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## Circulating Tumor Cell Detection

**—— The real-time tumor monitoring ——**

# Clinical Reference Manual for Circulating Tumor Cells Detection in Primary Lung Cancer

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# Preface

Despite the advancements in medical technology, lung cancer remains one of the most prevalent and deadliest malignancies in China. Due to its complex etiology, inadequate early screening and diagnostic techniques, and the propensity for metastasis, managing lung cancer presents numerous challenges. Particularly in the aspects of occurrence, progression, and post-treatment monitoring, conventional imaging techniques and serum tumor markers lack specificity or sensitivity. Clinicians and patients alike are anticipating new detection technologies or novel tumor markers to provide more information for accurate diagnosis and evaluation in clinical practice.

Circulating Tumor Cells (CTCs), commonly referred to as tumor cells present in the peripheral blood of cancer patients, were first observed by Australian team in 1869. However, it wasn't until around 2000, with the evolution of detection techniques, that the characteristics and clinical significance of CTCs were gradually recognized. They emerged as valuable tumor markers, offering substantial aid to clinical diagnosis and treatment. Since the FDA's approval of CTC detection for prognosis assessment in metastatic breast cancer patients in 2004, several countries have successively sanctioned various CTC detections for prognosis evaluation and adjunctive diagnosis in different cancers. Clinicians and researchers have utilized these detection technologies to explore the clinical significance and threshold of CTCs at different stages, including in lung cancer. The subsequent sections outline the clinical significance of CTC detection at different stages of lung cancer diagnosis and treatment.

## Clinical Application Reference of Circulating Tumor Cells (CTC) Detection in Lung Cancer Diagnosis and Treatment

### Prediction and Accurate Assessment of Therapeutic Efficacy on Late-Stage Non-Small Cell Lung Cancer (NSCLC).

Based on current acknowledged clinical research, recommendations for the timing and frequency of CTC detection in late-stage NSCLC patients undergoing comprehensive systemic therapy (including chemotherapy or targeted therapy) are proposed as follows:

1. Baseline CTC testing one week before comprehensive systemic therapy (including chemotherapy and targeted therapy): Consider poor prognosis if CTC count exceeds the threshold. Evaluate the necessity for continued treatment or explore new drug clinical trials.
2. For patients undergoing chemotherapy, perform a second CTC test before starting the third treatment cycle: If both CTC tests show counts above the threshold, suggest poorer treatment efficacy and consider changing the treatment plan. If the second CTC test is below the threshold or shows a significant decrease (over 50%) from baseline, indicate the current treatment's effectiveness. Consider maintaining the treatment plan and perform a third evaluation.
3. For targeted therapy, consider conducting a second CTC test in patients after 2-3 months of treatment: If both CTC tests show counts above the threshold, suggest poorer treatment efficacy and consider changing the treatment plan. If the second CTC test is below the threshold or shows a significant decrease (over 50%) from baseline, indicate the current treatment's effectiveness. Consider maintaining the treatment plan and perform a third evaluation.
4. After changing the treatment plan, conduct a CTC test after two treatment cycles (or two months) under the new plan and compare the results with the previous two tests. If CTC count remains above the threshold, reconsider the necessity for continued treatment or explore new drug clinical trials.
5. After completing all systemic therapies, if CTC count exceeds the threshold during follow-up, it suggests poor treatment efficacy and prognosis. Reevaluate the necessity for continued treatment or explore new drug clinical trials.



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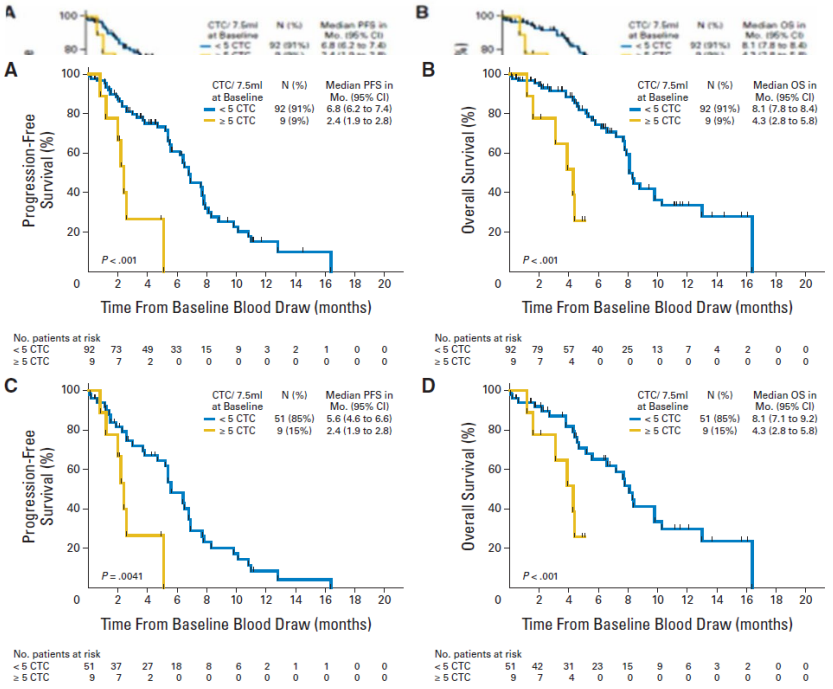
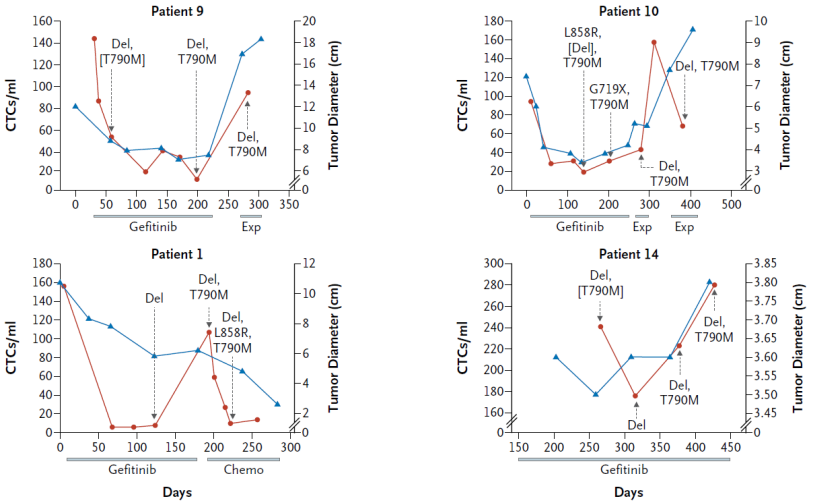
Late-stage non-small cell lung cancer typically encompasses locally advanced and metastatic stages, where patients often lack the conditions for surgical resection and must resort to comprehensive systemic treatment to control tumor growth. At this stage, specific biomarkers are necessary to evaluate patient prognosis and treatment efficacy. In 2011, Krebs et al. from the UK reported the use of the CellSearch system to detect circulating tumor cells (CTCs) in 101 stage III-IV non-small cell lung cancer patients before and after one course of chemotherapy. They found that patients with pre-treatment CTC counts exceeding 5 cells/7.5 mL of peripheral blood demonstrated significantly worse prognosis (PFS 6.8 months vs. 2.4 months, OS 8.1 months vs. 4.3 months, see figure below). Patients with post-treatment CTC counts surpassing this threshold also exhibited significantly worse prognosis. Conversely, patients experiencing a decrease in CTCs post-treatment showed significantly prolonged PFS and OS. Thus, at various stages pre- or post-treatment, the quantity of CTCs in peripheral blood effectively predicts patient prognosis, aiding clinicians in accurately predicting treatment response or assessing treatment efficacy.




A team from the United States reported in 2008 the detection of CTCs before and after EGFR-TKI drug treatment in 27 metastatic non-small cell lung cancer patients. They observed that patients with decreasing CTC counts exhibited significant tumor shrinkage, while those with increased CTCs showed disease progression (see figure below). Similar research outcomes were reported by Isobe et al. in 2012, where 24 metastatic non-small cell lung cancer patients undergoing EGFR-TKI treatment showed significantly worsened prognosis if CTCs increased.

In recent years, numerous clinical researchers, including Muinelo-Romay (2014), Qian and Wang (2017), Zhou (2017), Lindsay (2017), among others from various countries, conducted similar studies on different late-stage non-small cell lung cancer treatment regimens. They consistently found that baseline CTC counts before treatment could predict the prognosis of patients undergoing chemotherapy. Moreover, an increase in post-treatment CTC counts corresponded to a poorer prognosis.

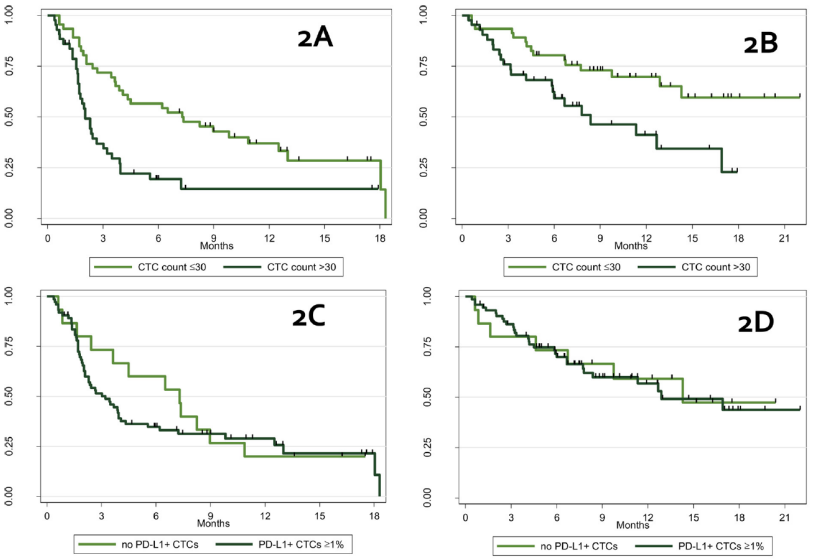
## Assessment of Immunotherapy Efficacy in Advanced NSCLC

Based on current recognized clinical research, for non-small cell lung cancer patients planning PD-1/PD-L1 monoclonal antibody (mAb) therapy, the recommended timing and frequency for circulating tumor cell (CTC) detection are as follows:

1. Baseline CTC quantity and PD-L1 expression testing should occur one week before immunotherapy initiation: Elevated CTC counts above the threshold suggest poor treatment efficacy, necessitating evaluation for the patient's continued treatment or participation in new drug clinical trials. Additionally, if the proportion of PD-L1-positive CTCs exceeds 1%, it also indicates poor treatment efficacy, prompting reevaluation of the patient's treatment continuation or exploration of new drug clinical trials.
2. After completing 3-5 treatment cycles, conduct the second CTC assessment before the next cycle: If both CTC assessments indicate counts above the threshold, it suggests poor treatment efficacy, prompting consideration of treatment plan alterations. Notably, a substantial increase (50% or more) in PD-L1-positive CTC counts during the second assessment signifies poor treatment efficacy, requiring reevaluation of the treatment plan. However, if the second CTC assessment shows counts below the threshold or a significant decrease (50% or more) compared to baseline, it indicates current treatment effectiveness, prompting consideration for maintaining the treatment plan and further assessment.
3. Upon completion of all treatments, detecting CTC counts above the threshold during follow-up indicates poor treatment efficacy and adverse prognosis. Therefore, reassessment for the patient's continued treatment necessity or exploration of new drug clinical trials is recommended.

PD-1/PD-L1 monoclonal antibodies are key immunotherapy drugs, offering hope to many advanced lung cancer patients unresponsive to chemotherapy. FDA-approved for various NSCLC treatments, these drugs show remarkable effects. Yet, assessing their effects accurately remains a challenge due to issues like pseudo-progression and immune-related events. Seeking new tumor markers aids in better evaluating PD-1/PD-L1 drug effects beyond traditional evaluations.

France research team published a clinical study in 2018 involving 96 advanced NSCLC patients treated with Nivolumab. They detected CTCs and PD-L1 protein expression before treatment, finding that patients with elevated pre-treatment CTC counts above the threshold showed similarly poor prognosis for immunotherapy. Moreover, a prognosis significantly worsened if the proportion of PD-L1-positive CTCs exceeded 1% (refer to figure below).




A pioneer presented the latest findings in 2019. Their study demonstrated that patients benefiting from PD-1 mAb treatment experienced reduced or unchanged CTC counts, whereas those developing resistance showed increased CTCs. Notably, in five cases with eventual disease progression, all showed an increase in PD-L1-positive CTC counts (refer to table below). Similar conclusions were reached by the Fifth Medical Center of the PLA General Hospital in China and several other global research groups.

| ID | Line | Drug | CTC Classification | CTC Numbers | | | Best Response |
|---|---|---|---|---|---|---|---|
| | | | | start | #3–5 | PD | |
| IT_1 | 1st line | pembrolizumab | total CTCs | 2 | 0 | na | PR |
| | | | PDL1+CTCs | 0 | 0 | | |
| IT_2 | 1st line | pembrolizumab | total CTCs | 0 | 2 | na | PR |
| | | | PDL1+CTCs | 0 | 0 | | |
| IT_3 | 2nd line | atezolizumab | total CTCs | 11 | 2 | na | PR |
| | | | PDL1+CTCs | 10 | 2 | | |
| IT_4 | 1st line | pembrolizumab | total CTCs | 4 | 4 | na | PR |
| | | | PDL1+CTCs | 4 | 4 | | |
| IT_5 | 2nd line | nivolumab | total CTCs | 4 | 1 | na | SD |
| | | | PDL1+CTCs | 4 | 1 | | |
| IT_6 | 2nd line | nivolumab | total CTCs | 3 | 1 | na | SD |
| | | | PDL1+CTCs | 3 | 1 | | |
| IT_7 | 1st line | pembrolizumab | total CTCs | 0 | 0 | 1 | PR |
| | | | PDL1+CTCs | 0 | na | 1 | |
| IT_8 | 2nd line | nivolumab | total CTCs | 2 | 0 | 1 | PR |
| | | | PDL1+CTCs | 1 | 0 | 1 | |
| IT_9 | 3rd line | nivolumab | total CTCs | 4 | 0 | 2 | SD |
| | | | PDL1+CTCs | 2 | 0 | 2 | |
| IT_10 | 2nd line | pembrolizumab | total CTCs | 0 | 3 | 3 | na |
| | | | PDL1+CTCs | 0 | 3 | 3 | |
| IT_11 | 1st line | pembrolizumab | total CTCs | 0 | 14 | 14 | na |
| | | | PDL1+CTCs | 0 | 14 | 14 | |

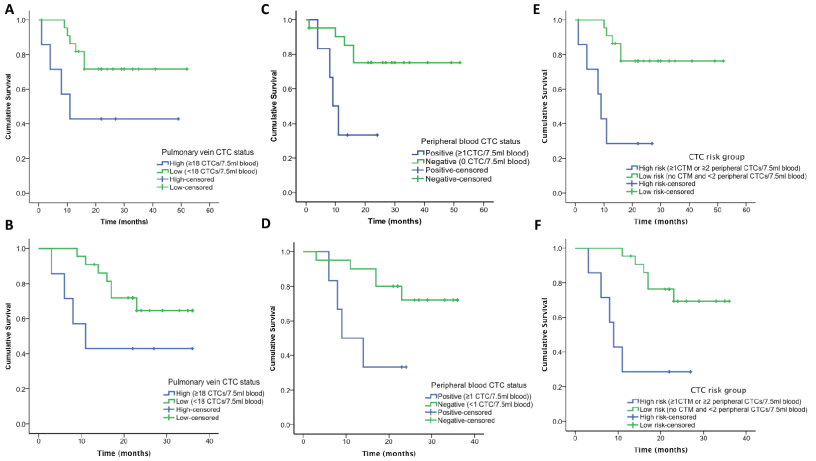
## Assessment and Monitoring of Recurrence/Metastasis Risk in Early to Mid-Stage NSCLC

Based on current recognized clinical research, when providing curative treatment for early to mid-stage lung cancer patients, including surgery and radiotherapy, clinicians are advised to conduct circulating tumor cell (CTC) testing at specific times and frequencies:

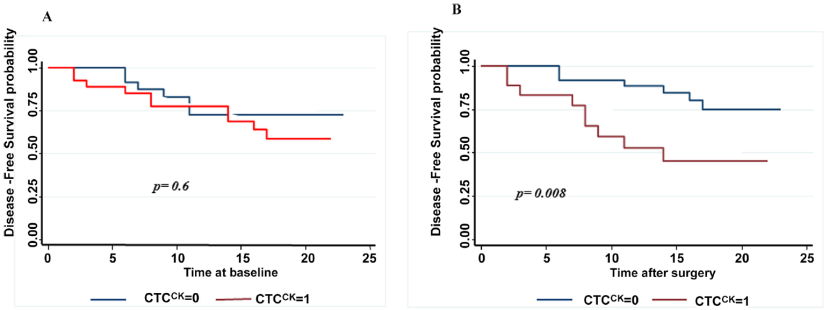
1. Within one week before surgery (or radiotherapy).
2. Between one week and one month after surgery (or radiotherapy).
3. Consider CTC testing every 6-12 months post-treatment completion, with a suggested interval of every 2-3 years post-surgery for up to five years.
4. If CTCs are detected post-surgery (or post-radiotherapy) beyond the threshold, consider additional treatment to lower the risk of recurrence/metastasis.
5. During planned adjuvant therapy post-surgery (or post-radiotherapy), refer to the systemic treatment section for testing timing and frequency.
6. In follow-ups, if CTC counts exceed the threshold, intensify follow-up frequency and conduct CTC retesting. If two consecutive CTC tests show counts above the threshold, it indicates a higher risk of recurrence, prompting detailed examinations or preventative interventions to reduce the risk of recurrence/metastasis.

Early to mid-stage non-small cell lung cancer (NSCLC) involves relatively small lesions, and most patients can be cured through treatments like surgery and radiotherapy. Specific markers are necessary post-surgery or radiotherapy to assess the risk of recurrence/metastasis, enabling decisions about additional or neoadjuvant therapies to mitigate risk. However, post-treatment, imaging is challenging due to the absence of lesions. Clinicians often employ serum tumor markers (e.g., CEA, Cyfra-21, SCC, CA125) for monitoring during follow-ups, yet these combinations still lack sensitivity and specificity. Thus, the advent of circulating tumor cell (CTC) detection offers an avenue to assess early to mid-stage NSCLC patients' post-operative risks accurately and monitor tumor recurrence/metastasis.

In 2016, A team studied 30 patients who underwent curative surgery for stages I-III lung cancer at the Manchester Thoracic Oncology Center. They detected CTCs in tumor-draining pulmonary veins and peripheral veins in 43% (13/30) and 22% (6/27) of samples, respectively. Patients with more CTCs during surgery or pre-operative peripheral blood had significantly shorter disease-free survival (DFS) and overall survival (OS) compared to those without detected CTCs (Figure below).




Similarly, in 2016, a team at Virgen de las Nieves University Hospital in Spain recruited 56 NSCLC patients undergoing curative surgery. Blood samples were taken pre-operatively (2-16h) and one month post-operation. Results showed 51.8% (29/56) had baseline CTCs (CTC1), decreasing to 32.1% (18/56) after one month (p = 0.034). Of the 29 patients with CTCs, 25 showed post-operative declines. Follow-up results indicated a strong association between post-operative CTCs and rapid recurrence. Fifty percent of post-operative CTC-positive patients experienced recurrence compared to 18.4% of CTC-negative patients (p = 0.018). Moreover, post-operative CTC-positive patients had significantly shorter DFS than CTC-negative patients (p = 0.008) (Figure below).




In 2017, a team at Beijing Chest Hospital also published similar research, demonstrating that post-operative detection of CTCs in peripheral blood indicates a significantly higher risk of recurrence, requiring intensified treatment and close post-operative follow-up.

## Prognostic Assessment and Monitoring in Small Cell Lung Cancer

Based on current clinical studies, the recommended timing and frequency for circulating tumor cell (CTC) detection in small cell lung cancer patients planning chemotherapy are as follows:

1.Baseline CTC testing should occur one week before chemotherapy initiation; elevated CTC counts above the threshold suggest poor prognosis, prompting assessment for the patient's continued treatment or participation in new drug clinical trials.

2.For chemotherapy patients, perform the second CTC assessment after two cycles: Elevated counts suggest ineffective treatment, prompting possible treatment plan alteration. Conversely, reduced counts or a significant decline from baseline indicate current treatment effectiveness, warranting maintenance and a subsequent assessment.

3.If treatment plan adjustment occurs, perform another CTC assessment after two treatment cycles (or two months) on the new regimen, comparing it with the prior assessments. Persistently high CTC counts may prompt reconsideration of ongoing treatment or search new drug trials.

4.After completing all systemic treatments, detecting CTC counts above the threshold in follow-up suggests poor treatment effectiveness and an unfavorable prognosis. Therefore, reevaluation for continued treatment necessity or consideration of new drug trials is advised.

For both limited and extensive small cell lung cancers, common treatments involve chemotherapy or a combination with other modalities. However, these patients often experience relapses or treatment resistance, underscoring the importance of prognostic evaluation and efficacy monitoring.

Studies on CTCs in small cell lung cancer precede those in non-small cell lung cancer due to the greater ease of detection. In 2012, Dr. Hou from Professor Caroline Dive's team in the UK published research in JCO involving 97 patients with limited and extensive-stage small cell lung cancer, revealing CTC detection in 85% of cases. Patients exceeding a specific CTC threshold (CTCs >= 50/7.5mL) showed significantly poorer prognoses (as depicted in the Figure below).

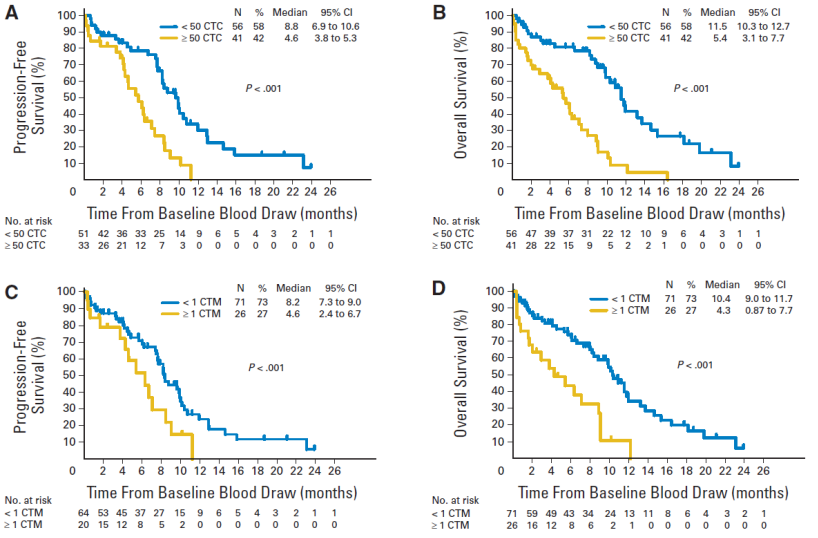
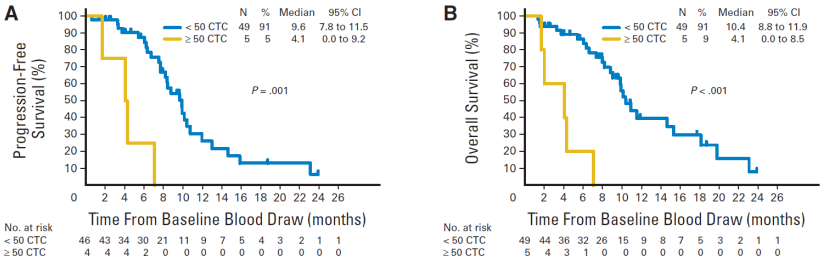



Fig 1. Kaplan-Meier curves for progression-free survival and overall survival of patients (A, B) with fewer than 50 and ≥ 50 circulating tumor cells (CTCs) per 7.5 mL of blood at baseline, (C, D) without the presence of circulating tumor microemboli (CTM) and with the presence of CTM at baseline, and (E, F) without the presence of apoptotic CTCs (ApopCTC) and with the presence of ApopCTCs at baseline (n = 97).

Furthermore, post one cycle of chemotherapy, if CTC counts persist above the threshold, the patient‘s prognosis deteriorates (data provided below), with the HR for PFS increasing from 2.70 to 6.28 and for OS from 3.55 to 8.63, suggesting that patients with poor chemotherapy responses (CTCs >= 50/7.5mL) exhibit significantly worse prognoses (data below).



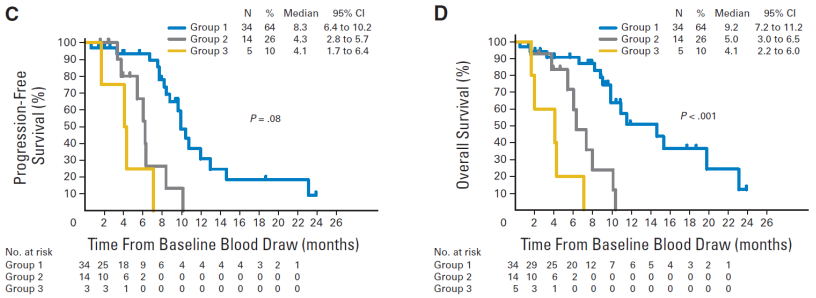



**Fig 2.** Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) of patients (A, B) with fewer than 50 and ≥ 50 circulating tumor cells (CTCs)/7.5 mL of blood at second time point (after one cycle of standard chemotherapy). When both time points (before and after one cycle of chemotherapy) are considered, (C) PFS and (D) OS of patients with fewer than 50 CTCs at both time points (group 1), ≥ 50 CTCs at baseline, fewer than 50 CTCs at second time point (group 2), and ≥ 50 CTCs at both time points (group 3) are shown.

In subsequent studies by Naito (2012), Hiltermann (2012), Shen (2017), and Messaritakis (2017), similar research findings were published. These studies included both limited and extensive-stage small cell lung cancer (SCLC) patients, encompassing different treatment phases such as pre-treatment, after one treatment cycle, and post-relapse. Results consistently indicated that CTC count was an independent prognostic factor for patient outcomes.

In 2016, Professor Ying Cheng from Jilin Province Cancer Hospital reported analogous multicenter research results. This study specifically involved 91 extensive-stage SCLC patients and conducted CTC detection at three different time points: before treatment, after the second chemotherapy cycle, and at disease progression. The findings demonstrated that changes in CTC counts post-treatment effectively distinguished between treatment-resistant and treatment-sensitive patients (data shown in the Figure below). Professor Cheng's research revealed that patients with persistently high CTC counts pre and post-treatment had the worst prognosis (both in terms of PFS and OS). Patients with consistently low CTC counts before and after treatment had the best overall survival.

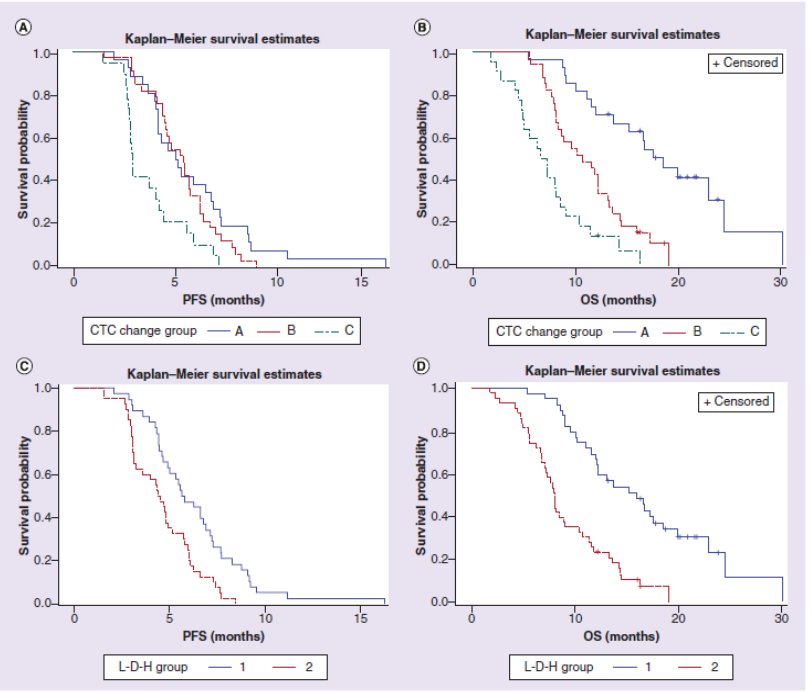



**Figure 4. Kaplan–Meier curves for progression-free survival and overall survival with circulating tumor cell changes. (A & B)** Show the PFS and OS after grouping according to the CTC change: <10 CTCs per 7.5 ml at both baseline and after the second cycle of chemotherapy (group A, blue); CTCs ≥10 per 7.5 ml at baseline and <10 per 7.5 ml after the second cycle of chemotherapy (group B, red); and CTCs ≥10 (or <10) per 7.5 ml at baseline and ≥10 per 7.5 ml after the second cycle of chemotherapy (group C, green). **(C &D)** Show the PFS and OS after grouping according to the absolute value of the CTC change: <10 CTCs per 7.5 ml at baseline and after the second cycle of chemotherapy or a CTC drop >150 per 7.5 ml (L-D-H = 1; blue); and ≥10 CTCs per 7.5 ml at baseline and after the second cycle of chemotherapy or a CTC drop ≤150 per 7.5 ml (L-D-H = 2; red).
CTC: Circulating tumor cell; L-D-H: Low-drop-high; OS: Overall survival; PFS: Progression-free survival.

# Afterwords

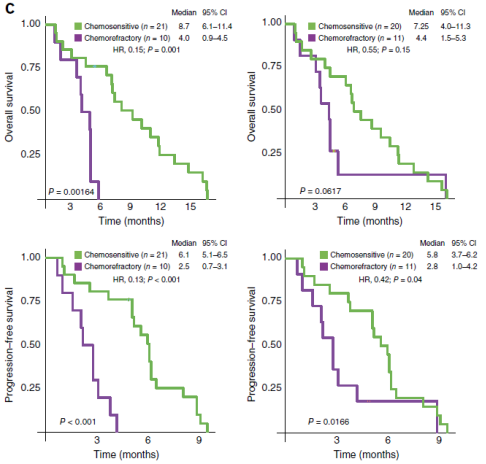
Whether in early or late stages of both non-small cell lung cancer and small cell lung cancer, the presence of CTCs significantly predicts poorer patient prognosis, including shorter progression-free survival and overall survival. Monitoring CTC count variations pre- and post-treatment can effectively reflect or predict treatment efficacy. Dynamic CTC monitoring across different stages of lung cancer provides precise information for physicians and patients, aiding in more accurate treatment planning.

However, the clinical significance of circulating tumor cells goes beyond their numbers. Various proteins and genetic information carried on the cell surface and within can offer additional clinical insights. For instance, CTC PD-L1 detection can provide more accurate results for the emerging field of tumor immunotherapy efficacy assessment. A study published in 《Nature Medicine》 in 2017 suggested that abnormal copy number variations (CNVs) in CTCs of SCLC patients could accurately predict resistance to chemotherapy (Figure below). With ongoing advances in organoid culture technique, capturing live CTCs can enable the establishment of CTC-derived organoids for metastatic lung cancer patients, facilitating the testing of drug combinations and the more effective treatment strategies targeting metastatic lesions.

b

| | Patient | Clinical status | CNA classifier | Accuracy |
|---|---|---|---|---|
| Training set | P1 | Sensitive | Sensitive | 100% (13/13) |
| | P2 | Sensitive | Sensitive | |
| | P3 | Sensitive | Sensitive | |
| | P4 | Sensitive | Sensitive | |
| | P5 | Sensitive | Sensitive | |
| | P6 | Sensitive | Sensitive | |
| | P7 | Sensitive | Sensitive | |
| | P8 | Refractory | Refractory | |
| | P9 | Refractory | Refractory | |
| | P10 | Refractory | Refractory | |
| | P11 | Refractory | Refractory | |
| | P12 | Refractory | Refractory | |
| | P13 | Refractory | Refractory | |
| Testing set | P14 | Sensitive | Sensitive | 83.3% (15/18) |
| | P15 | Sensitive | Sensitive | |
| | P16 | Sensitive | Sensitive | |
| | P17 | Sensitive | Sensitive | |
| | P18 | Sensitive | Refractory | |
| | P19 | Sensitive | Sensitive | |
| | P20 | Sensitive | Sensitive | |
| | P21 | Sensitive | Sensitive | |
| | P22 | Sensitive | Sensitive | |
| | P23 | Sensitive | Sensitive | |
| | P24 | Sensitive | Sensitive | |
| | P25 | Sensitive | Sensitive | |
| | P26 | Sensitive | Refractory | |
| | P27 | Sensitive | Sensitive | |
| | P28 | Refractory | Sensitive | |
| | P29 | Refractory | Refractory | |
| | P30 | Refractory | Refractory | |
| | P31 | Refractory | Refractory | |
| | | | | 90.3% (28/31) |

c




Medical progress stems from the continuous development of scientific technology and the willingness of clinical professionals to learn and experiment with new techniques. Whether it's the detection of circulating tumor cells or circulating tumor DNA, many challenges remain. However, clinical research should not stagnate but actively employ these new technological tools in clinical diagnosis, treatment, and research. Experience gained from this usage should prompt higher expectations for developers while offering better assistance to patients.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