

# Clinical utility of circulating tumor cells in patients with non-small-cell lung cancer

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**Abstract:** Several different studies have addressed the role of the circulating tumor cells (CTC) in non-small-cell lung cancer (NSCLC). In particular, the potential of CTC analysis in the early diagnosis of NSCLC and in the prediction of the outcome of patients with early and advanced NSCLC have been explored. A major limit of these studies is that they used different techniques for CTC isolation and enumeration, they employed different thresholds to discriminate between high- and low-risk patients, and they enrolled heterogeneous and often small cohort of patients. Nevertheless, the results of many studies are concordant in indicating a correlation between high CTC count and poor prognosis in both early and advanced NSCLC. The reduction of CTC number following treatment might also represent an important indicator of sensitivity to therapy in patients with metastatic disease. Preliminary data also suggest the potential for CTC analysis in the early diagnosis of NSCLC in high-risk individuals. However, these findings need to be confirmed in large prospective trials in order to be transferred to the clinical practice. The molecular profiling of single CTC in NSCLC might provide important information on tumor biology and on the mechanisms involved in tumor dissemination and in acquired resistance to targeted therapies. In this respect, xenografts derived from CTC might represent a valuable tool to investigate these phenomena and to develop novel therapeutic strategies.

**Keywords:** Biomarkers; circulating tumor cells (CTC); non-small-cell lung cancer (NSCLC); prognosis; metastasis

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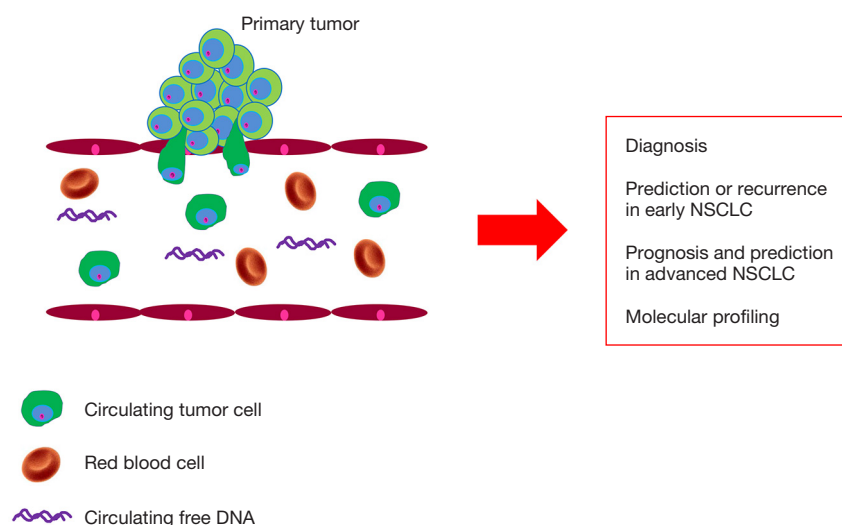
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## Introduction

Experimental evidence suggests that dissemination of tumor cells is an early event in the progression of solid tumors (1). Through a process called epithelial-mesenchymal transition (EMT), cancer cells acquire the ability to invade the basal membrane and to enter the systemic circulation. Disseminated tumor cells (DTC) have indeed been

identified at early stages of tumor progression in regional lymph nodes, peripheral blood, and in bone marrow of cancer patients using highly sensitive detection methods. In this respect, the term DTC refers mainly to cells that home in the bone marrow, whereas circulating tumor cells (CTC) are rare tumor cells that can be isolated from the peripheral blood (2). Only a small fraction of CTC will eventually form a metastasis at distant organs, where tumor cells



**Figure 1** Potential clinical applications of CTC. CTC, circulating tumor cells; NSCLC, non-small-cell lung cancer.

regain the epithelial phenotype (mesenchymal to epithelial transition, MET).

Identification of CTC can provide relevant prognostic and predictive information (*Figure 1*). CTC might allow early detection of tumor development in high-risk individuals. In patients with limited, surgically resectable disease the presence of CTC might suggest a metastatic spread, thus providing information that could help to guide treatment decisions before the onset of overt metastases. The levels of CTC have been shown to be associated with prognosis in different malignancies, in which they reflect the tumor burden and the biological aggressiveness of the disease (3). CTC variation during systemic treatment of patients with metastatic disease treatment has also been shown to reflect drug sensitivity. Finally, molecular characterization of CTC might provide new insights into the mechanisms of tumor metastatization and allow identifying actionable genetic alterations.

All the above potential applications of CTC detection have been explored in non-small-cell lung cancer (NSCLC). NSCLC is the most frequent form of lung cancer, accounting for approximately 85% of all lung cancers (4). Diagnosis of NSCLC occurs frequently in advanced stage of the disease and, therefore, the prognosis of this tumor is extremely poor (5). In this respect, biomarkers that might allow early detection of NSCLC, assessment of prognosis and identification of potential targets for therapeutic intervention are definitely needed in order to improve the prognosis of this deadly disease.

This review article will summarize the different applications of CTC in NSCLC, from early diagnosis to risk assessment in patients with early or advanced NSCLC. By doing this, we will try to draw a complete picture of current and future developments of CTC research and clinical applications in NSCLC.

### CTC as a diagnostic marker in NSCLC

Because tumor cell dissemination is a relatively early event in cancer progression, identification of CTC might allow early diagnosis of NSCLC. To this end, it is mandatory to demonstrate that the detection method employed to identify CTC is able to discriminate between healthy subjects, individuals with non-malignant lung disease and lung cancer patients.

Several studies have addressed the potential of CTC identification in the diagnosis of NSCLC. These studies used different approaches, based on either the identification of epithelial cell markers in the cellular fraction of peripheral blood or the isolation and characterization of putative epithelial cells.

Sheu and collaborators (6) found that a 17-gene membrane array-based assay could detect circulating cancer cells in 90% of NSCLC patients at different stages of disease and in 6% of healthy controls. The test was positive also in 3/20 breast cancer patients, 3/15 colorectal cancer patients and 2/12 gastric cancer cases. The sensitivity of the assay was significantly correlated with the presence

of distant metastases and disease stage, with the lowest sensitivity (78.8%) found in stage I patients.

Different studies have used the folate receptor transcript as a marker to identify the CTC (7-9). In fact, folate receptor is often expressed in NSCLC cells whereas its levels of expression are usually low in blood cells. These studies have shown that by using folate receptor as a marker it is possible to discriminate between NSCLC patients, individuals with benign lung disease and healthy subjects. A sensitivity up to 77.7% and a specificity up to 89.5% for the diagnosis of NSCLC was reported (7,9). However, it must be emphasized that the sensitivity was higher for stage IV patients, while it was <70% in stage I.

The isolation by size of epithelial tumor cells (ISET) technology coupled with cytomorphological evaluation of isolated cells can allow the identification of CTC, which have been also defined as circulating non-hematologic cells (CNHCs). ISET identified CHNCs with malignant features at a significantly higher frequency in patients with malignant disease as compared with subjects with non-malignant pathologies (10). By using this approach, Ilie and coworkers (11) examined the presence of CTC in 168 patients with chronic obstructive pulmonary disease (COPD). These patients had no clinically detectable lung cancer and the presence of CTC was explored in complement to CT scan for early lung cancer diagnosis. CTC with malignant features were detected in 5/168 patients (3%) of COPD patients. CT-scan screening detected lung nodules 1 to 4 years after CTC detection in all CTC-positive COPD patients. Histopathological diagnosis following surgical resection confirmed the diagnosis of early-stage lung cancer. The patients were without tumor recurrence and without CTC 16 months after surgery thanks to this early diagnosis. Importantly, patients with COPD without CTC (n=160) as well as patients with CTC with benign features (3/168; 1.8%) did not develop lung nodules during the follow up. Although preliminary, these findings confirm the potential of CTC detection for early diagnosis of NSCLC in patients at high risk to develop lung cancer.

More recently, a size-based microfluidic chip for detecting single CTC and CTC clusters allowed for the detection of CTC in lung cancer patients with apparent high sensitivity and specificity when compared to healthy controls (82.7% sensitivity and 100% specificity) (12). Interestingly, the levels of CTC in stage I-II patients were lower as compared with patients in advanced stage or with local recurrence of the disease, thus reinforcing the need for high sensitive techniques for early diagnosis of lung cancer

through CTC detection.

### Detection and prognostic value of CTC in early NSCLC

Although the number of CTC generally correlates with tumor stage, CTC can be detected in early NSCLC. A number of studies have addressed the role of surgical manipulation on the dissemination of CTC as well as the prognostic significance of CTC detection before and/or after surgical resection of lung tumors.

It has long been hypothesized that any manipulation of the tumor mass might facilitate the release of tumor cells in the bloodstream, although the implications of this phenomenon in the dissemination of the disease have not been elucidated. In this respect, a recent study analyzed CTC in peripheral vein blood samples obtained before and after lung tumor biopsy using flexible fiber-topic bronchoscopy. In 2/6 patients, CTC were absent before bronchoscopy but they were detected after lung biopsy (13). In early lung cancer, CTC can be detected in the pulmonary vein (PV) blood in addition to the peripheral blood. A number of studies have shown that the levels of CTC are usually higher in PV blood samples as compared with peripheral draws, suggesting that CTC might be in part removed by the circulation after shedding from the tumor mass (14-17). Interestingly, Reddy *et al.* found that PV CTC were more frequently detected in patients who underwent preoperative bronchoscopic biopsy (8/9 cases), compared with patients who underwent CT-guided biopsy (4/12) (16). Although the limited number of patients included in these studies prevents any firm conclusion, these findings confirm the possibility that biopsy of tumor mass might lead to tumor cell dissemination.

Surgical manipulation of the primary lung tumor might also contribute to CTC dissemination. In this respect, Hashimoto and collaborators (15) found that the median number of CTC in PV samples increased from 4 to 60 in 30 consecutive primary lung cancer patients who underwent lobectomy through open thoracotomy. An increase in CTC during and/or after surgery has also been observed in studies that assessed peripheral blood samples using different detection methods (18-21). Interestingly, CK19 and CEA transcripts have shown a different peri-operative kinetic when used as markers of CTC dissemination (20). The value of CK19 mRNA during the operation was significantly higher than that of pre-operation and post-operation; in contrast, the post-operative levels of CEA

mRNA were significantly higher than those of pre-operation and those of operative day. Because CK19 mRNA is expressed in all epithelial cells, this might indicate that surgery leads to release of CK19 mRNA from non-transformed cells and, therefore, it might be not an adequate marker for CTC detection.

The correlation between surgical techniques and CTC dissemination has been also explored. Hashimoto and collaborators (15) reported that the increase in CTC count after surgery was not significantly associated with the sequence of vessel interruption (pulmonary artery first versus PV first). In contrast, Ge and co-workers (20) found higher levels of CEA mRNA in patients had the ligation of the pulmonary artery first as compared with PV first. However, it must be emphasized that no difference in survival using different sequences of pulmonary vessel ligation has been described (18). A more frequent increase in CTC has also been reported in patients undergoing limited resection as compared with pneumonectomy (22), as well as in those receiving video-assisted lobectomy as compared with open lobectomy (18).

Taken together, these studies have shown that surgical manipulation might indeed induce CTC dissemination. However, the observation that CTC can be detected before surgical manipulation in selected early NSCLC patients, suggests that tumor dissemination might precede surgical intervention. In this respect, the above summarized studies did not provide a formal demonstration that the surgical techniques might affect patients' prognosis through CTC dissemination.

A number of studies have addressed the prognostic value of CTC detection in early NSCLC (*Table 1*). Different mRNA markers have been employed to track CTC in the peri-operative period in patients undergoing surgical resection of primary NSCLC with curative intent. In a prospective study of 103 consecutive NSCLC patients who underwent a curative lobectomy, pre- and post-operative CEA mRNA levels were assessed in peripheral blood samples (23). At multivariate analysis, lack of CEA mRNA expression was an independent prognostic factor for overall survival (OS) with a hazard ratio (HR) of 0.21. Yie and collaborators (24) evaluated the levels of expression of survivin in pre-operative peripheral blood samples obtained from 67 stage I-III NSCLC patients. With a median follow-up period of 18 months (range 3–36 months), the detection of survivin-expressing CTC was found to be an independent negative predictor for cancer recurrence (HR 43.5) and survival (HR 1.35) at multivariate analysis.

Thyroid transcription factor-1 (TTF-1) is expressed only in epithelial cells of the thyroid and in bronchioloalveolar cells of the lung. Because expression of TTF-1 is frequent in lung adenocarcinoma (31), its expression has been used to identify neoplasms with a pulmonary origin. In this respect, the CTC status in pre- and post-surgery peripheral blood samples from 79 early NSCLC patients was investigated using TTF-1 and CK19 mRNA markers (25). The presence of post-surgery TTF-1 positive CTC was the strongest prognostic markers, resulting associated with earlier disease progression (P=0.004) and shorter progression-free survival (PFS) (P=0.004) at univariate analysis. In addition, patients with post-surgery TTF-1 positive CTC—but not pre-surgery—showed shorter PFS than other patients (P<0.001). However, these findings were not confirmed at multivariate analysis. Assessment of CTC at multiple time points might increase the predictive value of the assay. Li and collaborators (26) assessed the CTC status in 68 stage I-III NSCLC patients before surgery, after surgery and after completion of adjuvant chemotherapy. The LUNX mRNA whose expression is strictly limited to normal lung tissue and NSCLC was used as marker. Patients with LUNX mRNA-positive CTC at 3 time points had a shorter DFS and OS than those with positive pre-operative samples but negative post-operative and post-adjuvant therapy samples. Multivariate analysis confirmed that the presence of LUNX mRNA-positive CTC either after surgery or after the completion of adjuvant therapy were independent factors for worse DFS (HR 2.14 and 2.17, respectively) and OS (HR 2.17 and 2.25, respectively).

Few studies have addressed the role of CTC in early NSCLC by using the CellSearch system to isolate and detect CTC. In a small study by Sawabata and collaborators (21) that enrolled only 9 patients, 1 patient was positive for CTC before surgery and 3 were positive immediately after the operation. However, CTC were not detected in any of these patients 10 days after surgery. With a median follow-up of 14 months, no cancer recurrence was observed in any of the cases. The CellSearch was also used to detect CTC in the peripheral and PV blood samples of 30 consecutive lung cancer patients who underwent thoracotomy (14). Both peripheral and PV CTC status did not correlate with recurrence of the disease observed during a median follow-up of 13 months. The limited number of patients and the short follow up period of these two studies might have significantly affected the reported results. In this respect, completely different conclusions were drawn by Crosbie and co-workers (17) who assessed the CTC and the

**Table 1** Prospective studies evaluating CTC in early stage NSCLC

Author	Detection method (marker)	Time point of draw	No. pts	Stage	Main findings
Yamashita (23)	RT-PCR (CEA)	Pre- and post-operative	103	I-III	Preoperative CEA mRNA expression was an independent prognostic factor for OS (HR 0.21), at multivariate analysis
Yie (24)	RT-PCR (Survivin)	Pre-operative	67	I-III	Positivity for survivin mRNA-CTC was an independent negative predictor for cancer recurrence (HR 43.5) and survival (HR 1.35) at multivariate analysis
Yoon (25)	Nested RT-PCR (TTF-1)	Pre- and post-operative	79	I-III	Post-surgery TTF-1 <sup>+</sup> CTC-positive status was associated with earlier disease progression (P=0.004) and shorter PFS (P=0.004) than TTF-1 <sup>+</sup> CTCs-negative status
Li (26)	RTqRT-PCR (LUNX)	Pre- and post-operative	68	I-III	On multivariate analysis, LUNX mRNA-positive CTC after surgery and after the completion of adjuvant therapy were an independent factor for worse DFS (HR 2.14 and 2.17) and OS (HR 2.17 and 2.25)
Sawabata (21)	CellSearch	Pre- and post-operative	9	IA-IB	Detection of CTC before or after surgery was not correlated with recurrence
Okumura (14)	CellSearch	Intra-operative (peripheral and PV)	30	I-IV	No significant correlation between CTC count in pulmonary vein and peripheral blood with recurrence of the disease
Crosbie (17)	CellSearch	Pre- and intra-operative (peripheral and PV)	30	I-III	Peripheral CTC count $\geq 1$ and pulmonary vein CTC count $\geq 18$ were independent risk factors for disease recurrence. Patients with at least one CTM detected in PV samples or $\geq 2$ CTC in peripheral had an HR 8.44 for recurrence and HR 7.53 for death at multivariate analysis
Hofman (27)	ISET (Vimentin, pan cytokeratin)	Pre-operative	208	I-IV	Preoperative CTC number $\geq 50$ was correlated with a shorter DFS (HR 2.631) and OS (HR 2.096) at multivariate analysis
Hofman (28)	CellSearch & ISET (vimentin, pan-cytokeratin)	Pre-operative	210	I-IV	Presence of CTC as detected by CellSearch (HR 1.564) or ISET (HR 1.372) or by both methods (HR 1.235) was a significantly independent prognostic factor for shorter DFS in the multivariate analysis
Sienel (29)	Ficoll-Hypaque (Pan-cytokeratin)	Pre-operative (PV)	62	I-III	Presence of CTC was an independent prognostic factor of shorter cancer-related survival in patients without involvement of mediastinal lymph nodes at multivariate analysis (P=0.004)
Bayarri-Lara (30)	MACS (EpCAM)	Pre- and post-operative	56	I-III	At multivariate analysis, CTC status after surgery was significantly correlated with a shorter DFS (HR =5.75)

No. pts, number of patients; OS, overall survival; PFS, progression-free survival; DFS, disease-free survival; HR, hazard ratio; PV, pulmonary vein blood; RT-PCR, reverse transcriptase-polymerase; RTqPCR, real-time quantitative polymerase chain reaction; ISET, isolation by size of epithelial tumor cells; CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1; CTC, circulating tumor cells; CTM, circulating tumor microemboli.

circulating tumor microemboli (CTM) in both peripheral and PV blood from 30 patients with early NSCLC. A CTC number  $\geq 1$  in peripheral vein and  $\geq 18$  in PV samples were independent risk factors for lung cancer recurrence (P=0.01 and P=0.001, respectively). PV CTC count was also an independent risk factor for death (P=0.01). CTM were only detected in 20% of PV blood samples. Interestingly, the

combination of CTC analysis in both peripheral and PV increased the predictive value of the test. High-risk patients with at least one CTM detected or  $\geq 2$  CTC in peripheral blood demonstrated a significantly shorter DFS and OS. Multivariate analysis confirmed the predictive value of combined CTM and CTC detection. In fact, in the high-risk group the HR for lung cancer recurrence and death



were 8.44 and 7.53, respectively.

By using the ISET technology, CTC were found in pre-surgery peripheral blood samples from 102/208 (49%) patients undergoing curative surgery for NSCLC (27). A number of CTC  $\geq 50$  was significantly associated with shorter OS and DFS. Multivariate analysis confirmed that the presence of  $\geq 50$  CTC in the pre-operative sample was correlated with a shorter DFS (HR 2.631) and OS (HR 2.096). The same research group compared the ISET and CellSearch technologies in 210 consecutive patients undergoing radical surgery for NSCLC (28). Survival data demonstrated that patients without CTC had a significantly longer DFS compared to patients with CTC detected by either CS alone, ISET alone or both techniques. At multivariate analyses, the presence of CTC detected by either the CellSearch (HR 1.564) or ISET (HR 1.372) methods or by both techniques (HR 1.235) was an independent significant prognostic factor for shorter DFS.

Disseminated cancer cells were observed in pulmonary venous blood of 11/62 patients with resected primary NSCLC, using immunocytochemical staining of cytopins with a pancytokeratin antibody (29). Detection of CTC was an independent significant prognostic factor of shorter cancer-related survival in patients without involvement of mediastinal lymph nodes. Finally, Bayarri-Lara and collaborators (30) assessed CTC in peripheral blood samples obtained from 56 NSCLC patients who underwent radical surgery, before and one month after surgery. CTC were enriched with MACS technology and identified by immunocytochemical methods. The presence of CTC 1 month after surgery was significantly associated with a shorter DFS as confirmed at multivariate analysis (HR 5.75).

### Prognostic and predictive value of CTC in advanced NSCLC

Several different studies have assessed the role of CTC count in patients with advanced NSCLC. Although the CellSearch system was the most frequently used technique for CTC detection in this setting (Table 2), several studies used different methods based either on antibody-mediated identification of CTC or expression of tumor-specific transcripts (Table 3). One of the major drawbacks of CTC studies in metastatic NSCLC is that heterogeneous cohorts of patients have been enrolled. Therefore, we tried to group and discuss together clinical studies that enrolled patients with similar pathologic features and treatments.

Several studies have addressed the prognostic value of

CTC in chemo-naïve advanced or metastatic NSCLC patients that received standard chemotherapy as first-line systemic therapy. Krebs and co-workers assessed the prognostic value of CTC count with the CellSearch System in 101 NSCLC patients (14 stage IIIA, 27 stage IIIB and 60 stage IV) who received standard chemotherapy (32). Notably, before administration of chemotherapy only 9 patients had  $\geq 5$  CTC. Nevertheless, at univariate analysis a count  $\geq 5$  CTC before administration of chemotherapy was associated with a shorter PFS (6.8 versus 2.4 months;  $P < 0.001$ ) and OS (8.1 versus 4.3 months;  $P < 0.001$ ) as compared with  $< 5$  CTCs. The prognostic value of CTC count at baseline was confirmed at multivariate analysis (HR for OS 7.92). When the CTC levels before and after one cycle of chemotherapy were considered, a CTC count  $< 5$  at both time points was an even stronger predictive marker (HR for OS 15.65). Interestingly, a reduction in CTC count after administration of chemotherapy was also predictive of longer PFS (5.4 versus 1.9 months; log-rank test  $P < 0.001$ ) and OS (8.3 versus 3.3 months; log-rank test  $P < 0.009$ ) (32).

A prospective trial of 43 stage IV NSCLC patients confirmed at univariate analysis that a CTCs count  $\geq 5$  before administration of first-line chemotherapy correlated with worse PFS and OS ( $P = 0.034$  and  $P = 0.008$ , respectively) (33). However, at multivariate analysis only the correlation of CTC count with PFS was statistically significant (HR 4.3;  $P = 0.016$ ). An exploratory analysis also suggested that an increase of CTC count after chemotherapy was predictive of progressive disease and shorter PFS (33).

In contrast, the prognostic value of CTC count with the CellSearch system was not confirmed by Hirose and collaborators (34). Among 33 chemo-naïve stage IV NSCLC patients, 12 (36.4%) had at least 1 CTC in the peripheral blood and 5 (15.2%) had  $\geq 5$  CTCs. Patients with one or more CTC showed an higher rate of progressive disease (66.7%) as compared with CTC-negative patients (23.8%;  $P = 0.02$ ) following chemotherapy treatment. However, the presence of CTC did not correlate with PFS and OS (34).

The correlation with survival of CTC counts was not reported by Xu and collaborators, who identified peripheral blood CTCs in 47/66 (71.2%) chemo-naïve stage IV NSCLC patients by using the CellSearch (35). However, they found that the number of patients with  $\geq 3$  CTCs decreased significantly after two cycles of chemotherapy. In this respect, a count  $\geq 3$  CTCs after two cycles was associated with an higher rate of progressive disease ( $P < 0.05$ ) (35).

Two additional studies have investigated the prognostic

**Table 2** Studies of CTC in advanced and metastatic NSCLC using the CellSearch system

Author	Blood sample collection	Stage	Treatment	No. pts	Threshold level	Main findings
Krebs (32)	Before and after cycle 1	III-IV	First-line CT	101	5	Multivariate analysis showed the prognostic value of a CTC count <5 at day 0 (HR for OS 7.92; P<0.001) or at both day 0 and after cycle 1 (HR for OS 15.65; P<0.001)
Muinelo-Romay (33)	Before cycle 1, 2 and 5	IV	First-line CT	43	5	Multivariate analysis showed the prognostic value of a CTC count <5 at day 0 for PFS (HR 4.3; P=0.016) but not OS (HR 2.9; P=0.11)
Hirose (34)	Before cycle 1 and after cycle 2	IV	First-line CT	33	1	The presence of CTC at day 0 did not correlate with PFS and OS
Xu (35)	Before cycle 1, 2 and 3	IV	First-line CT	66	3	A CTC count $\geq 3$ was associated with higher rate of PD (P<0.05)
Juan (36)	Before cycle 1 and 3	IIIB-IV	CT	37	2	No significant difference between patients with CTCs <2 and CTCs $\geq 2$
Tarumi (37)	At surgery (PV)	IIIA	I-CT+RX	9	2	The patients who achieved complete pathological response after I-CT+RX were negative for PV-CTC, whereas those with major/minor response were positive
Punnoose (38)	Before treatment and at days 4, 28 and 56	IV	EGFR TKI	41	5	Higher baseline CTC counts were associated with response to treatment (P=0.009).
Isobe (39)	Before treatment	IV	EGFR TKI	24	1	Presence of at least one CTC was associated with a shorter OS (HR 2.9, P=0.012) at univariate analysis

No. pts, number of patients; CT, chemotherapy; TKI, tyrosine kinase inhibitor; PV, pulmonary venous; I-CT+RX, induction chemoradiotherapy.

role of CTC on chemo-naïve patients receiving standard chemotherapy using different methods for CTC count (Table 3). Zhang and collaborators used a gradient-centrifugation method (Cytel) to investigate CTC in 46 stage III-IV NSCLC patients (40). Patients with a CTC count <8 had a better OS (21.3 versus 9 months) and PFS (7.4 versus 5.3 months) as compared with those having  $\geq 8$  CTC. Multivariate analysis confirmed the correlation between low CTC count and both PFS (HR 0.36; P=0.022) and OS (HR 0.316; P=0.014) (40).

By using survivin mRNA as surrogate biomarker for CTC detection, CTC were detected in 54/78 (69.2%) stage IIIB and IV NSCLC before administration of chemotherapy, in 41 (52.6%) after cycle 1 of chemotherapy and in 38 (50.7%) after cycle 3 (41). The presence of survivin transcripts before chemotherapy was significantly associated with shorter PFS (P=0.004) and OS (P=0.007) at univariate analysis. Multivariate analysis confirmed that the presence of CTC at any time (before chemotherapy, cycle 1 and 3) or at cycles 1 and 3 was associated with a shorter PFS (HR 1.87 and 1.46, respectively) and OS (HR 2.18 and 1.97,

respectively) (41).

The prognostic value of CTC in patients receiving chemotherapy has been addressed in other studies that enrolled both chemo-naïve and pre-treated patients.

Nieva and co-workers assessed CTC by three color immunofluorescence imaging in 28 either chemo-naïve or previously treated NSCLC patients who received cytotoxic chemotherapy or EGFR kinase inhibitor (42). A CTC count  $\geq 5$ /mL predicted a HR for death four times higher as compared with lower CTC count (P=0.0084) at univariate analysis.

CTC assessment with the CellSearch system was not prognostic in a study that analyzed 37 fragile patients (36). In fact, no significant association between CTC count and both PFS and OS was observed using a threshold of two CTC to discriminate between high- and low-CTC count.

The probability to detect CTC is increased by using multiple markers for their identification. For example, when transcripts for tumor specific antigen 9 (TSA-9), keratin 19 (KRT-19), and pre-progastrin releasing peptide (Pre-proGRP) were analyzed by nested reverse

**Table 3** Prospective studies of CTC in advanced and metastatic NSCLC using different methods for CTC enrichment and detection

Author	Detection method (marker)	Blood samples	Stage	Therapy	No. pts	CTC threshold level	Main findings
Zhang (40)	Cyttl	Before and after cycle 1	III-IV	First-line CT	46	8	At multivariate analysis a CTC count <8 was significantly correlated with better PFS (HR 0.36; P=0.022) and OS (HR 0.316; P=0.014)
Du (41)	RTqPCR (survivin)	Before cycle 1; after cycle 1 and 3	IIIB-IV	First-line CT	78	Marker positive	At multivariate analysis the presence of CTC at any time or at cycles 1 and 3 was associated with a shorter PFS (HR 1.87, P=0.011 and HR 1.46, P=0.023, respectively) and OS (HR 2.18, P=0.006 and HR 1.97, P=0.013, respectively)
Nieva (42)	IF (pan-cytokeratin)	Before cycle 1; at week 3, 6 and 9; at 12 months	IV	CT/TKI	28	5	A CTC count $\geq 5$ /mL predicted a HR for death 4 times higher as compared with lower CTC count (P=0.0084) at univariate analysis
Liu (43)	Nested RT-PCR (TSA-9, KRT-19, Pre-proGRP)	Before cycle 1	III-IV	CT	103	$\geq 0$ marker positive	Multi-marker positive patients had a significant shorter OS as compared with negative patients at multivariate analysis (P<0.05)
Sher (44)	Nested RTqPCR (KRT19, UCH-L1, FN precursor, TRIM28)	Before cycle 1	IIIB-IV	CT	35	$\geq 5$ marker positive	Higher load of cancer cells was correlated with a shorter OS (P=0.006) at univariate analysis
Chen (45)	Nested RT-PCR (CK19)	Before and after treatment	I-IIIB	CT+RX	67	Marker positive	At multivariate analyses the positivity of CK19 mRNA after CT+RX was an independent unfavorable prognostic factor for both OS and PFS (HR 3.717 and 3.534, respectively)
He (46)	Flow cytometry (CK18)	Before and after treatment	IV	TKI	66	Median value (68.5)	The response rate, PFS and OS were superior in the low CTC group (CTC count < median value) as compared with the high CTC group

No. pts, number of patients; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; PD, progression of disease; CT, chemotherapy; TKI, tyrosine kinase inhibitor; RTq-PCR, real-time quantitative polymerase chain reaction; IF, immunofluorescence; PET, positron emission tomography; RX, radiotherapy.

transcriptase (RT)-PCR, 113/134 (84.3%) blood samples from lung cancer patients were positive for at least one of these markers (43). Patients who were positive for more than one marker had a significant shorter OS as compared with negative patients (P<0.01). The predictive value of positivity for multiple marker was confirmed at multivariate Cox regression analysis (P<0.05) (43). However, it must be emphasized that a heterogeneous population of patients was enrolled in this study.

Sher and co-workers used homo sapiens keratin 19 (KRT19), ubiquitin thiolesterase (UCH-L1), highly similar to HSFIB1 for fibronectin (UCH-L1), and tripartite motif-containing 28 (TRIM28) as marker transcripts to investigate CTC by semi-quantitative RT-PCR in 54 patients with advanced NSCLC. The detection rate by using this

approach was 72%. At univariate analysis, the presence of a high CTC load correlated with a shorter OS in the 35 stage IIIB and IV patients who received systemic chemotherapy (P=0.006) (44).

The predictive value of CTC has been explored in studies in which NSCLC patients received multimodality treatments. A small study assessed both peripheral and PV CTC in locally advanced patients who received either induction chemoradiotherapy followed by surgery or surgery alone, by using the CellSearch system (37). The patients who achieved complete pathological response after induction chemoradiotherapy were negative for PV CTC, whereas those achieving major/minor response were positive. In addition, all patients treated with surgery alone were positive for PV CTC. However, no correlation with



survival was described in this study.

A fraction of patients with early or locally advanced NSCLC are treated with chemo-radiation when surgery is not feasible because of co-morbidity or for the extension of the disease. In this regard, CK19 transcript was measured by nested RT-PCR in peripheral blood from 67 stage I–IIIB NSCLC patients (6 stage I–II, 16 stage IIIA, 45 stage IIIB) before and after chemo-radiation (45). Although CK19 status before chemo-radiation did not permit correlate with survival, the patients with CK19 mRNA expression after treatments had worse OS ( $P < 0.001$ ) and PFS ( $P < 0.001$ ) as compared to those with negative CK19 expression. Positivity of CK19 mRNA after chemo-radiation was confirmed to be an independent unfavorable prognostic factor for both OS and PFS at multivariate analyses (HR 3.717 and 3.534, respectively).

Finally, CTC count has been evaluated in studies of EGFR tyrosine kinase inhibitors (TKI) in NSCLC. A single-arm phase II trial of erlotinib and pertuzumab enrolled 41 patients with relapsed or refractory metastatic NSCLC in whom CTC were assessed with the CellSearch (38). Importantly, patients were not selected for the presence of EGFR mutations. At least one CTC was identified before treatment in 28/37 (76%) patients with evaluable samples. A high baseline CTC count was correlated with response to treatment by RECIST ( $P = 0.009$ ) and with radiographic response ( $P = 0.02$ ), but not with PET response and PFS. However, a decrease in CTC count following treatment predicted a longer PFS (38).

Isobe and co-workers assessed the prognostic value of CTC in EGFR mutant patients who progressed following treatment with EGFR TKI. At least one CTC was detected in 8/24 (33.3%) cases using the CellSearch system. Patients with CTC had a significantly shorter OS as compared with those without CTC (3.0 months *vs.* not reached; HR 2.9) (39).

CTC were assessed by flow cytometry in 66 EGFR mutant advanced NSCLC patients (46). Patients were divided in two groups (high CTC count *vs.* low CTC count) based on the median CTC count of 68.5. The response rate, PFS and OS were superior in the low CTC group as compared with the high CTC group at univariate analysis ( $P < 0.05$ ,  $P < 0.005$  and  $P < 0.01$ , respectively).

### Conclusions and future perspectives

It is difficult to derive conclusions on the clinical utility of CTC count in NSCLC. An overview of the studies summarized in this article suggests that the detection

of CTC has potential for early diagnosis as well as for prognostic assessment in early and advanced NSCLC. In agreement with this conclusion, a previous meta-analysis found that the presence of CTC indicates a poor prognosis in patients with NSCLC (47). However, after more than 15 years of research in this field, assessment of CTC did not enter in the routine clinical management of NSCLC patients.

A number of issues are indeed limiting the transfer of CTC count to the clinical scenario.

One of the major drawbacks is represented by the fact that several different methods and thresholds have been employed in studies of CTC detection in NSCLC, thus limiting the possibility to compare the findings of these analyses. Reproducibility and sensitivity of CTC detection is still a major issue. Automated systems for CTC isolation and identification such as the CellSearch warrant a higher standardization of the procedure. However, the use of an epithelial cell marker such as EpCam for CTC isolation might lead to an underestimation of the CTC count because of a potential loss of epithelial markers during the EMT that is a phenomenon frequently associated with the metastatic cascade (48). Methods that are more sensitive have been recently demonstrated to capture a higher number of CTC in NSCLC patients (12,49). Sensitivity of CTC detection is an important issue also for early diagnosis in high-risk patients as well as for prognostic assessment in early lung cancer. However, the sensitivity in these settings needs to be counterbalanced by an appropriate specificity in order to limit the false positive rate.

Several different studies have demonstrated that the detection of CTC in the pre-operative and/or post-operative period in early lung cancer patients correlate with tumor recurrence. Some studies suggest that the assessment of PV CTC might increase the sensitivity of detection of CTC and the predictive value of the analysis in this setting. In this respect, recent studies have demonstrated that detection of somatic mutation in the circulating free DNA (cfDNA) after surgery predicts tumor recurrence in early breast and colorectal carcinoma (50,51). Therefore, evaluation of minimal residual disease in early tumor stages might represent one of the most interesting fields of application of CTC in lung cancer in the next future.

Although several different trials have demonstrated a prognostic role of CTC count in patients with advanced NSCLC, few studies have confirmed these findings at the multivariate analysis (Tables 2,3). In addition, low numbers of patients have been enrolled in these studies conducted

with different methods for CTC detection. Both qualitative methods assessing transcripts for epithelial markers as well as methods that allow isolation and quantification of CTC have been employed. The advantage of the latter techniques is that they permit to follow better the changes in CTC count during treatment, which might reflect the sensitivity of the tumor to chemotherapy. The utility of this information for the development of novel therapeutic strategies needs to be evaluated in prospective randomized clinical trials.

A number of studies have shown that CTC can be used for molecular profiling of the tumor and, in particular, for the identification of predictive biomarkers. For example, molecular alterations of EGFR, ALK and ROS1 are detectable in CTC isolated from NSCLC patients and they correspond to the mutational status of the primary tumor (52-57). However, we have to acknowledge that the field of molecular profiling of the tumor through liquid biopsy is currently dominated by the analysis of circulating tumor DNA (ctDNA). Indeed, ctDNA analysis has the advantage of lower costs and complexity for the extraction of tumor-derived nucleic acids as compared with CTC. In addition, the low number of CTC that are usually isolated in patients with NSCLC makes particularly complex the molecular analysis. Nevertheless, analysis of CTC has some advantages over ctDNA. First, analysis of CTC might allow the identification of protein biomarkers that cannot be revealed by analysis of nucleic acids. For example, CTC isolation can be optimized for the assessment of PD-L1 expression as potential biomarker of response to checkpoint inhibitors (58). Both analysis of ctDNA and CTC might result in false negative results due to the relative low sensitivity of these approaches. In this respect, it has been shown that contemporary analysis of CTC and ctDNA increases the possibility to detect predictive biomarkers in NSCLC patients, therefore improving the sensitivity of the liquid biopsy test (59). Finally, molecular profiling of single CTC might provide information on tumor heterogeneity that cannot be derived from ctDNA. Increasing evidence shows that the majority of solid tumors, including NSCLC, are highly heterogeneous (60,61). As a consequence of such intra-tumor heterogeneity, also driver mutations might be subclonal in selected patients (60,62,63). Evidence also suggests that the tumor heterogeneity is likely to increase over the time with the different lines of therapy. For example, significant fractions of patients that carry the T790M resistance mutations also have additional resistance mechanisms that can significantly affect the response to

T790M inhibitors (64). In this scenario, analysis of single CTC will clear whether these molecular alterations occur in the same clone of neoplastic cells or in different clones, thus allowing the development of specific therapeutic strategies. The possibility to perform multiplex molecular profiling of single CTC up to RNAseq analysis will also allow deriving important information on tumor pathogenesis as well as on the mechanisms involved in tumor dissemination (65).

Recent studies have also demonstrated that CTC from lung cancer patients can be used to generate patient's derived xenografts in immunocompromised mice also defined as xenopatients (48,66). Xenopatients are a valuable tool to gain information on the mechanisms regulating the formation of metastasis as well for studying the sensitivity or resistance to chemotherapeutics and to develop novel therapeutic strategies.

In conclusion, prospective clinical trials in adequate cohorts of patients are needed to confirm the prognostic and predictive value of CTC in NSCLC. Nevertheless, the study of CTC plays a fundamental role to gain novel information on the molecular evolution of the disease and to design novel therapeutic strategies in NSCLC.

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### Footnote

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