Review Article

Molecular characterization and prognostic significance of circulating tumor cells in patients with non-small cell lung cancer

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Abstract: Circulating tumor cells (CTCs) are rare epithelial cells that can be found in the peripheral blood of cancer patients. A growing body of evidence indicates that CTCs may play a role in non-small cell lung cancer (NSCLC) for diagnosis, therapy monitoring and prognostic purposes. CTCs evaluation could be particularly relevant in this clinical setting, considering that physicians often have difficulty in obtaining an adequate tumor tissue and that patients are not always suitable to receive a re-biopsy. In the current review, we will focus on the molecular characterization and prognostic significance of CTCs in NSCLC patients.

Keywords: Non-small cell lung cancer; circulating tumor cells (CTCs); liquid biopsy; epidermal growth factor receptor (EGFR); anaplastic lymphoma kinase fusion gene

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Introduction

Circulating tumor cells (CTCs) are rare epithelial cells that can be found in the peripheral blood of cancer patients (1). Over the last few years several methods have been developed with the aim of detecting CTCs, though the CellSearch® (CS, Veridex LLC, Raritan, NJ, USA) and the isolation by size of epithelial tumor cells (ISET, RareCell Diagnostics, Paris, France) represented up to now the most commonly used techniques (2-4).

Recently, Hodgkinson *et al.* demonstrated that CTCs isolated from small cell lung cancer patients can form tumors in immunocompromised mice with preserved morphological and genetic characteristics, providing evidence for their tumorigenicity (5). Furthermore, Chinese researchers demonstrated that CTCs collected from an individual patient, regardless of the cancer subtypes, exhibit reproducible copy number variation (CNVs) patterns similar to those of the metastatic tumor of the same patient (6).

An increasing body of evidence suggests that CTCs may play a role in non-small cell lung cancer (NSCLC) for diagnosis, biological characterization, disease monitoring and prognostic purposes (7-9). CTCs could be particularly relevant in this clinical setting considering that with standard procedures physicians have difficulty in obtaining an adequate tumor tissue for a comprehensive histopathological-molecular analysis and that patients are not always suitable to receive a re-biopsy, now highly recommended to identify the mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs). In the current review, we will focus on the molecular characterization and prognostic significance of CTCs in NSCLC patients.

Molecular characterization of CTCs

CTCs can be characterized by immunolabeling and

molecular analysis such as reverse transcription polymerase chain reaction (RT-PCR), reverse transcription quantitative PCR (RT-qPCR), fluorescent *in situ* hybridization (FISH), microarray or sequencing (10). In particular in NSCLC patients, especially with adenocarcinoma, molecular characterization of CTCs may permit the assessment of druggable alterations such as EGFR mutations or anaplastic lymphoma kinase (ALK) translocation. Maheswaran *et al.* performed EGFR mutational analysis on DNA recovered from CTCs using the Scorpion amplification refractory mutation system (SARMS) technology and compared the results with those obtained from concurrently isolated free plasma DNA and from the original tumor-biopsy specimens (11). The expected EGFR activating mutations were observed in CTCs in 11 out of 12 patients (92%) and in free plasma DNA in 4 out of 12 patients (33%). In addition T790M, the main mechanism of acquired resistance to TKIs, was detected in CTCs of patients who had a response to TKIs (33%) and who had clinical progression (64%). The possibility to detect T790M has become clinically relevant in NSCLC patients considering the recent development of specific anti-T790M drugs, such as osimertinib (12). Sundaresan *et al.* evaluated the presence of T790M mutation in tumor biopsies, CTCs and circulating tumor DNA (ctDNA) of 40 EGFR-mutant NSCLC patients with progressive disease during TKI therapy (13). For all three analytic platforms the overall T790M mutation-positive rate was approximately 50%, with concordance among them ranging from 57% to 74%. However, the combination of CTC and ctDNA analysis permitted to identify the T790M mutation in 14 (35%) patients in whom the concurrent biopsy was negative or indeterminate. Punnoose *et al.* conducted a molecular characterization of CTCs and ctDNA in 41 NSCLC patients (14). In this study mutations detected in CTCs, ctDNA and in matched tumor samples were strongly concordant, even though it was observed a greater sensitivity in ctDNA than in CTCs. Breitenbuecher *et al.* developed a novel highly sensitive and specific assay based on real-time PCR and melting curve analysis to identify the presence of activating EGFR mutations in blood cell fractions enriched in CTC (15). They achieved a 100% detection rate in a pilot cohort of 8 patients with EGFR positive NSCLC. Ran *et al.* employed magnetic beads labeled with antibody against leukocyte surface antigens to deplete leukocytes and enrich native CTCs independent of epithelial marker expression level (16). Then, they performed a laser cell microdissection to isolate individual CTCs, followed by whole-genome amplification of the DNA for exon 19 deletion, L858R and T790M mutation detection by PCR sequencing. Using this method EGFR mutations were correctly identified in 85% of the CTCs for L858R, 55% for exon 19 deletion and 45% for T790M. In addition, Italian researchers analyzed EGFR mutations by next generation sequencing (NGS) in CTC-enriched samples of 37 advanced NSCLC patients (17). They demonstrated that the CS system coupled with NGS is a very sensitive and specific diagnostic tool for *EGFR* mutation analysis in CTCs. Several studies showed also the ability to detect ALK rearrangements in CTCs (18-20). Ilie *et al.* reported that ALK status can be determined in CTCs isolated from patients with lung adenocarcinoma using a dual immunocytochemistry with an anti-ALK antibody-FISH assay (18). Researchers of the Institute Gustave Roussy (Villejuif, France) evaluated the feasibility to detect ALK rearrangement in CTCs of 32 patients with metastatic NSCLC (18/32 ALK positive) using a FISH method optimized for filters (19). Using a cut-off value of 4 ALK-rearranged CTCs per 1 mL of blood, ALK rearrangements were found in all of the ALK-positive patients and variations in ALK-rearranged CTCs levels were detected in patients being treated with crizotinib. Tan *et al.* reported high concordance (>90%) of ALK rearrangements assessed by ALK FISH testing in CTCs and tumor tissue samples of 14 ALK positive NSCLC patients (20). We monitored the presence of ALK positive CTCs, using the CS assay and a customized test with an anti EML4-ALK antibody (clone 5A4 abCam), in an ALK positive NSCLC patient during his targeted treatment (21). In this case we observed a correlation between the levels of ALK positive CTCs and the clinical response to crizotinib therapy or the development of resistance to the drug.

Recently the so-called immune checkpoint inhibitors, demonstrated to be able to interrupt inhibitory immune signals and to restore antitumor immune responses due to their interaction with the programmed cell death protein 1 (PD-1), the programmed death-ligand 1 (PD-L1) and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) in several tumors, including NSCLC (22,23). Among these new drugs, pembrolizumab, a humanized IgG4 PD-1 blocking antibody, received approval by the main regulatory authorities for the treatment of NSCLC patients whose tumors express PD-L1. Preliminary experiences have already demonstrated the feasibility of assessing the PD-L1 status in CTCs (24-26).

Prognostic significance of CTCs in NSCLC

CTCs can be detected at all stages of disease in NSCLC patients, though Krebs *et al.* found that CTC count was higher in NSCLC patients with stage IV compared to patients with stage III (27). The same authors indicated that the presence of >5 CTCs at baseline is predictive of poor prognosis in NSCLC patients receiving standard chemotherapy regimens. Hofman *et al.* evaluated the prognostic relevance of CTCs detected both by ISET and CS in 210 patients undergoing radical surgery for NSCLC (28). The authors demonstrated a significantly shorter disease free survival (DFS) in patients with preoperative detectable CTCs and suggested that ISET and CS should be considered as complementary methods. Recently, Chinniah *et al.* reported the results of a CTC monitoring conducted in 48 patients with locally advanced NSCLC treated with chemoradiotherapy (29). At a median follow-up of 10.9 months, 22 patients (46%) experienced a disease recurrence; moreover, 15 out of the 20 evaluable patients showed elevated CTC counts after treatment and two-thirds of them demonstrated a rise in CTCs counts an average of 6 months before radiographic evidence of recurrence. Muinelo-Romay *et al.* investigated the prognostic significance of CTCs count in 43 advanced NSCLC patients (30). The analysis showed the presence of CTCs in 41.9% of patients at baseline (with 23.2% of them having >5 CTCs); moreover, patients with CTCs >5 at baseline as well as patients presenting increased levels of CTCs during the treatment reported worse progression free survival (PFS) and overall survival (OS). CTCs were assessed also in patients with relapsed NSCLC enrolled in a phase II study of pertuzumab plus erlotinib (14). The authors reported a significant correlation either between higher baseline CTC counts and response to treatment or between decreases in CTC counts and radiographic response evaluated using both by 2[18F]fluoro-2-deoxy-D-glucose positron emission tomographic (FDG-PET) and computed tomographic (CT) imaging. Xu *et al.* detected CTCs in 47/66 (71.2%) NSCLC patients with the CS system, but they didn't find any CTC in the control group including healthy volunteers and patients with benign lung disease (31). In addition the authors reported a statistically significant correlation between CTC changes after two courses of chemotherapy and disease progression. Qi *et al.* investigated the presence of CTCs in 100 patients with locally advanced squamous cell lung cancer (32). In the univariate analysis, CTC count >5 at baseline and CTC count >5 at both time points (before and after one cycle of chemotherapy) were significantly associated with a poor PFS and OS outcome. Other Chinese researchers evaluated the prognostic significance of CTC count in 46 patients with advanced NSCLC (33). In this experience CTCs, which were measured at baseline in all patients and before every chemotherapy cycle in 23 patients, were found in 40 patients (87%). The authors also reported a relationship between a CTC count of >8 at a baseline and a worse prognosis. Recently Milaki *et al.* showed in a large clinical trial that the detection of CK19mRNA+ CTCs before and after chemotherapy is an adverse prognostic factor in patients with stage IIIB/IV NSCLC (34). Finally, also two meta-analyses explored the prognostic role of CTCs count in lung cancer patients (35,36). In the first meta-analysis, that included data extracted from 27 articles (12 containing survival outcomes) published between the year of 1997 and 2012, both pre and post-treatment CTCs detection in peripheral blood were associated with poor prognosis (35). The second meta-analysis, including data of more than 1,500 NSCLC patients enrolled in 20 studies, confirmed that the presence of CTCs has a negative prognostic significance in terms of OS and PFS (36).

Conclusions

A real time liquid biopsy could be extremely useful for individualized therapy of advanced NSCLC, considering that tissue sampling in lung cancer presents different issues compared to other tumors and that analysis of CTCs/ctDNA, which originated from all potential lesions, could overcome the disadvantages of single site biopsy (37). Moreover, in these patients molecular characterization of CTCs may permit the assessment of druggable genetic abnormalities or the discovery of a molecular disease evolution, while the enumeration of CTCs could become a prognostic biomarker, as well as has already been proved in metastatic breast, colorectal and prostate cancer. On the other hand further advances, regarding in particular standardization of CTCs detection methods, are still needed to improve the use of CTCs in lung oncology practice.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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