

Detection and Characterization of Circulating Tumor Cells in Lung Cancer: Why and How?

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INTRODUCTION

Lung cancer is the leading cause of death by cancer in the world. For example, in 2014, the number of deaths in Europe due to lung cancer was estimated to be 271,000.¹ The disease has a poor prognosis, with a survival rate of 15% after 5 years for all pTNM stages of disease. The poor prognosis is mainly due to the fact that diagnosis is made at a late inoperable stage. Chemotherapy and/or radiotherapy or therapy targeting genomic alterations (which are present in a limited number of patients) are proposed to patients with inoperable disease. However, despite some therapeutic advances, patient survival remains short.²⁻⁴ Preliminary results of clinical trials using immunotherapy targeting the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) axis have recently demonstrated promise in prolonging the survival of patients with metastatic lung cancer.^{5,6} Unfortunately, immunotherapy will not be able to cure these patients. In this context, new approaches aimed at improving the quality and length of life among these individuals are urgently needed. These advances are expected to come from noninvasive, predictive, and personalized new tests, recently referred to as “liquid biopsies,” which are used for diagnostic, prognostic, and theragnostic purposes.^{7,8}

The term “liquid biopsy” refers to a blood sample used for analysis of a certain number of biomarkers and of rare circulating nonhematological cells (CNHCs) of interest. These biomarkers can be present in either the serum or plasma (free circulating DNA of tumor origin and free circulating RNA, in particular circulating microRNA).⁹⁻¹¹ CNHCs include circulating cancer cells (CCCs), also called CNHCs with malignant features or circulating tumor cells (CTCs), and other noncancer cells such as CNHCs with benign features, CNHCs with uncertain malignant features, and circulating endothelial cells.¹²⁻¹⁴ The data obtained from biomarkers and from CNHCs are expected to provide complementary information.¹⁵ Over the last 15 years, many different techniques have been proposed to evaluate the different blood biomarkers and CNHCs.^{12,16,17} These methods hold advantages and disadvantages and different sensitivities and specificities.^{12,16,17}

Ideally, the new domain of liquid biopsy should allow for improvement in the practice of thoracic oncology by providing: 1) better screening approaches for the diagnosis of lung cancer, in particular in high-risk populations; 2) earlier diagnosis; 3) better prognostic assessment; and 4) better prediction of the response to targeted therapies (based on genomic alterations and/or immune-mediated mechanisms). Finally, liquid biopsy-based approaches should permit the earlier detection of therapeutic resistance and tumor recurrence, and possibly the identification of targeted treatments that are effective against escape mutants.¹⁸

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Translational research applied to the field of liquid biopsy has already improved our knowledge of the natural history and pathophysiology of lung cancer.^{12,17-19} Recently, genomic data have allowed better characterization of CTCs.¹⁹⁻²¹ However, despite the substantial interest in liquid biopsy analysis applied to the area of thoracic oncology, to our knowledge its routine clinical application remains inexistent or exceptional for the majority of clinicians and therefore has not yet been transferred to daily clinical practice.^{12,17,19} This situation can be explained by several key issues: 1) the absence of standardized assays; 2) the difficulty in controlling and standardizing the preanalytical steps required to obtain reliable, robust, and sensitive and specific reproducible results; and 3) the substantial cost of innovative tests, which prevent introduction into hospital practice.

Detection of CNHCs

CNHCs can be detected in patients with lung cancer by indirect and direct methods.^{12,15-17} The most used and most cited indirect method is the CellSearch Epithelial Cell Kit (Janssen Diagnostics LLC, Raritan, NJ), which is intended for the enumeration of CTCs of epithelial origin. To our knowledge to date, this is the only method that has received authorization from the US Food and Drug Administration for the prognostic detection of epithelial CTCs in patients with metastatic breast, colon, or prostate cancers. However, the US Food and Drug Administration does not recommend this method for patients with metastatic lung cancer. Thus, although some studies have shown that this technique is of potential interest for patients with metastatic disease (in particular, better survival was reported for patients with a rapid decrease in the number of epithelial CTCs after chemotherapy), the CellSearch system reportedly does not perform well in patients with lung cancer.²² In this setting, the CellSearch system appears to be relatively insensitive and has poor specificity. This is expected to be due to several factors: 1) the majority of CCCs in patients with lung cancer rarely express epithelial markers and therefore cannot be captured by the anti-EpCAM (epithelial cellular adhesion molecule) antibody of the CellSearch system; 2) the CNHCs may correspond to epithelial nontumor cells and can be incorrectly counted with the CellSearch system, which does not afford in-depth morphological identification; and 3) the tumor cells of lung cancers often form large aggregates composed of numerous tumor cells and these masses of cells (so-called

circulating tumor microemboli) are rarely or incorrectly counted with the CellSearch system (Fig. 1). Many other indirect techniques using reverse transcriptase-polymerase chain reaction or microfluidic systems have been or are used in thoracic oncology.^{12,15-17} However, to the best of our knowledge, none to date meet the requirements for use in daily clinical practice.

Direct methods can detect CNHCs in patients with lung cancer and, among them, can distinguish CCCs, which are tumor cells isolated without bias related to the use of antibodies and are identified as cancer cells by cytopathology.^{12,14,23} Currently, the direct method that is the most used and the most cited is the ISET method (Isolation by Size of Tumor Cells; Rarecells Diagnostics, Paris, France).^{12,24} ISET avoids the use of antibodies for cell isolation and is based on the filtration of 10 mL of whole blood through filters with 8- μ m calibrated pores. It allows for the elimination of the majority of hematological cells and the collection on a filter of CNHCs due to their larger size. These CNHCs can include CCCs of epithelial and nonepithelial (mesenchymal, stem) origin. Some hematological cells occasionally may be retained on the filter and be visible (Fig. 1). More often, they are blood cells associated with CNHCs (platelets and less often lymphocytes and/or neutrophils). In rare cases, circulating megakaryocytes can be detected on the filters. This direct filtration permits very precise and in-depth cytomorphological analyses.^{12,14,23}

The morphological criteria used to characterize CCCs have been defined and blindly validated by a team of 10 cytopathologists on 808 blood samples analyzed using the ISET method.²⁵ They are the same criteria used to identify cancer cells in other domains of cytopathology.¹⁴ The ISET method is being used in several research laboratories around the world, and has generated several independent scientific publications in the domain of thoracic oncology.^{12,23} ISET allows the analysis of 10 spots (1 mL of blood per spot) on the filter and holds several advantages. In particular, it permits multiplexing (multiple tests from the same blood sample) and the precise morphological identification of CNHCs with classification into benign cells, cells of uncertain malignancy, and malignant cells, following criteria used for the application of cytopathology to other settings (eg, Papanicolaou test or fine-needle aspiration [FNA] biopsy) and to other biological liquids (ascites, urine, pleural liquid, etc).¹⁴ Accordingly, the key criteria used to assess the malignancy of CNHCs

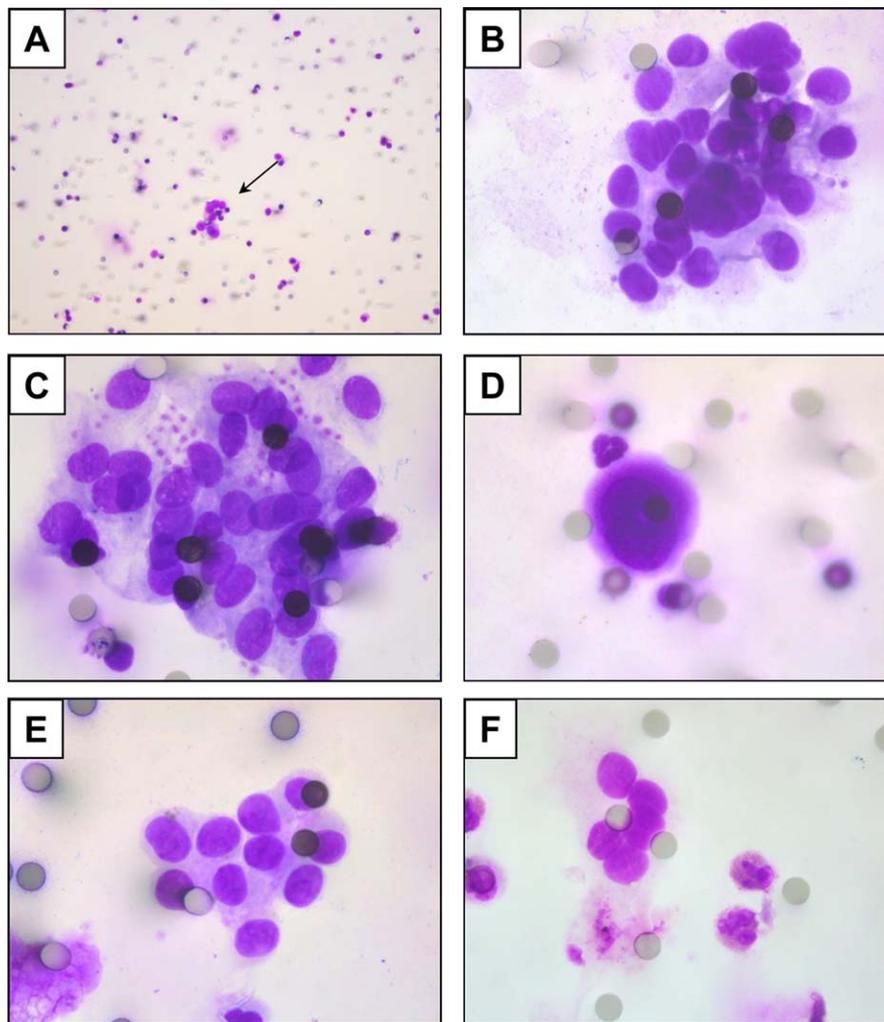


Figure 1. Cytomorphology of circulating tumor cells from patients with lung cancer. (A) Aggregates of circulating nonhematological cells (CNHCs) of small size retained on the surface of the filter (indicated by arrow) (May-Grunwald-Giemsa stain, $\times 40$). (B) Microemboli of malignant CNHCs from a lung squamous carcinoma (May-Grunwald-Giemsa stain, $\times 1000$). (C) Microemboli of malignant CNHCs from a lung adenocarcinoma (May-Grunwald-Giemsa stain, $\times 1000$). (D) Malignant CNHC (May-Grunwald-Giemsa stain, $\times 1000$). (E) Aggregates of CNHCs of uncertain malignancy (May-Grunwald-Giemsa stain, $\times 1000$). (F) Morphologically benign CNHCs (May-Grunwald-Giemsa stain, $\times 1000$).

are an irregular nucleus, an increase in the nuclear-cytoplasmic ratio, the presence of multiple and large nucleoli, the size of the cell ($>24\ \mu\text{m}$), basophilic nuclei, and the presence of touching tridimensional aggregates and anisocytosis (Fig. 1).¹⁴ However, these criteria hold the same limits as those used in “classic” cytology. There is also a need for specific biomarkers to formally assert the malignancy of some cells under evaluation.¹⁴ Previous studies have shown that approximately 50% of patients with metastatic lung cancer have CNHCs with cytological criteria of malignancy whereas CNHCs with a benign appearance are rare. It is interesting to note that a certain

number of patients with a benign thoracic pathology (eg, pneumothorax or infectious diseases) have CNHCs without a malignant appearance, which is expected to correspond to circulating benign bronchial epithelial cells (unpublished data). Based on epithelial markers, these cells may be assessed as non-CTCs by indirect methods. The false detection of CTCs has been reported with the Cell-Search system, which does not allow for the use of in-depth cytological analyses in patients with benign intestinal pathologies.²⁶ Some patients with operable lung cancer (stage I-IIIa disease, according to the IASCLC/AJCC TNM staging system) have been found to have malignant

CNHCs (CCCs) before surgery when analyzed by the ISET method.¹⁴ This cytological approach for the analysis of blood therefore may be of diagnostic interest.

With regard to “classic” cytopathology, there are many pitfalls and limits to the analysis of CNHCs within the context of certain pathologies, in particular thyroid, parathyroid, and pancreatic pathologies.^{25,27-29} In this regard, it is possible to detect CNHCs in benign pathologies of the thyroid and parathyroid.²⁵ Similarly, a recent article demonstrated the presence of circulating epithelial cells that were detected morphologically using a size-selective filtration device in patients with benign pancreatic lesions.³⁰ The authors of this study alerted the readers to the finding that the exact nature of these circulating epithelial cells (eg, whether they are benign or malignant cells) cannot be stated by morphological analysis alone. However, in this context, it is interesting to note that many pitfalls for diagnosis exist both at the histological and cytological levels to distinguish benign from malignant pancreatic lesions.²⁷⁻²⁹

The detection of CCCs by the ISET system holds prognostic interest for patients with stage I to stage IIIA lung cancer because disease recurrence after surgery is more frequent in those with preoperative detection of CCCs and detection is associated with worse survival.³¹ The presence and number of aggregates of CCCs (so-called circulating tumor microemboli) is a poor prognostic factor, in particular for patients with small cell lung cancer.³² The ISET method also allows for better characterization of the phenotype of the CNHCs and demonstrates the existence of panels of circulating cells that express epithelial and mesenchymal markers or only mesenchymal markers.³³ These different phenotypes represent the phenomena of epithelial-mesenchymal transition.¹² Thus, the ISET method certainly appears to have a better specificity than the CellSearch method for lung cancer with regard to the detection of tumor cells that have lost their epithelial phenotype.^{33,34}

Cytological analysis of CCCs also implies the performance of immunocytochemistry (ICC) and cellular visualization of immunolabeled targets (nuclear, cytoplasmic, and/or membranous) (Fig. 2).³⁵ Consistently, it is possible to envision the use of ICC on filters to assess the labeling intensity (absent, low, moderate, or intense), although to our knowledge these analyses have never been reported in the literature. It is also possible to detect theranostic markers such as rearrangements of anaplastic lymphoma

kinase (*ALK*) and *ROS-1*, and the expression of PD-L1 or c-MET.³⁵⁻³⁸ This can be performed by ICC and/or by in situ hybridization (Fig. 2). It is interesting to note that a correlation has been demonstrated with regard to *ALK* gene rearrangement status in CCCs and in bronchial tumor biopsies obtained from the same patients.³⁵ Other filtration methods allowing for the morphological assessment of CNHCs such as the ScreenCell method (ScreenCell, Paris, France) are available from other companies. Filtration methods vary according to the type of blood pretreatment, filter type, and mechanism of filtration used, with an effect on the cell capture sensitivity, quality of cell morphology, and possibility of multiplexing.^{39,40}

General Perspective

Those methods based on blood filtration and isolation of CNHCs according to size hold advantages, but also necessitate optimization before they can be used as a daily routine test. Blood filtration is a very complex approach due to the extremely high cellularity of blood and variability of essential components such as coagulation factors. It has to be performed rapidly after sampling (generally within 3 hours). In this context, the development of a reagent allowing for the long-term stabilization of very rare organ-derived cells in blood before filtration should make use of the ISET platform by distant blood collection sites easier. Along with the phenotypic characterization of CCCs by ICC or in situ hybridization, protocols allowing for the collection of fixed or fresh CCCs on filters for genomic and transcriptomic analyses or for culture and injection into mice need to be better optimized. In this regard, to the best of our knowledge, the characterization by next-generation sequencing technology of CCCs detected among patients with lung cancer has not yet been described using this filter approach. One of the recurring issues raised concerning the methods of filtration is related to the possibility that small-sized and potentially highly invasive cancer cells may pass through the pores of the filter. Nevertheless, it should be stressed that cytological analyses of FNA specimens or spread cells on smears do not identify cancer cells measuring <7 μm to 8 μm, suggesting that together the different cytological approaches have a relatively similar specificity. Finally, filtration methods would benefit substantially from automatization before being adopted by clinical pathology laboratories as a routine diagnostic approach.

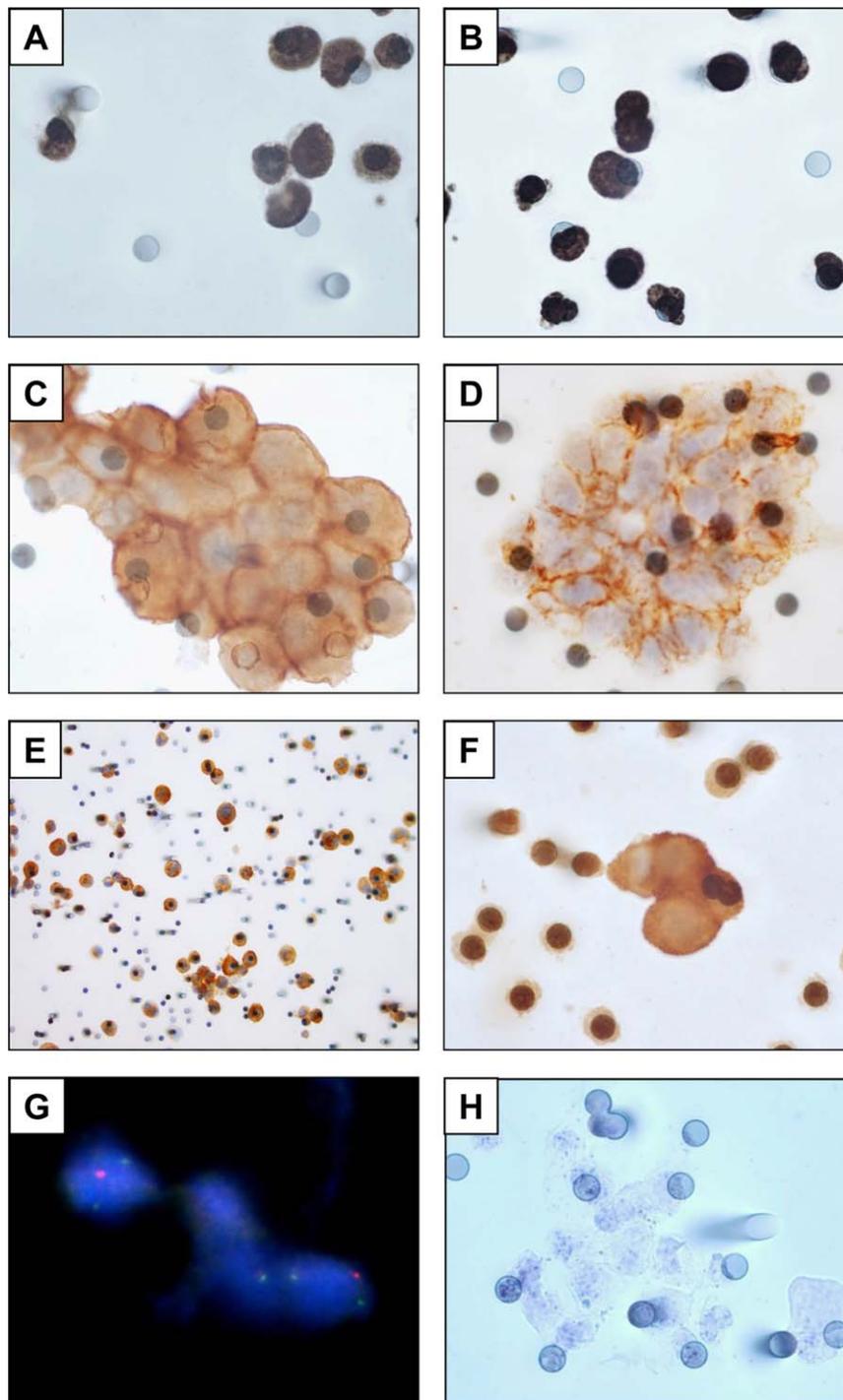


Figure 2. Examples of the expression of different biomarkers on circulating nonhematological malignant cells from patients with lung cancer. (A) Expression of the nuclear antigen thyroid transcription factor 1 on circulating cells isolated from a lung adenocarcinoma (immunoperoxidase stain, $\times 1000$). (B) Expression of the nuclear antigen P40 on circulating cells isolated from a lung squamous carcinoma (immunoperoxidase stain, $\times 1000$). (C) Expression of cytokeratins on circulating cells isolated from a lung adenocarcinoma (immunoperoxidase stain, $\times 1000$). (D) Expression of the epithelial membrane antigen on circulating cells isolated from a lung squamous carcinoma (immunoperoxidase stain, $\times 1000$). (E) Expression of C-met (immunoperoxidase stain, $\times 1000$). (F) Expression of C-met (immunoperoxidase stain, $\times 1000$). (G) Expression of the anaplastic lymphoma kinase (ALK) antigen (immunoperoxidase stain, $\times 1000$). Rearrangement of *ALK* (fluorescence in situ hybridization, $\times 1000$). (H) Expression of microRNA 21 (in situ hybridization, $\times 1000$).

It is important to consider the analysis of CCCs within the context of recent developments using immunotherapy for patients with lung cancer, particularly that targeting the PD-1/PD-L1 axis.^{5,6} The quantification of CCCs in patients treated with immunotherapy is expected to be important for evaluation of the efficacy of these new treatments. It also will be interesting to evaluate the protein expression of PD-L1 among CCCs to assess a possible correlation with the expression of PD-L1 on bronchial biopsies and/or possibly on surgical and metastatic specimens. Moreover, the analysis of PD-L1 in CCCs should benefit patients with small cell carcinoma because it is difficult to quantify the expression of PD-L1 on tissue biopsies due to the number of artifacts classically associated with this type of tissue. Finally, many other markers of interest also could be analyzed in CCCs extracted from blood by filtration by using ICC or in situ hybridization for patients with metastatic lung cancer (Fig. 2).⁴¹⁻⁴³

Conclusions

The analysis of CNHCs as well as the analysis of molecular markers (free circulating DNA of tumor origin, free circulating RNA, and microRNA) are generally comprehensively referred to as “liquid biopsy.” However, from an etymological point of view, the term “biopsy” is derived from the ancient Greek word *bios* (“life”) and *opsis* (“view”), which should, *stricto sensu*, be applied to blood sampling allowing cytomorphological analysis, by analogy with a “solid” tissue biopsy. Cytologists currently are generally unaware of the blood cytomorphological analysis of CNHCs in patients with solid cancers. This domain has not been communicated and is not taught in pathology laboratories, and more generally in universities. However, it appears legitimate that this type of approach will become part of the routine work of professional cytopathologists, as is the case for “classic” cytopathology performed on smears or for liquid-phase cytology and on FNA. Cytological analysis of CNHCs is a very promising and complementary approach to that of tissue biopsies, smears, and FNA in thoracic oncology. Moreover, only the detection and characterization of CCCs is expected to provide an early assessment of tumor invasion. This cytological approach is certainly complementary to approaches targeting molecular markers in plasma, in particular for the detection of genomic alterations (such as mutations in epidermal growth factor receptor) from free circulating tumor DNA.¹⁵

In addition to its potential as a diagnostic, prognostic, and predictive marker of therapeutic response, a liquid biopsy also could be used as a screening tool for patients with lung cancer. In this setting, some recent studies have opened the way to very promising investigations, for example by evaluating a profile of microRNA in plasma, which is potentially associated with the presence of lung cancer.⁴⁴ Furthermore, a prospective study performed on a population at high risk of lung cancer (ie, smokers who develop chronic obstructive pulmonary disease) was performed using the ISET method to isolate and characterize sentinel circulating malignant cells, which can spread into the blood before the lung cancer becomes detectable by imaging.⁴⁵ This study revealed CCCs in the blood of approximately 5% of patients with chronic obstructive pulmonary disease 1 to 4 years before the tumor nodule was detected by imaging.⁴⁵ These exciting data need to be confirmed with a larger series of patients and validated independently in a multicenter study. This result is consistent with results obtained in animal models that demonstrate that highly invasive tumors spread cancer cells into the blood at the very beginning of their development.⁴⁶ Thus, similar to cervical cancer screening by the Papanicolaou test, the cytological analysis of blood could provide the exciting perspective of the early identification of invasive lung cancers through the accurate cytological detection of CCCs.

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