Detection of Circulating Tumor Cells from Lung Cancer Patients in the Era of Targeted Therapy: Promises, Drawbacks and Pitfalls

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Abstract: Interest in biomarkers in the field of thoracic oncology is focused on the search for new robust tests for diagnosis (in particular for screening), prognosis and theragnosis. These biomarkers can be detected in tissues and/or cells, but also in biological fluids, mainly the blood. In this context, there is growing interest in the detection of circulating tumor cells (CTCs) in the blood of lung cancer patients since CTC identification, enumeration and characterization may have a direct impact on diagnosis, prognosis and theragnosis in the daily clinical practice. Many direct and indirect methods have been developed to detect and characterize CTCs in lung cancer patients. However, these different approaches still hold limitations and many of them have demonstrated unequal sensitivity and specificity. Indeed, these methods hold advantages but also certain disadvantages. Therefore, despite the promises, it is currently difficult and premature to apply this methodology to the routine care of lung cancer patients. This situation is the consequence of the analysis of the methodological approaches for the detection and characterization of CTCs and of the results published to date. Finally, the advent of targeted cancer therapies in thoracic oncology has stimulated considerable interest in non-invasive detection of genomic alterations in tumors over time through the analysis of CTCs, an approach that may help clinicians to optimize therapeutic strategies for lung cancer patients. We describe here the main methods for CTC detection, the advantages and limitations of these different approaches and the potential usefulness and value of CTC characterization in the field of thoracic oncology.

Keywords: Cancer screening, circulating tumor cells, diagnosis, lung cancer, personalized medicine, prognosis, targeted therapy.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in both genders worldwide, with an estimation of more than 225 000 new cases, and more than 160 000 deaths related to lung cancer per year in the USA alone, about 80% of them being non-small cell lung cancer (NSCLC) [1]. Currently, patients with advanced stages of lung cancer are incurable and have a very short overall survival [2]. Of all patients diagnosed with NSCLC, only about 15% survive more than 5 years [2]. Most patients (>75%) are diagnosed at advanced stages or suffer significant comorbidity precluding surgical treatment, and thus chemotherapy and/or radiation is/are needed [2]. Unfortunately, despite recent advances in the management of resected lung cancers and many new treatments of metastatic tumors, the cure rate of patients with lung cancer remains globally very low. In this context, it is clear than a better understanding of lung carcinogenesis and the development of new diagnostic and therapeutic strategies based on reliable biomarker analysis are the current challenges in thoracic oncology.

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In this field, the growing development of targeted therapies and of the concept of personalized medicine call for quick and significant changes in the biologist's and pathologist's practice to optimize detection and follow-up of genomic alterations of lung cancers [3-5]. There is an urgent need to discover new biomarkers for earlier diagnosis of lung cancer and for development of better prognostic and predictive factors for lung cancer patients. Among the various approaches that have been or are currently developed in order to make an early diagnosis and/or to assess the prognosis of lung cancer, interest has focused on imaging techniques [6]. Different techniques, in particular the spiral CT scan, can allow early detection of lung cancer. However it is currently difficult to deploy this approach in a systematic way in all populations or in all countries. Finally, it is possible that even if these techniques can already allow detection of small-size lung cancer (few millimeters in larger diameter), they are not accurate enough for the the initial diagnosis of very small cancers and especially to detect the first metastatic steps [6, 7]. Many biomarkers are being analysed in order to assess their clinical potential in the diagnosis and/or prognosis of cancers, including lung cancer [8-11]. However none of these circulating biomarkers is used routinely to date to make the diagnosis of lung cancer or metastases and/or to assess the prognosis of these cancers [8-11].

Among different promising biomarkers, the detection and the characterization of circulating tumor cells (CTCs) in blood of cancer patients are being explored in an increasing number of clinical and translational studies. It is the subject of different research and development activities in the lung cancer field. This demonstrates the substantial interest of researcher scientists and physicians in the CTC domain, in particular for potential optimization of lung cancer patient care. The theoretical possibility of substituting a tissue sampling (lung biopsy or surgical resection specimen) with a blood sample (so called a « liquid biopsy ») is very attractive for physicians and patients [12]. Indeed, this non-invasive test gives access to tumor cells and can be repeated, which allows patient monitoring of treatment efficacy and early detection of mutations in the primary tumor or in distant metastases. However, the effort made in the development of techniques and tools for CTC detection and/or identification and the high number of scientific publications regarding this topic contrast with the real benefit to the care of lung cancer patients in the daily practice. This may be due to: *1*) the absence of a common consensus concerning the optimal/ideal method for detection of CTCs, *2*) the uncertainties concerning both the sensitivity and the specificity of the different proposed approaches, in particular for characterization of genomic alterations of CTCs, *3*) the current weakness of a business model and socio-economic development in hospitals, at least in certain countries, for strategic research and development and large test validation in the CTC field. However, new opportunities have been created to set up such developments in the health care era, including different calls for national and international research that support programs in the field of CTCs. The results obtained through these programs may allow, in the near future, the routine implementation of extensively validated CTC detection in hospital biological laboratories, and thereafter discussion of possible reimbursement through health care institutions.

In this review, after covering some points on terminology, we will describe some new aspects concerning the pathophysiology of CTCs and then the main methods for their detection, giving emphasis to their advantages and limitations. Finally, we will summarize and comment on published clinical studies on CTC detection in lung cancer patients.

TERMINOLOGY AND DEFINITIONS

A number of biomarkers can be detected in the blood of lung cancer patients, but are outside the scope of the CTC field and thus will be not developed in the present review. These include the molecular biomarkers detected from DNA, mRNA and microRNA extracted from blood and other cellular biomarkers such as circulating endothelial cells [13-17].

The term « circulating metastatic cell » is not adapted to the field of CTC as, at present, it is not possible to assess the "metastatic potential" of CTCs, in particular using immunological, morphological, phenotypical and/or genotypic analyses. The term "circulating tumor cells" is largely employed in the literature, but the word "tumor" may not refer to a "diagnostic" analysis in this context. In fact, several methodological approaches use "epithelial-specific" and not "tumor-specific" markers [18-20]. As an example, the widely used CellSearch method isolates and identifies cells from blood using epithelial-specific antibodies and markers. The test is thus not "diagnostic" for tumor cells in blood and the term "circulating tumor cells" is inappropriate. In this setting the term "Circulating Epithelial Cells" (CEpC) is definitely more adapted to the method used for cell detection. This situation has historical reasons. At the beginning of the "circulating tumor cell" era, in the years 1985-2000, the view was that "epithelial cells do not circulate in blood unless they become "tumorous". It was thus "plausible" that detection of circulating epithelial cells could be, in some way, referred to as CTCs. However, our knowledge in the field of circulating rare cells has evolved since then. It is now established that non tumor epithelial cells having benign epithelial morphological characteristics may circulate in the blood of patients [20]. In addition, we now know that the most malignant CTCs lose their epithelial antigens, which additionally questions the use of the term "circulating tumor cells" applied to "circulating epithelial cell" detection and of course the related potential clinical utility of the approach [19-22]. Cytopathology is used in clinical oncology to study and identify morphological features associated with the characteristics of a "tumor". It is a difficult domain, especially when used for diagnostic purposes (for examples for cervix cancer, bladder cancer etc.), as it

cannot be backed up by aspects of the "tissue" structure that are related to cell transformation. However, we have shown that cytopathological criteria can be applied to cells isolated from blood, in combination with immunolabeling for cell characterization, provided that the isolation pre-analytic step carefully maintains the cell morphology [23, 24]. We have also shown that the classical limitations of cytopathology (e.g. cells from thyroid and parathyroid adenomas) are still valid in the context of cells isolated from blood [25]. To clarify this point, we have used the term "circulating non haematological cell" (CNHC) referring to circulating cells without blood- or bone marrow-specific characteristics [24]. In this group we then identified cells with clearly defined "tumor" morphological and/or immune-morphological characteristics, which can be defined as true "circulating tumor cells". It is clear that the future advent of validated high throughput alternative approaches to identify a cell as a "tumor cell", like molecular genetic assays, could bring benefit to the domain of CTCs. However, as yet we do not have alternative diagnostic markers or approaches for the identification of tumor cells in blood [26]. The cytomorphological study of CTCs reveals that they can form aggregates or sheets of several cells. In this case, the term "circulating tumor microemboli" (CTMs) should be employed instead of "blood micrometastasis" as the metastatic potential of these sheets of CTCs, while probable, is not demonstrated. Finally, we have to underline that the difficulty of culturing CTCs isolated from the blood of patients has hampered, up to now, investigations aimed at studying their proliferative, invasive, transforming and tumorigenic potential. In this review, we will use the term "circulating epithelial cells" (CEpC) and Circulating Tumor Cells (CTCs) according to the method used for their detection.

NEW INSIGHTS INTO LUNG CANCER DISSEMINATION

It has been well-established for a couple of years that the metastatic dissemination of lung cancer takes place through spread into the blood of tumor cells that invade the vessels after migration from the primary tumor. The transendothelial migration of tumor cells and their subsequent blood vessel intravasation is an early event in the natural history of carcinogenesis and may occur in lung tumors of a small size. As a matter of fact, CTCs can be detected in stage I NSCLC before surgical resection [27]. During surgery for lung cancer, a large number of tumor cells can be shed into the blood stream but the behavior of these CTCs is unknown, in particular it has not yet been demonstrated that these cells may be involved in development of future metastases [28-31]. A large number of tumor cells circulate into the bloodstream but a variable proportion of them are apoptotic or die because of shearing forces and/or are eliminated by the immune system. In agreement with this view, animal models have shown that within 24 hours of intravenous administration of tumor cells, around 0.1% of the cells are still viable and that 0.01% of these surviving CTCs may give rise to metastases [32].

The transition of CTCs into the blood stream benefits substantially from a change in the tumor cell phenotype. This phenotypical change is characterized by a loss, more or less complete, of the epithelial markers (in particular cytokeratin filaments and E-cadherin) and the increase or the gain of some mesenchymal markers such as vimentin. This progressive transformation, called the epithelio-mesenchymal transition (EMT) phenomenon, gives substantial plasticity to the tumor cells and increases their migration and invasion (Fig. **1**). It probably also gives CTCs a better resistance to high blood pressure and to anoïkis, which is "an apoptosis that is induced by inadequate or inappropriate cell-matrix interactions" [33, 34]. As recently described for breast carcinoma, the population of mesenchymal CTCs in lung cancer patients could also be associated with disease progression [35]. CTCs may migrate as clumps of different sizes and these clusters of cells, called circulating tumor microemboli (CTMs) can probably survive better in the bloodstream than isolated CTCs. The study of the phenotypical profile of tumor cells belonging to CTMs shows that some of them do not express any epithelial biomarkers, but have gained a mesenchymal phenotype, which could help collective migration. In fact, when organized as CTMs, the CTCs may benefit from a survival advantage over single CTCs *via* cell-cell contact-related survival signaling. They can also bring their own "soil" helping them to metastasize to distant organs [36, 37]. In this context it is possible that the CTMs may bring passenger stroma cells from the primary tumor to the secondary site in the same host to provide a provisional stroma and facilitate growth and metastasis development in one or several distant organs. As an example, the ectopic presence of tumor-associated fibroblasts in brain metastases in patients indicates a potential clinical relevance of this phenomenon.

The interaction between the CTCs and blood leukocytes in the blood stream has been poorly investigated to date. Different studies have demonstrated that neutrophils may facilitate the transendothelial migration of tumor cells [38]. Neutrophils could also help the formation of CTMs. A relationship seems to exist between the number of lymphocytes, the number of CTCs and the overall survival of patients [39-41]. Moreover, some cytokines released by lymphocytes may indirectly have an impact on the behavior of CTCs. An elevated neutrophil to lymphocyte blood ratio has been associated with worse prognosis in cancer patients, in particular in lung cancer patients who present with lower disease-free survival, early recurrence and worse overall survival [42]. However, direct impact of leukocytes on CTC behavior has not been established.

Few studies have demonstrated that platelets may play a role in survival of CTCs and in their metastatic behavior [43, 44]. In fact, platelet-tumor cell interactions may prime tumor cells for subsequent metastatic

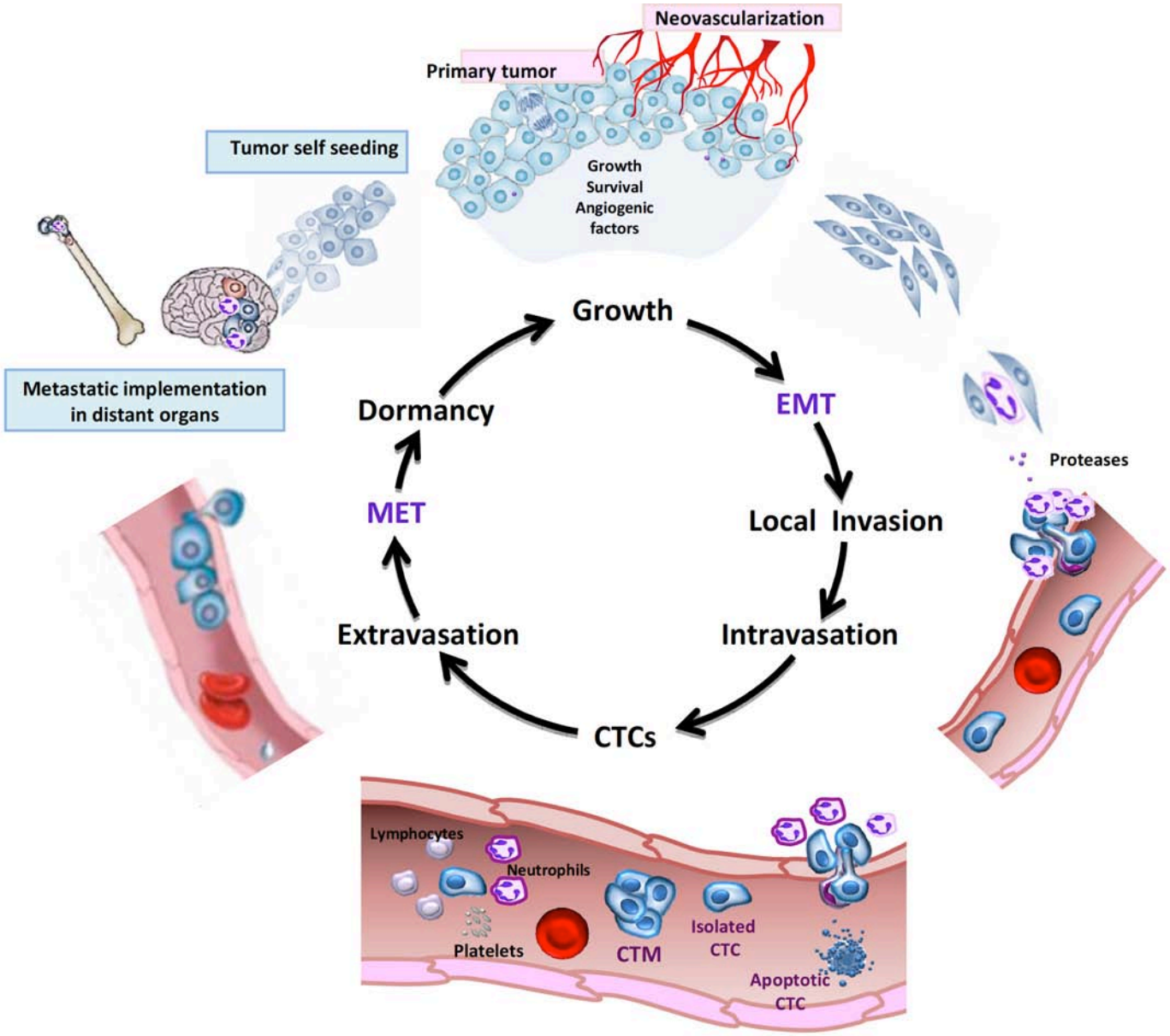

Fig. (1). Schematic steps of lung cancer metastases and biological interaction of CTCs in the blood stream. EMT: epithelio-mesenchymal transition; MET: mesenchymal to epithelial transition; CTCs: circulating tumor cells; CTM: circulating tumor microemboli.

formation *in vivo* by activating the TGFβ/Smad and NF-κB pathways in cancer cells and transition to an invasive and metastatic mesenchymal-like phenotype [44]. Once located in distant organs, the CTCs may return to the epithelial phenotype, thereby allowing them to cross the endothelial barrier and to proliferate in the parenchyma. At this stage they undergo a switch called the mesenchymal to epithelial transition (MET) phenomenon [19, 20]. So a subpopulation of CTCs having a high "plasticity" should exist and may be able to switch from EMT to MET and vice-versa depending on a combination of conditions including re-expression of E-cadherin and/or EMT-inhibitory miR-200 and local microenvironment-dependent factors [45]. Additionally, the expression of chemokine receptor on CTCs could favor specific metastatic implementation in a distant organ [46]. Finally, neutrophils may also facilitate CTC implantation in metastatic sites [47].

It has been highlighted recently that CTCs can re-colonize the primary tumor site from which they were initially detached [48, 49]. This phenomenon, called "tumor self seeding", allows the primary tumor to keep growing (Fig. **1**). CTCs interact preferentially with the tumoral stroma *via* certain integrins [49]. Tumor reactivation following an apparently successful treatment of the primary mass with initial therapies (such as surgery or systemic therapy) is a well-known phenomenon. This metastatic rebirth is preceded by an interlude, termed "dormancy", when the cancer "sleeps" and remains undetected for periods that can last years or even decades until reactivation of tumor cell proliferation occurs, thereby revealing long-term tumor

recurrence [50, 51]. In this setting, it has been postulated that the CTCs could be involved in this phenomenon of dormancy [50, 52].

METHODS USED FOR CTC DETECTION IN LUNG CANCER PATIENTS

General Principles of the Detection and Characterization of CTCs

The methods for CTC detection are numerous and are based on many different technical approaches [19, 20, 53, 54]. Most detection techniques are preceded by enrichment steps (removal of red blood cells, then separation of CTCs from leukocytes using immunomagnetic beads or immunofluorescence-based separation of mononuclear cells). In fact, the most current methods can be distinguished according to three general principles for CTC isolation: methods based on antigen expression by the target cells, also called "immunological methods" for CTC isolation (they use antigens presumably not expressed on blood cells, but not specific to tumor cells (e.g. epithelial antigens); methods based on cell density, which is thought to be higher in CTCs than in blood cells; and, methods based on cell size, which is defined by cytopathological criteria and is distinctly larger in CTCs compared to blood cells [19, 20, 53, 54]. Additionally, two main approaches to CTC detection can be distinguished: 1) direct methods based on CTC cytomorphology, complemented when needed by immunomediated characterization, which allow diagnostic identification of CTCs and, 2) indirect methods based on immunological and/or biochemical cell characterization without cytopathological analysis [19, 20, 53, 54].

Following their isolation by different approaches, CEpCs and CTCs can be further characterized using different complementary tools. Indeed, different genomic alterations or molecular characteristics can be performed on CEpCs and CTCs after nucleic acid (DNA, RNA, microRNA) extraction and characterization using several molecular biology analyses (PCR, RT-PCR, microarrays) [19, 20, 53, 54]. Immunocytochemical and *in situ* hybridization methods performed on CEpCs and CTCs can also detect expression of a number of macromolecules of interest [19, 20, 53, 54]. Finally, some methods are able to isolate non fixed viable CEpCs and CTCs, to grow them in a culture medium and to further check their phenotype, including drug resistance or sensitivity.

The different methods for CTC isolation and detection hold advantages and disadvantages [19, 20, 53, 54]. Some of these methods are quite easy to perform and "low cost", but others are more complex and expensive (both for equipment and consumables). Some methods have been widely used and are commercially available, while others are still « confidential » and under investigation and/or not commercialized, and thus difficult to set up for routine use in a laboratory [19, 20, 53, 54].

The cellular and molecular characterization of the detected CTCs is certainly the most critical step at this stage. However, this step is mandatory for optimization of this field of interest in thoracic oncology, especially with the increasing development of targeted therapies based on the identification of different genomic alterations. Indeed, this step of CTC characterization is crucial to help lung cancer diagnosis, to better define prognosis and to optimize personalized treatment during the time of disease progression.

We briefly describe here the main methods for CEpC and CTC isolation and detection while highlighting both their advantages and their limitations. Most of these different direct and indirect methods have been applied in the past to detect occult CTCs in patients with lung cancers. However, the more currently used methods for CTC detection in NSCLC patients are certainly the CellSearch system, RT-PCR methods and ISET technology.

CTC Isolation Using Indirect Methods: CellSearch and RT-PCR

Indirect methods are based on immunological and/or biochemical approaches.

CellSearch Method

This method is based on an immunological approach that uses antibody-coated ferrofluids directed against an epithelial antigen (Epcam) [19, 20]. This method allows immunomagnetic enrichment of captured CTCs. The captured CTCs are then permeabilized, prefixed and detected simultaneously with a nuclear fluorochrome (DAPI), a fluorescent anti-CD45 antibody (anti-pan leukocytes) and fluorescent antibodies raised against intracytoplasmic cytokeratins of different molecular weights (cytokeratins 8, 18 and 19) (Fig. **2**). This technology is widespread and widely used in different countries, notably in the USA. It has been approved by the Food Drug Association (FDA) in the USA for CTC detection in metastatic breast, colon and prostatic cancer patients, but not in metastatic lung cancer patients [19, 20]. Several advantages are associated with this method: *1*) the blood sample kept in an appropriate buffer (called the "cellsave buffer") can be analyzed until 96 hours after venous puncture; *2*) this method is sensitive (especially for metastatic breast, colon and prostatic cancer patients) and allows the quantification of detected CTCs by analyzing a gallery of images. However, this approach presents some disadvantages, in particular some false negative results can be obtained as detection is only based on EpCAM and cytokeratin antigens (epithelial antigens) expressed by the targeted cells [55-57]. False positive results from circulating benign epithelial cells, which may circulate in patients, especially those with pathologies, such as thyroid, parathyroid, colon or other diseases [25, 56, 58]. These non malignant epithelial cells can be released into the bloodstream from inflammatory foci, benign tumors and other non tumoral sources [25, 41]. As an example, in case of pneumothorax, numerous epithelial non tumor cells can penetrate into vessels and be counted as « CTCs » when using the Cellsearch method. Additionally, among the different blood cells, some monocytes may

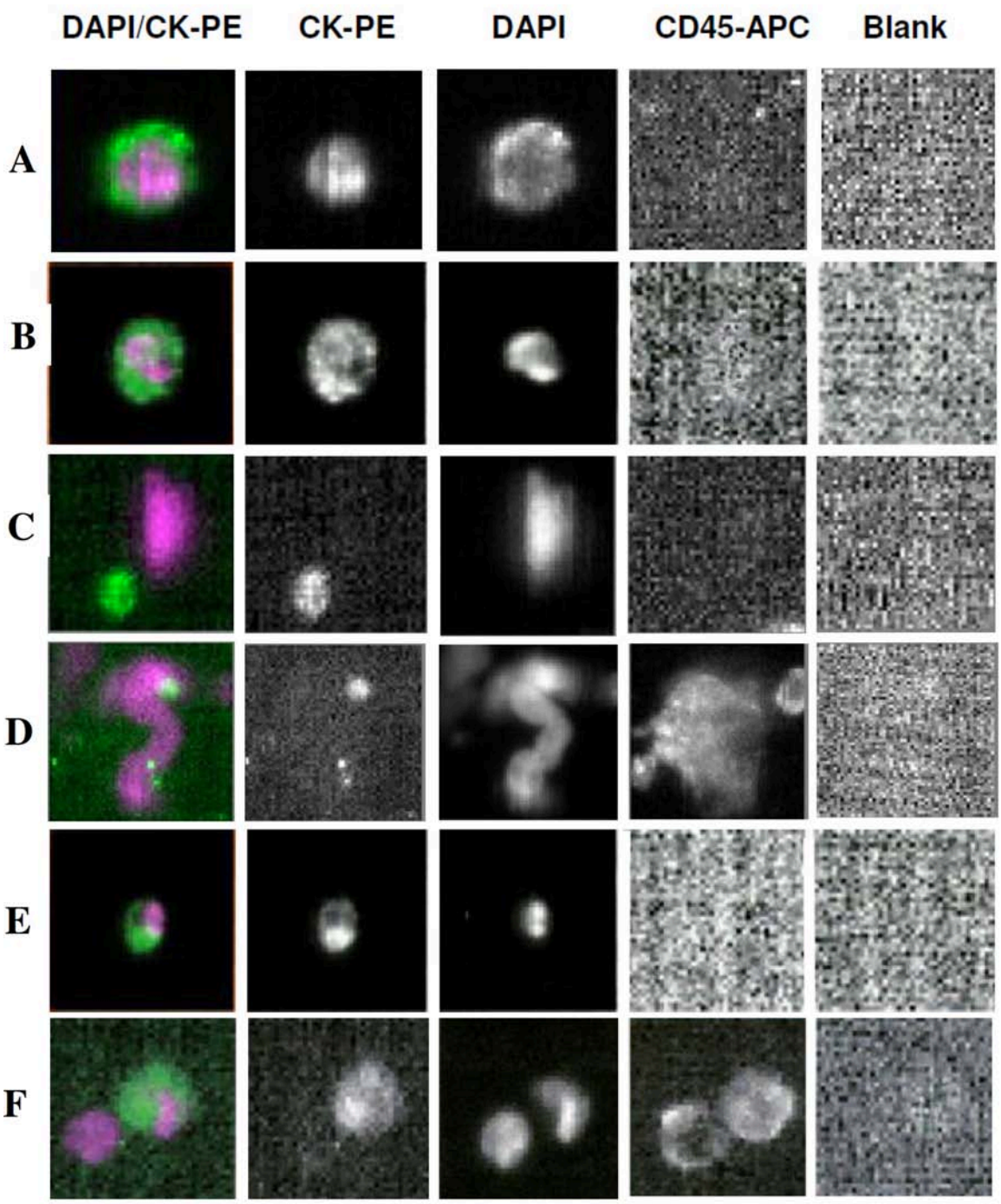

Fig. (2). Cellsearch technology. An example of an indirect method for CTC detection in lung cancer patients. Gallery of circulating tumor cells (CTCs) from the CellSpotter Analyzer. Cells captured with an anti-epithelial cell adhesion molecule (Ep-CAM) antibody were stained with 4',6-diamino-2-phenylindole (DAPI), with an anti-cytokeratin antibody conjugated with phycoerythrin (CK-PE) and with an anti-CD45 antibody conjugated with allophycocyanin (CD45-APC). (**A**, **B**) Cells with a round morphology, a visible DAPI-positive nucleus, positive CK-PE staining in the cytoplasm and negative staining for CD45-APC are considered as typical intact CTC. (**C**, **D**, **E**) Images of cells not included in the CTC count, but frequently observed in the CTC analysis of NSCLC patients. (**F**) The CD45-positive cells were not considered as CTC even when cells were positively stained for DAPI and CK-PE.

express cytokeratin filaments and thus may be counted as CTCs, especially if they show weak CD45 expression [19, 20]. Conversely, false negative results can also be obtained using CellSearch, in particular if CTCs undergo complete EMT. CTCs in EMT are not detected by the CellSearch technology, which is based on expression of epithelial (EpCAM and cytokeratin) molecules [20, 23, 59, 60]. Moreover this approach is quite expensive (equipment and consumables), limiting its routine use, especially to certain countries.

RT-PCR and QPCR Methods

The main advantage of the RT-PCR approach is its high sensitivity, which is considered higher than that of immunodetection methods [61, 62]. This technology consists in the following steps: blood sampling and immediate enrichment of nucleated cells using density gradient centrifugation and/or immunological or immunomagnetic methods, RNA extraction, cDNA synthesis and amplification, and PCR product analysis. While the RT-PCR method can be very sensitive, specificity remains a critical issue as no transcript or antigen with absolute specificity for tumor cells from solid tumors is known at present. One of the major limitations of this method is the fact that the entire population of CTCs is destroyed, precluding their quantification and/or their individual analysis. Moreover the choice of the RNA marker of interest (the targeted transcript expected to indicate the presence of CTCs in blood) is difficult. An ideal target would be a transcript expressed in all tumor cells, but not in normal blood cells and not in non tumor epithelial cells. This transcript (or combination of transcripts) has not yet been identified. Thus, if the targeted transcripts are expressed by epithelial cells (such as cytokeratins), false positives may result if non tumor epithelial cells are present in the bloodstream. As a matter of fact, the mRNA of cytokeratin 19, a biomarker used by many investigators, has been found in 4% of blood samples from healthy donors [63-67]. This result has been attributed to illegitimate transcription of cytokeratin 19 in leukocytes [63, 66, 67]. In this regard, a large number of so called "tumor biomarkers" (such as prostatic specific antigen, mammaglobin, alpha-foetoprotein, epidermal growth factor receptor, etc.) are not completely tumor-specific as they are also expressed in non tumoral circulating epithelial cells and/or in activated leukocytes, which markedly lower their potential as tumor-specific and/or organ-specific biomarkers when they are detected in blood samples using RT-PCR [68, 69]. Some RT-PCR and real time RT-PCR adapted methods have been applied to the CTC field. In this regard, CTCs have been isolated from lung cancer patients by using a QPCR method targeting several epithelial biomarkers [70]. These methods also have some weaknesses for CTC detection and do not solve the issue of "tumor-specificity" [71]. Furthermore, the high sensitivity of RT-PCR is susceptible of being associated with false positive results, which therefore require rigorous controls.

CTC Isolation Using Direct Methods

Isolation by Size of Epithelial Tumor Cell (ISET) Method

ISET is based on the isolation of CTCs according to their size, which is larger than any normal blood cell (the majority of CTCs have a size superior to 20 microns while the size of blood lymphocytes and neutrophils is 8-10 microns). Moreover this method allows accurate cytomorphological evaluation of the isolated cells [24, 72]. Briefly, 10 ml of whole blood is taken in a tube containing EDTA and then filtered through polycarbonate filters with 8 micron pores [72]. The non haematological circulating cells (which may correspond to CTCs) are retained on the filter surface and the vast majority of normal blood cells cross the pores [72]. The main advantage of this approach is the possibility to analyze cytomorphologically the isolated CTCs and to distinguish them from other non-tumor circulating rare cells [24]. Three large categories of circulating rare cells have been defined based on morphological criteria: *1*) a group of cells showing cytological malignant features (anisocytosis, high nucleo-cytoplasmic ratio, large nucleoli, tridimentional sheets, nuclear irregularities), *2*) a group of cells showing cytological benign features, and, *3*) a group of cells showing uncertain cytological features of malignancy [24, 25] (Fig. **3**). In this context, the morphological criteria used are those also used for conventional cytological evaluation (in fine needle aspiration or cytological smears). The ISET method distinguishes the "true CTCs" (with malignant features) from other rare cells that are not CTCs and from non tumor circulating epithelial cells [25]. Moreover, additional analyses can be performed on the isolated cells to better characterize them. Immunocytochemical and/or an *in situ* hybrization approaches can thus be associated with morphological analyses to identify biomarkers of interest [73, 74] (Fig. **3**). These analyses can be performed to explore the primitive organ of origin of these cells, their invasive potential and/or the presence of genomic alterations, which may be of interest for administration of targeted therapy. Evaluation of genomic alterations can also be done on cells identified as CTCs after their selective laser capture microdissection and DNA extraction [72] (Fig. **4**). However, the ISET method has some particularities and constraints. There is debate about the size of tumor cells, which could, according to some discussions, be smaller than 8 microns, thus smaller in size than mature lymphocytes. However, for now, no demonstration has been provided that cytopathologically validated tumor cells can be smaller than mature lymphocytes, and that elements smaller than 8 micron and presenting genomic abnormalities are intact tumor cells, rather than apoptotic debris. Thus, the possibility of loosing CTCs through the 8 micron pores is still an open question. The blood has to be filtered as soon as possible after collection in order to guarantee the unparalleled sensitivity of ISET [20]. In fact, circulating rare cells are fragile and can be destroyed if they remain in the blood for a long time. Furthermore, the cytomorphological analysis requires the maintenance of morphological details. In our experience, a maximum delay of two hours gives the best morphological results. Studies are ongoing to improve this aspect. Finally, CTC counting could be speeded up by the use of automated image analysis, which rapidly identifies the rare cells for morphological analysis by the cytopathologist.

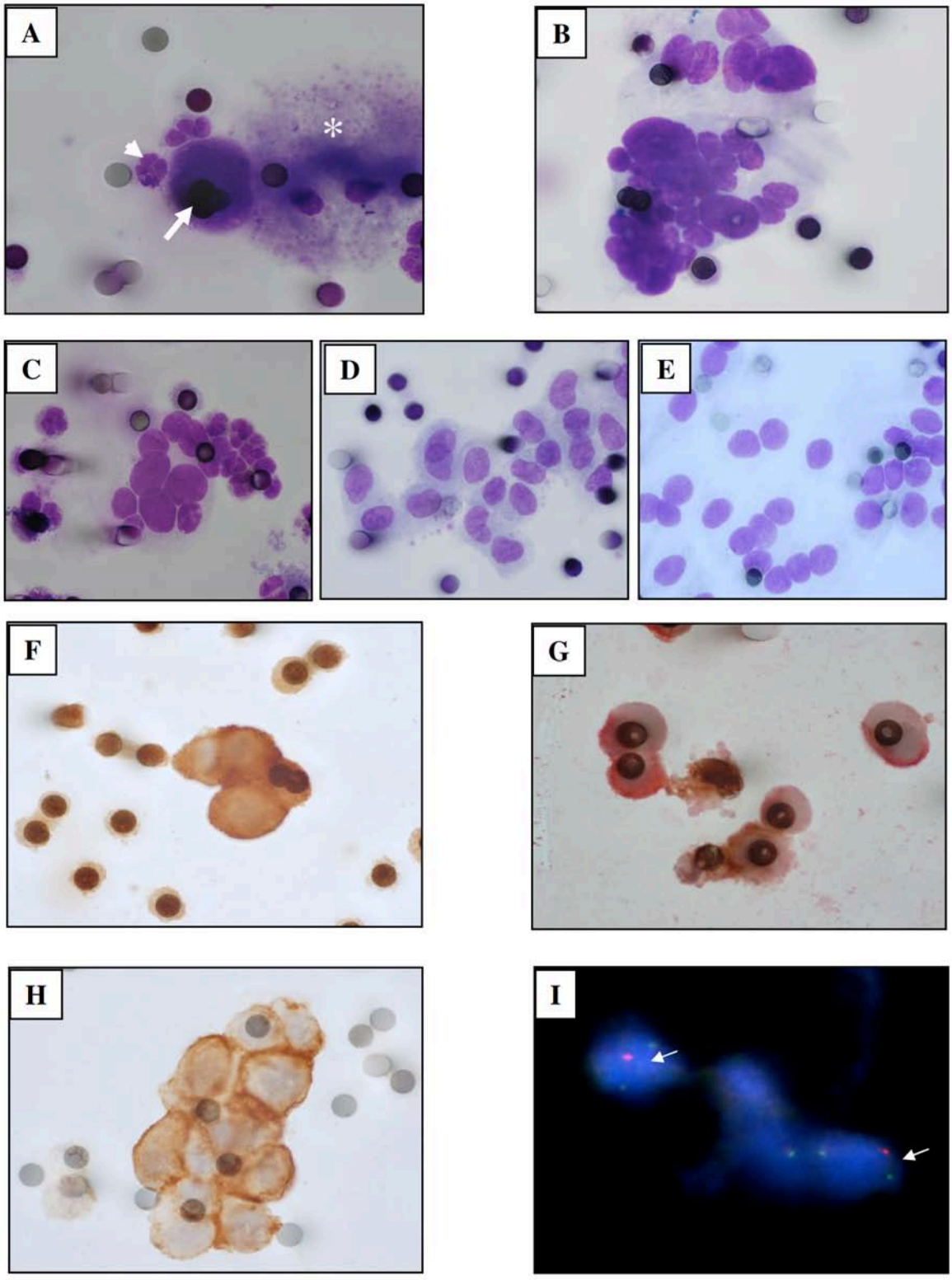

Fig. (3). ISET method. An example of a direct method for CTC detection in lung cancer patients. Different images after CTC detection and characterization. (**A**) Isolated malignant CTC (arrow) associated with neutrophils (arrowhead) and platelets (asteriks). (**B**) CTM composed of different CTCs from a lung adenocarcinoma showing malignant features. (**C**) A CTM from a squamous cell carcinoma of the lung composed of different circulating non haematological cells showing malignant features. (**D**) Circulating non haematological cells showing uncertain malignant features. (**E**) Circulating non haematological cells showing benign features. (**F**). CTCs expressing the pancytokeratin antigen. (**G**) CTCs expressing mostly the vimentin antigen. (**H**) Immunostained CTCs with an anti-ALK antibody. (**I**) FISH analysis on CTCs showing the EML4-ALK rearrangement (arrow). (A-I, original magnification x 800; A-E: May Grunwald Giemsa staining; FG: double immunophosphatase and immunoperoxidase staining; H: immunoperoxidase staining; I: immunofluorescence analysis).

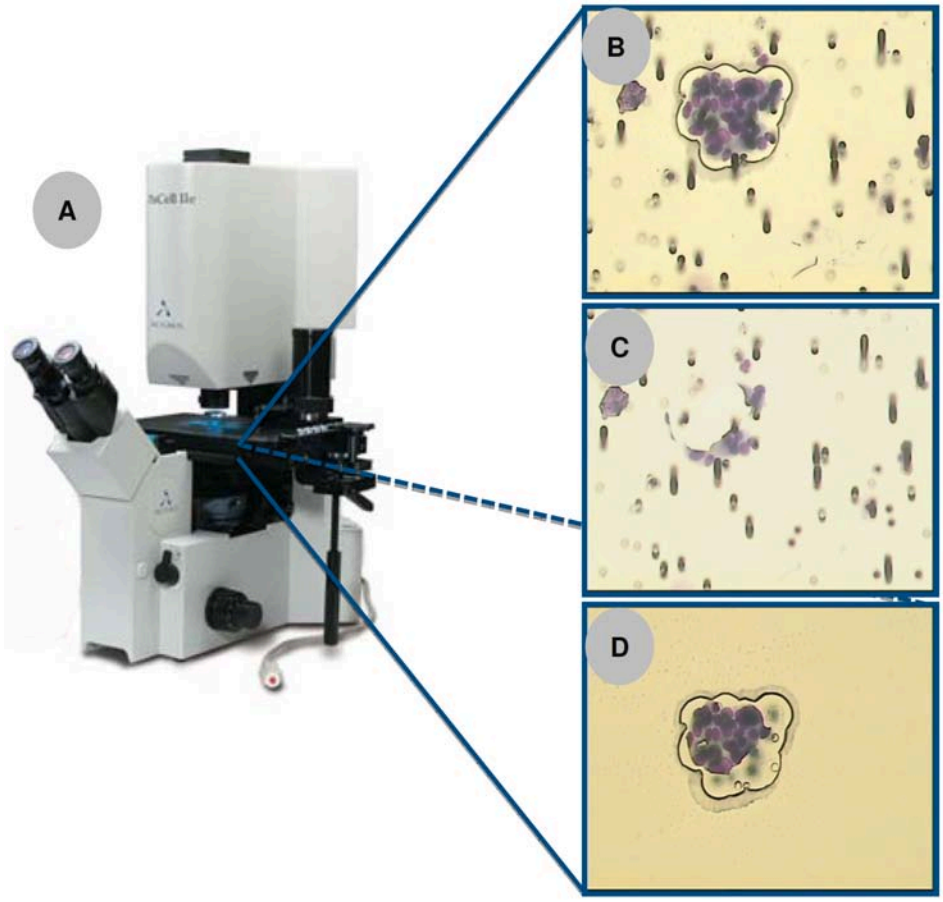

Fig. (4). (**A**) Example of a CTM trapped on a polycarbonate filter, laser capture microdissected using an Acturus Pix Cell II microscope. (**B**). CTCs before microdissection. (**C**) Filter after microdissection. (**D**) Microdissected CTCs.

Other Methods

Fiber-optic array scanning technology (FAST) does not require initial CEpCs enrichment for their detection [75-77]. CEpCs are isolated with a laser scanner at a speed superior to one thousand times that of a digital microscope. The CTC observation is done by immunofluorescence after morphological analysis using anti-cytokeratin antibodies and nuclear DAPI staining [75-77]. A new method called microcavity array system (MCA) for size-based enrichment of CTC allows the isolation of small tumor cells based on differences in size and deformability between tumor and blood cells [78]. In particular, this method can be use for the detection of SCLC CTC [78].

For brevity, we cite here only the EPISPOT, approaches using chips (CTC-chip and CTC-chip Ephesia), magnetic beads, telomerase activity detection, and other methods such as the Maintrac, Ariol, MagSweeper, Ikoniscope, Vita-Assay, dean flow fractionation and DEP-FFF techniques [69, 79-98]. Thus, the number of methods for CTC isolation and characterization in patients with lung cancer is rapidly expanding. However, we still have to define which method will be more appropiate for routine practice and accessible to all patients, while being sufficiently sensitive and specific.

IMPACT OF CTC DETECTION FOR DIAGNOSIS AND PROGNOSIS OF LUNG CANCER PATIENTS

Most of the studies related to CTC detection in lung cancer patients have targeted patients developing NSCLC (Table **1**). A few studies have demonstrated that CTC detection can be diagnostic for these lung cancer patients [27, 99] (Table **1**). In NSCLC patients, recent studies have demonstrated that the efficiency of the chemotherapy directly correlates with a decrease in the number of detected CEpCs following treatment [100, 101]. The presence of CEpC/CTCs has been shown by several studies, using different technologies, to be an independent bad prognostic factor in NSCLC patients [27, 102-109] (Table **1**). In this regard, CEpC/CTCs detected before, during or after resected tumors as well as CEpC/CTCs detected in non resected lung cancer correlated with worse prognosis [27, 102-109] (Table **1**). Finally, several studies have demonstrated the interest of CEpCs detection for diagnosis and prognosis of small cell lung carcinoma [103, 110-113] (Table **1**).

The field of CTCs is potentially relevant in patients with lung cancer. However, there is a strong need of method standardization of sensitive CTC isolation without loosing CTCs in EMT and of diagnostic CTC identification and characterization. In fact, there are

Table 1. Main Studies Related to CTC Detection in Lung Cancer Patients

Study [Ref.]	Cancer Cases	Control Size	CTC Isolation Method	Sampling Time	Histology	Results
Isobe K, *et al.* 2012 [14]	24	—	CellSearch	After development of EGFR-TKI resistance	Metastatic III/IV NSCLC	Presence of CTCs was correlated with the positivity of EGFR mutation in cfDNA
Hofman V, *et al.* 2011 [23]	210	40	CellSearch and ISET	Before surgery	Resectable NSCLC (I-IV)	Prognostic biomarker
Hofman V, *et al.* 2012 [24]	250	59	ISET	Before surgery	Resectable NSCLC (I-IV)	Diagnostic biomarker
Okumura Y, *et al.* 2009 [30]	30	—	CellSearch	Before surgery	Resectable NSCLC	Prognostic biomarker
Farace F, *et al.* 2011 [56]	20	—	CellSearch and ISET	Before treatment	Stage IV NSCLC	Discrepancies between the number of CTC enumerated by the CellSearch and the ISET systems
Lecharpentier A, *et al.* 2011 [57]	6	6	ISET and triple fluorescent labeling	Unknown	Metastatic NSCLC	Exploratory study demonstrates that the majority of isolated or clusters of CTCs in patients with advanced metastatic NSCLC harbor a dual epithelial–mesenchymal phenotype
Krebs MG, *et al.* 2011 [59]	101	—	CellSearch	Before and after administration of one cycle of standard chemotherapy	Untreated, stage III/IV NSCLC	Prognostic and predictive biomarker
Krebs MG, *et al.* 2012 [60]	40	—	CellSearch and ISET	Before chemotherapy	Metastatic III/IV NSCLC	Complementary dual technology approach to CTC analysis
Hayes DC, *et al.* 2006 [61]	49	25	RT-PCR	Before treatment	NSCLC NOS	Diagnostic and prognostic biomarker
Guo Y, *et al.* 2009 [64]	83	30	Nested RT-PCR	Before treatment	NSCLC NOS	Diagnostic biomarker
Huang TH, *et al.* 2007 [65]	51	40	ICC and RT-PCR	Before treatment	Chemotherapy-naïve NSCLC (I-IV)	Diagnostic and prognostic biomarker
Devriese LA, *et al.* 2012 [70]	46	46	CellSearch and multi-marker qPCR	Before treatment	Metastatic III/IV NSCLC	Diagnostic biomarker
Ilie M, *et al.* 2012 [74]	87	—	ISET	Before treatment	Lung adenocarcinoma	ALK feasibility on CTCs
Hou HW, *et al.* 2013 [86]	20	20	Dean Flow Fractionation	Before and after treatment	Metastatic III/IV NSCLC	Feasibility (5 to 88 CTCs/mL)
Nieva J, *et al.* 2012 [92]	28	—	Enrichment free fluorescent labeling with automated digital microscopy	Before chemotherapy	Metastatic IV NSCLC	Higher numbers of detected CTCs were associated with an unfavorable prognosis
Sheu CC, *et al.* 2006 [94]	100	147	Membrane array-based multimarker assay	Before treatment	NSCLC (I-IV)	Diagnostic and prognostic biomarker
Tanaka F, *et al.* 2009 [99]	125	25	CellSearch	Before treatment	Chemotherapy-naïve NSCLC (I-IV)	Diagnostic and prognostic biomarker
Hirose T, *et al.* 2012 [100]	33	—	CellSearch	Before treatment	Metastatic III/IV NSCLC	Predictive biomarker for the effectiveness of cytotoxic chemotherapy (gemcitabine and carboplatin)

(Table 1) contd…..

Study [Ref.]	Cancer Cases	Control Size	CTC Isolation Method	Sampling Time	Histology	Results
Funaki S, *et al.* 2011 [102]	94	—	RosetteSep® Human CD45 Depletion Cocktail (Stemcell Technologies, Inc.)	During treatment	NSCLC (I-IV) without preoperative chemo- and/or radiation therapy	Prognostic biomarker
Hou JM, *et al.* 2012 [103]	97	—	CellSearch	Before and after chemotherapy	SCLC	Prognostic and predictive biomarker
Sher YP, *et al.* 2005 [104]	54	24	Density gradient centrifugation and nested RT-PCR assay	Before treatment	NSCLC (I-IV) without preoperative chemo- and/or radiation therapy	Prognostic and predictive biomarker
Sienel W, *et al.* 2003 [105]	62	—	ICC	During treatment	Resectable NSCLC (I-III)	Prognostic biomarker
Wu C, *et al.* 2009 [106]	47	31	CellSearch	Before chemotherapy	34 NSCLC (I-IV) and 13 SCLC	Predictive biomarker
Yamashita J, *et al.* 2002 [107]	103	Unknown	Density gradient centrifugation and RT-PCR assay for carcinoembryonic antigen	Before and after surgery	Resectable NSCLC	Prognostic biomarker
Yie SM, *et al.* 2009 [108]	143	172	RT-PCR ELISA	Before and after treatment	NSCLC (I-IV) without preoperative chemo- and/or radiation therapy	Prognostic biomarker (Survivin-expressing CTCs)
Yoon SO, *et al.* 2011 [109]	79	—	Nested (for CK19) or semi-nested (for TTF-1) real-time RT-PCR	Before surgery and 1 month after surgery	Resectable NSCLC (I-III)	Prognostic biomarker
Hiltermann TJ, *et al.* 2012 [110]	59	—	CellSearch	Before, after one cycle, and at the end of chemotherapy	SCLC	Predictor for response on chemotherapy and survival
Hou JM, *et al.* 2009 [111]	88	85	CellSearch	Before and after chemotherapy	SCLC	Prognostic and predictive biomarker
Naito T, *et al.* 2012 [113]	51	—	CellSearch	At the baseline, after chemotherapy, and at relapse	SCLC	Prognostic and predictive biomarker
Punnoose EA, *et al.* 2012 [116]	41	—	CellSearch	Before and after treatment (erlotinib and pertuzumab)	Metastatic III/IV NSCLC	Decreases in CTC numbers during treatment of patients with advanced NSCLC with targeted therapies are associated with clinical responses measured by FDG-PET and CT imaging
Das M, *et al.* 2012 [123]	17	—	Cytophotometry	Before and after treatment (platinum-based therapy)	Metastatic III/IV NSCLC	Low expression of ERCC1 on CTCs correlates with PFS in patients with metastatic NSCLC receiving platinum-based therapy.
Wendel M, *et al.* 2012 [136]	78	—	HD-CTC technology	Before treatment	Chemotherapy-naïve NSCLC (I-IV)	Prognostic biomarker
Liu L, *et al.* 2008 [144]	134	186	Density gradient centrifugation and nested RT-PCR assay	Before treatment	NSCLC (I-IV) without preoperative chemo- and/or radiation therapy	Diagnostic and prognostic biomarker

clear limitations in using CEpC as a prognostic biomarker in certain lung cancer patients as some patients, although negative for CEpCs, still develop metastases. Moreover, a significant fraction of patients with overt metastasis have surprisingly low CEpCs counts. Thus, CEpCs that are capable of forming metastasis may remain undetected by some detection methods.

MOLECULAR CHARACTERIZATION OF CTCS IN LUNG CANCER PATIENTS: HOW AND WHY?

The current interest in molecular characterization of CTCs isolated from lung cancer patients is strongly linked to the advent of targeted therapies and to the concept of personalized medicine in thoracic oncology. It is now well-established that genomic alterations (mutations and gene rearrangements) occurring in lung tumor cells can be "druggable", thus driving targeted treatments. The list of the potential genomic alterations of interest is long and concern mostly NSCLC, reflecting the great molecular heterogeneity of NSCLC. Some genomic alterations are detected mostly in lung adenocarcinomas whereas others are more frequent in lung squamous cell carcinomas. Among the druggable mutations for targeted therapy, those occurring in exons 19 and 21 of the epidermal growth factor receptor (*EGFR*) gene are currently routinely evaluated in the tumor tissue of patients with a pTNM IIIb/IV adenocarcinoma. Moreover, some reports have shown that *EGFR* mutations can also be detected either in CEpCs or in plasma free DNA [114, 115]. However, some *EGFR* mutations detected in plasma free DNA have been reported to be associated with false negative results, thus highlighting the interest of detecting mutations specifically in CTCs [114]. The analysis of *EGFR* mutations in CEpCs isolated from lung cancer patients has been performed using different methods [115, 116]. These studies are of major potential interest in lung cancer patient care as the approach is non invasive, rapid and can be proposed in vulnerable patients. Moreover, it is possible to repeat the test during follow-up to adapt the therapeutic choice by screening the onset of secondary resistant mutations such as T790M. Among other druggable genomic alterations currently being actively looked for in adenocarcinoma lung cancer patients, the rearrangement of the *EML4-ALK* gene can be detected in CTCs by FISH and ICC analysis, thus guiding treatment with crizotinib [74, 117]. Our group found a good correlation between the results obtained in lung cancer tissues and in CTCs isolated by ISET [74].

The field of molecular characterization of CTCs is vast as virtually all genomic alterations detected in lung cancer tissues can be detected in CTCs. Among them, those occurring in the *BRAF*, *KRAS*, *Her2*, *PIK3CA/AKT1*, *ROS*, *FGFR1* and *MET* genes are of strong interest since clinical trials are currently investigating mutation_specific targeted drugs [118-122]. In the future, next generation sequencing methods applied to CTCs from lung cancer patients could probably explore several hundred mutations on different genes. Furthermore, the development of antibodies and probes specific to mutated nucleic acids and proteins could be used in FISH and immunolabeling assays to speed up detection in CTCs of druggable genomic alterations. In fact, the results obtained through these approaches have been published and in the future may guide the choice of targeted treatments [52, 53, 73, 123-126].

Finally, in addition to the theranostic impact, molecular characterization of CTCs, and in particular transcriptomic analyses may be of great help in studying the origin of occult lung cancer in patients without detectable imaging of the primary tumor [127].

CTC IDENTIFICATION IN THE CLINICAL DAILY PRACTICE FOR BETTER MANAGEMENT OF LUNG CANCER PATIENTS: FACT OR FANCY?

The increasing number of publications, comments and general reviews in the CTC field shows the growing interest in this subject by the scientific and medical community [12, 128-136]. Thus, the CTC research was performed in almost all solid cancers with the objective to make an early diagnosis and/or to appreciate their prognosis or predict the metastatic risk [12, 128-136]. However, the clinical impact of CTCs is still under question as contrasting results have been obtained due to technical differences in specificity and sensitivity and due to limitations including the high cost and labour needed, which limit the use of some approaches for large cohorts of patients and in the daily clinical practice [53, 133, 137]. Moreover, the results of these different studies of lung cancer patients are difficult to compare due to the different technologies. Moreover, the numbers of patients studied are often too small and the clinico-pathological characteristics are not homogenous between studies, which makes it difficult to reach robust conclusions. As mentioned above, the most diffused approach for CEpC detection, the CellSearch technology has been shown to have prognostic FDA approved value only in metastatic breast, colon and prostate cancer patients. However, as for other methods, the Cellsearch technical approach is based on epithelial markers (epithelial cell adhesion molecule and keratin), which prevents the detection of most CTCs since epithelial markers tend to be down regulated during tumor cell dissemination, especially in lung cancer patients.

The major challenging goals in oncology, especially in lung oncology, are early detection of the primary cancer; with or without metastasis, determining the prognosis of a patient and predicting the response of an individual to treatment [74, 101, 110, 115, 116, 129, 138]. In addition, the possibility of detecting early on secondary resistance to treatment so as to quickly adapt the therapy or to appreciate the treatment efficacy or disease recurrence through blood sampling is critical since access to tissue or cytological can be difficult in fragile patients. Moreover, the presence of CTCs in early stage operated patients may help to define the prognosis of these patients and for the positive population to propose an adjuvant treatment even if this strategy deserves to be validated with large prospective trials. Furthermore, in the case of carcinoma of unknown primitive origin, the molecular analysis of CTCs, in particular by using a new genomic sequencing method or a transcriptomic approach could provide informative results regarding the primitive tumor site [139-141].

Despite the objectives and the promises claimed by the scientific community concerning the value of CEpC/CTC detection and characterization in thoracic oncology, there is uncertainty concerning as to when and how CEpC/CTC translational results will be implemented into the daily health care [133, 137]. In this context, there is a striking contrast between the high number of international publications related to the CTC field and the real usefulness for oncologists in their daily decision-making and thus the benefit to lung cancer patients. Nonetheless, studies on CTCs have considerably increased knowledge concerning lung cancer pathophysiology and the mechanisms involved in metastasis.

In fact, doubts persist among some oncologists who question whether the results obtained with CTC detection in lung cancer patients are meaningful. Clearly, issues concerning specificity, sensitivity and reproducibility of the different technical approaches, their ability to isolate and identify the "most malignant" CTCs, the optimal blood volume for study, the possibility to find different genomic alteration in the tumor tissues *versus* the CTCs and the cost policy allowing implementation of CTC testing in public and private hospitals, are still to be addressed [131, 142]. An additional concern, related to the low number of CTC in blood, is the need to develop methods and tools compliant with ISO norms (such as ISO 15189) for their suitable transfer to the hospitals' biological laboratories. Thus avoiding the potential pitfalls that occur during the pre-analytical and analytical phases, which lead to technical errors with impact on the quality and robustness of the results, especially for interpretation of genomic alterations and detection in lung cancer patients [126, 143].

However, despite all these open questions and unmet needs, studies on CTCs have increased considerably the knowledge of lung cancer pathophysiology and of mechanisms involved in metastasis. In this setting, methods allowing the isolation of non fixed CTCs, which can be engaged in cell multiplication processes, although difficult to apply in current clinical practice, are being developed more and more. These viable cells could be further injected into mice to form xenografts, thereby allowing the study of their invasive potential *in vivo*.

Multicenter research projects using different technologies could provide important data to help detection of invasive, highly malignant CTCs when using high throughput approaches with socioeconomic impact in clinical lung oncology.

CONCLUSION

While pTNM staging is currently the only validated prognostic factor used in NSCLC patient follow-up and treatment, 25% to 50% of patients with early stage NSCLC show tumor recurrence, even following extensive tumor resection. Thus, the discovery and implementation of sensitive and robust prognostic and predictive biological markers is a competitive field in thoracic oncology, which reflects an important and urgent need. In this context, the isolation, development and characterization of CTCs as a non-invasive predictive biomarker have a huge clinical potential and can increase our knowledge of lung cancer pathophysiology. In early stage NSCLC patients the occult metastasic disease correlates with disease recurrence. Thus, sensitive and specific detection of CTCs in the blood has the potential to become a relevant prognostic biomarker for patients with resectable NSCLC. Indeed, the main goal of preoperative detection of CTCs is to identify NSCLC patients with a high risk of recurrence after surgery in order to adopt the best therapeutic strategy and follow-up [27].

However, the clinical use of CTC detection as a new biomarker has to be very carefully validated. Indeed, despite the report of a large number of studies in lung cancer patients on CTC detection, methodological aspects concerning sensitivity, specificity and reproducibility have prevented a clear appraisal of the clinical impact. Numerous technologies have now been set up to improve CTC detection in lung cancer patients, some of them appear to be very sophisticated. Despite the enthusiasm in method development, in the different translational research studies and some clinical trials, and despite the impressive number of recent publications concerning the CTC field, it is quite paradoxical to note that this approach is rarely taken into consideration by the clinical oncologists regarding their therapeutic choice for lung cancer patients. Indeed, the technology transfer from research to the hospital's biology laboratories is still difficult in 2013. CTC detection in lung cancer patients, especially for theragnostic application, is currently a very competitive domain with different approaches also targeting non cellular blood biomarkers. Thus, the identification of genomic alterations from free circulating DNA isolated from lung cancer patients could be an attractive and probably less costly option for some specific indications [144, 145]. Finally, the use of non-invasive approaches based on CTCs and/or other blood biomarkers to develop so called « liquid biopsy » applications could allow, in the future, monitoring of lung cancer patients on therapy in order to diagnose early recurrence or disease progression, but also to decipher the molecular mechanisms of resistance for rapid treatment adjustment.

The concept of a liquid biopsy needs to be considered in tight association with the different parameters obtained from the pathology laboratory (data from the surgical specimen, from bronchial and transparietal biopsies and/or from bronchial cytology). These latter parameters include the morphology, the immunohistochemical and the molecular pathology data. Only a combined approach that integrates the pathologist, the cytopathologists and the biologists will allow optimisation of the interpretation of the results of the liquid biopsy while avoiding the different pitfalls. Thus developing new training options of expertise as a "biopathologist" could be a good way to obtain a global view of the lung cancer disease both for diagnosis and

for a better understanding of the pathophysiology of the disease.

CONFLICT OF INTEREST

The authors declare that this article content has no conflicts of interest.

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