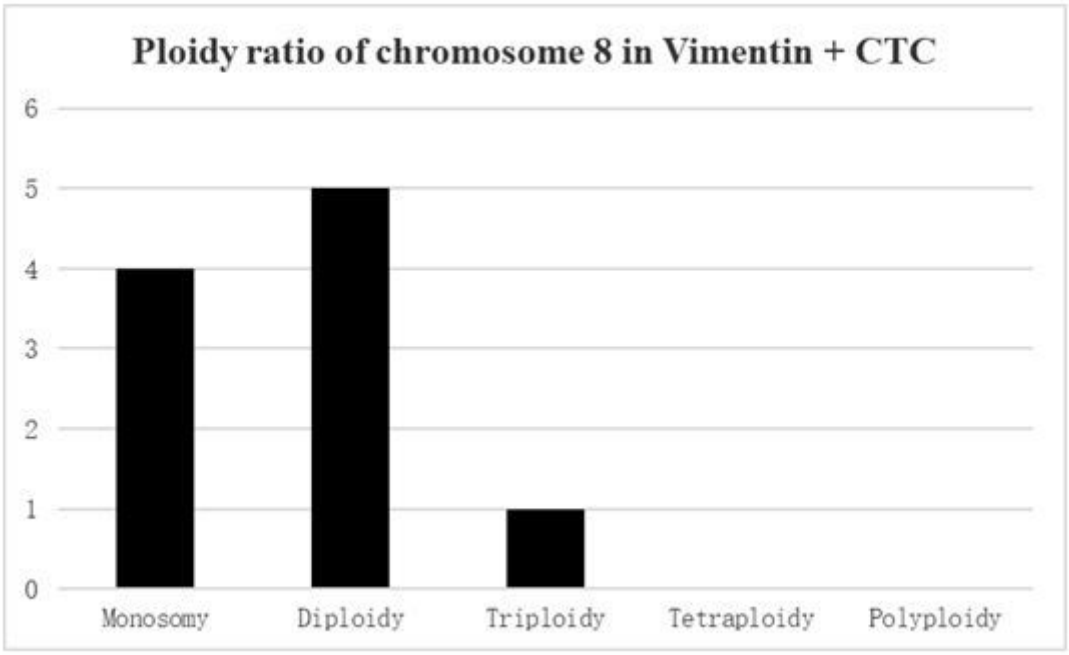


Figure 3

Ploidy ratio of chromosome 8 in Vimentin +/- CTC