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TIMELY REVIEWS

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Lung cancer samples preserved in liquid medium: One step beyond cytology

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Abstract

Lung cancer is one of the most common cancer types in men and women worldwide with a high mortality rate. World Health Organization (WHO) classification has accepted biopsy as the primary sample for lung cancer diagnosis, pathological classification and molecular testing for management of patients, yet, the use of alternative sampling procedures is highly encouraged. Bronchial cytological samples require a less invasive collection technique and may be suitable for pathological and molecular analysis and storage in liquid medium. Furthermore, the molecular analysis of bronchial cytological samples allows the detection of molecular biomarkers, which may be useful for the selection of molecular targeted therapies. Thus, the purpose of this review is to describe the usefulness of bronchial cytological samples preserved in liquid medium from lung cancer patients for pathological diagnosis and molecular investigation.

KEYWORDS

bronchial cytology, liquid medium, lung cancer, molecular biomarkers

1 | INTRODUCTION

Lung cancer is the major cause of cancer-related death among men and the second most common among women worldwide.[1] The International Agency for Research on Cancer (IARC) reported that almost 1.6 million deaths in 2012 were due to lung cancer and estimated 1.8 million new cases for 2016.[2] Only 15% of lung cancer cases are diagnosed at the initial stages of the disease, when the treatment is more effective, rendering a low overall survival rate of approximately 17%.[3,4]

In 2015, for the first time, the World Health Organization (WHO) classification proposed new criteria for the diagnosis of lung cancer based on small biopsies (bronchoscopic, needle or core biopsies) and cytology. For the collection of these biopsies, surgical resection is not required, resulting in a less invasive procedure for collection of specimens.[5] These specimens are used for pathological classification, allowing the diagnosis of advanced stages of the disease, as well as for molecular testing to guide the treatment of patients, especially in inoperable cases.[6,7] The major objective of biopsy is to establish the diagnosis and stage of disease and minimize the risk to the patient.[8] Based on the high demand for these small specimens with limited tissue, the use of alternative samples is highly encouraged.[8–10] Cytological smears and liquid-based cytology may be a less invasive option to avoid re-biopsy. This scenario was noted by the WHO in 2004, when the cytology was included for the first time as an acceptable diagnostic sample, and more recently by the Papanicolaou Society of Cytopathology Guidelines published for Standardized Terminology and Nomenclature for Respiratory Cytology.[11] However, since then, the employment of cytological samples has been mostly limited to ancillary samples for diagnosis. Herein, we will focus in the main clinical and molecular aspects of lung cancer as well as the advantages of liquid-based cytology (LBC) for lung cancer diagnosis.

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1.1 | Collecting bronchial samples

Before showing the main aspects of lung cancer and the advantages of liquid cytology for the study of neoplastic or potentially neoplastic disease and the investigation of a variety of benign diseases, including opportunistic infections,[12] we will briefly discuss the sampling procedure. The most common methods to obtain cytological specimens include fine-needle aspirates (FNAs) from lung (frequently coupled with core needle biopsies), bronchial brushings, bronchial washings, bronchoalveolar lavages and sputum, along other minimally invasive aspirations of distant, deep-seated or superficial metastatic lesions.[13]

Fine-needle aspirates (FNAs): as a collection method, the sensitivity and specificity for the diagnosis of lung cancer is high with an acceptable complication rate.[14]

Bronchial brushings: an useful option in endoscopically visible tumors, it can be performed before or after biopsy sampling, but it is advisable to be perform it before the bronchial biopsy to avoid contamination of the specimen with blood.[15,16] For adequate representativeness of the suspect malignancy area, the brush should be forward/reverse two or three times.[17]

Bronchial washing: it can also be taken before or after biopsy sampling. The exact volume to be recovered from this procedure has not been established; however, several studies recommend recovering as much fluid as possible after instilling 20-30 mL of sterile isotonic saline.[15] The material is centrifuged and the sediment is used to prepare the smears or liquid-based preparations, which is then stained.[17]

Bronchoalveolar lavage (BAL): this technique increases the diagnostic yield in pulmonary peripheral lesions, and it is primarily used to diagnose infections and interstitial lung diseases.[18] Unlike the other techniques, BAL has well-standardized guidelines.[17]

Sputum: it is not recommended as a suitable material for diagnosis of lung cancer due to its low sensitivity.[19] In patients who are unable to undergo bronchoscopy or other minimally invasive procedure and for whom a diagnosis of malignancy is required, sputum may be helpful. It is recommended to repeat sputum sampling over several days for increased sensitivity.[17,19]

First, we would like to highlight that cytological samples are considered an important tool for the diagnosis of lung cancer due to their low cost and less invasive collection methods.[17,20]

1.2 | Lung cancer: current aspects

The pathogenesis of lung cancer is complex and it may occur due to a combination of genetic and environmental factors.[21] Although smoking habits is the strongest factor linked to lung cancer, the incidence of the disease among non-smokers has increased.[22] In addition to smoking, other factors are also related to lung cancer such as occupational exposure, previous lung disease and diet among others are attracting the attention of the physicians to patients who do not smoke.[22–24] Based on this, we can suppose that additional factors might be the cause of the disease and they can function independently or in combination with the well-known factors.[23,25]

Regarding the histology, lung cancer is classified into two main groups: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC).[26,27] For many years, this histological classification of lung cancer was sufficient for the management of lung cancer patients in routine practice.[5]

1.3 | Small Cell Lung Cancer (SCLC)

Previously described as 'oat cell carcinoma' in a patient with asbestosis, SCLC exhibits neuroendocrine differentiation, strong association with smoking and genomic alterations enrolling genes with a key role in transcriptional regulation and chromatin modification.[28,29] Patients with SCLC have carcinomas which display a high growth fraction, rapid doubling time and early establishment of widespread metastatic lesions and response to first-line chemotherapy. Nonetheless, SCLC patients experience relapse, and there is no approved targeted drug as a second line therapy for these tumors.[30] However, this scenario may change since many promising advances with immune checkpoint inhibitors have emerged in the last few years.[31,32]

Genomic analyses have identified driver mutations in SCLC, leading to a better understanding about cellular features and molecular mechanisms involved in initiation, progression, metastasis and resistance.[33] However, limited tumor tissue for research due to small diagnostic biopsies represents a significant barrier to progress in the research field,[28,34] which can be circumvented using cytological samples.

Basically, lung carcinoma differentiation into SCLC and NSCLC subsets is important for the clinical management of the patients. The first step for the diagnosis is the observation of cytological features from tumor samples. The diagnosis of SCLC can be achieved through cytomorphology of FNA prepared using ThinPrep and conventional smears.[35] Indeed, the LBC of sputum specimens from 101 patients with lung cancer exhibited a significantly higher sensitivity for SCLC diagnosis ($P < .05$) than conventional smear, suggesting the LBC may be easily and widely applied for lung cancer diagnosis.[36] The diagnostic value of LBC was also shown using 4,380 brushing specimens from lung cancer patients. The accuracy was 75.6% and, compared with histopathology—gold standard, the LBC allowed lung cancer subtyping into squamous cell carcinoma, adenocarcinoma and small cell carcinoma with accuracy rates of 95.6% (351/367), 95.6% (351/367) and 100% (367/367), respectively ($P < .001$), with the highest value for SCLC. These results emphasize that LBC is an effective tool for the diagnosis and subtyping of lung cancer.[37] and should be better exploited.

1.4 | Non-Small Cell Lung Cancer (NSCLC)

The NSCLC group can be further subdivided into three major cell types: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Adenocarcinoma is the most common histological type and generally arises in the distal airways.[38] Squamous cell carcinoma usually arises in the proximal airways and in the hilum or close to it.[38,39] Large cell carcinoma is diagnosed by exclusion, when the tumor cells do not present glandular or squamous appearance and present with distinctive

histological elements, giant cells and clear cells.[39] Among all cases of lung cancer, approximately 80%-85% are diagnosed as NSCLC, and among all NSCLC cases, almost 70% are locally advanced or metastatic.[40] For metastatic NSCLC cases, chemotherapy, targeted therapy and/or radiation therapy are not effective for disease cure and they can only extend survival and palliate the symptoms.

In general, the diagnosis of lung cancer involves three important steps: (I) identification and classification of malignancy, (II) immunohistochemistry to predict the likely NSCLC subtype and (III) molecular testing, mainly in advanced adenocarcinoma to investigate oncogenic driver mutations.[17] The evidence of therapeutically relevant driver mutations in subsets of NSCLC and the emerging drugs as targeted therapies for some subtypes of NSCLC have rendered the classification of NSCLC subtypes clinically mandatory.[5]

1.5 | Lung cancer: molecular landscape

1.5.1 | EGFR

Mutations in the epidermal growth factor receptor *(EGFR)* gene activate this receptor, which leads to the activation of intracellular signaling pathways involved in cell proliferation, differentiation and cell cycle, triggering neoplastic progression. The most common mutations are located between exons 19 and 21 and they are frequently observed in some types of lung cancers especially adenocarcinomas. Among NSCLC, *EGFR* hotspot mutations are found in approximately 10%-15% of all tumors, being more common in female patients, non-smokers and Asian patients, in which the frequency of these mutations is improved.[41,42] The presence of *EGFR* mutations is related to the prognosis of NSCLC patients, sensitivity and improved response to tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib.[43–47]

1.5.2 | KRAS

Mutations in the kirsten rat sarcoma viral oncogene homolog (*KRAS*) oncogene have been associated with several malignancies including as pancreatic cancer, colorectal cancer and lung cancer.[48–50] *KRAS* mutations occur in approximately 20% of NSCLC patients and are commonly located at codons 12 and 13 of the gene. These mutations are associated with smoking habits, have a worse prognosis and are more common in the NSCLC subtype.[38,51,52] Patients harboring *KRAS*, unlike *EGFR*-mutated patients, do not benefit from TKIs, probably because *KRAS* itself is difficult to inhibit, and the effectiveness of agents targeting *KRAS* effectors have been blocked by the activation of offsetting pathways that limit their efficiency.[53,54]

1.5.3 | EML4-ALK

Anaplastic lymphoma kinase (*ALK*) is a tyrosine kinase receptor translocations of which trigger an oncogenic process in NSCLC tumors.[55] The echinoderm microtubule associated protein like 4 (EML4) gene is located near to ALK gene.[55] An inversion in the short arm of chromosome 2 results in a fusion gene comprising parts of the *ALK* and *EML4* genes. The protein encoded by this gene-fusion gene is highly oncogenic and associated with NSCLC pathogenesis in a subgroup of patients.[55] *ALK-EML4* translocations and *EGFR* and *KRAS* mutations are mutually exclusive.[56,57] The oral inhibitor of the ALK tyrosine kinase domain, crizotinib, was effective in decreasing in vitro cell proliferation and to improve overall and disease-free survival in NSCLC patients harboring *ALK-EML4* translocations.[58,59] However, the frequency of this alteration in patients with NSCLC is low, approximately 3%–7%.[55–57,60]

1.6 | Molecular biomarkers in body fluids

In the last few years, many studies have emerged to investigate lung cancer biomarkers, which have been defined for prognostic purposes, diagnosis and early detection of the disease. The detection of biomarkers can help to define tumor stage, recognition of recurrence, and to support disease and treatment monitoring.[61] These biomarkers may be proteins, RNA molecules, miRNAs, methylated DNAs and, more recently, circulating tumor cells.[62] Tumor biomarkers are secreted by the neoplastic tissue into body fluids and help to distinguish abnormal from normal conditions.[50,63] Body fluids are generally easily obtained and may be routinely collected for laboratory testing. Among body fluids in lung cancer patients, bronchial washes are obtained in a volume sufficient to allow its use in molecular biomarker identification.

1.6.1 | Molecular biomarkers and clinical relevance for lung cancer patients

In routine practice, patients with advanced adenocarcinomas are currently tested for *EGFR* mutations and *ALK* aberrations.[5] Since 2009, several clinical trials have reported better response rates and increased progression-free survival in patients with advanced NSCLC with mutations in *EGFR* gene who received TKIs compared with conventional therapy.[64–66] Since most patients with early-stage lung cancer will eventually experienced relapse and disease progression, the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) have developed a guideline encouraging *EGFR* and *ALK* testing for lung cancer at the time of diagnosis for patients presenting with advanced disease stage to guide clinical management with targeted therapies.[47,59,67]

ALK-EML4 translocations and *EGFR* and *KRAS* mutations are mutually exclusive.[33,34] Thus, when *EGFR* and *KRAS* mutations are absent, the detection of *ALK-EML4* translocations is crucial for a favorable response to crizotinib.[68] Until now, the only FDA-approved laboratory test to detect *ALK-EML4* translocations is fluorescence in situ hybridization (FISH), which allows direct visualization of multiple changes in chromosomes and in the number of gene copies.[69] However, new technologies have emerged, such as NanoString, to detect this alteration, including other gene fusions such as fusion comprising the *RET* and *ROS1* genes.[70]

Different from *EGFR*-mutated patients, those harboring *KRAS* mutations do not benefit from TKIs. The main reason for this problem is probably because *KRAS* itself is difficult to inhibit, and the effectiveness of agents targeting *KRAS* effectors has been blocked by the activation of offsetting pathways limiting their efficiency.[53,54] To date, there is no effective targeted therapy for patients harboring *KRAS* mutations.[71] Trametinib acts downstream of *KRAS* and suppresses the

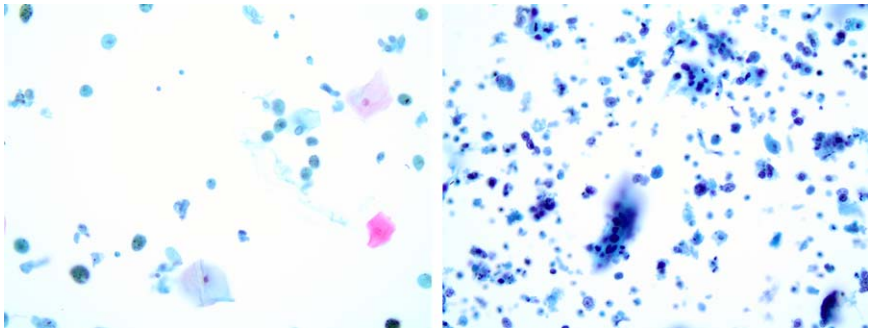

FIGURE 1 The Papanicolaou stain allows the cytologic analyses of squamous cell carcinoma features (40×) from two patients [Color figure can be viewed at wileyonlinelibrary.com]

MAPK signaling; however, it was recently demonstrated that trametinib provokes a compensatory response mediated by the fibroblast growth factor receptor 1 (*FGFR1*), leading to adaptive drug resistance and *FGFR1* inhibition increased sensitivity to trametinib. These data paved the way for a combinatorial approach to treat *KRAS*-mutant lung cancer and open opportunities for effective treatment for these patients.[72]

1.7 | Liquid medium beyond cytology

The concept of personalized medicine of lung cancer is based on the therapeutic decisions driven by the specific histology and genetic features of patient's tumor. However, the biopsy techniques traditionally performed provide limited specimens that usually are insufficient for all tests and frequently require re-biopsy the performance of which depends on the patient's clinical situation.[5]

In this sense, cytological samples are an attractive tool to help in the management of patients with lung cancer because it enables genomic and proteomic assays beyond cellular analysis, with the advantage of being enriched in tumor cells and not formalin-fixed.[20,73,74]

Thus, the maintenance of cytologic bronchial material in liquid medium, initially used for gynecological issue (LBC), represents an useful method. This is, especially true in cases where there is no option to immediately prepare the smear. These liquid-based methods, preserve the morphology of cells at room temperature for a long time until laboratorial processing can occur (Figure 1).

1.7.1 | Assessing molecular biomarkers in cytological samples

Among molecular laboratory tests for assessing *EGFR* mutations in cytological samples, PCR-based techniques can be highlighted including direct sequencing, real-time PCR, pyrosequencing, fragment length analysis and high resolution melting analysis, amplification-refractory mutation system, peptide nucleic acid-locked nucleic acid, low denaturation temperature co-amplification and next-generation sequencing (NGS).[20] FISH (fluorescence in situ hybridization), employed for the detection of *ALK* rearrangements, has been shown to be significantly more sensitive to detect cancer in brushed bronchial specimens than conventional cytology, and it allows the detection of tumors in early stages and those tumours peripherally located. In addition, there are reports describing immunocytochemistry (ICQ) applying anti-ALK antibodies as a faster and low-cost alternative to FISH assays in cytological samples.[73]

Beyond that, differentially expressed proteins can be easily detected using specific antibodies in ICQ technique, and the detection of these proteins reflects signaling pathways activation or inactivation, which contributes for the identification of molecular aspects of lung cancer in cytological samples.[75]

Likewise, flow cytometry is widely used to quantify cells and inflammatory molecules. This technique may be carried out using cytology samples from BAL specimens. Several studies have shown that flow cytometry is useful to differentiate CD4+, CD8+ and CD56 + lymphocytes, which are present in BAL specimens from both in lung cancer and in pulmonary diseases such as asthma.[76] BAL provides non-cellular components of the fluid covering the respiratory epithelium and allows the quantification of cytokines (e.g., IL-6 and IL-1 b) employing cytometry beads-assay.[77,78]

Another approach that highly fits employing cytological samples is next-generation sequencing (NGS), which uses a simple platform to assess several variations such as single-nucleotide polymorphisms (SNPs), insertions and deletions, as well as gene amplifications and rearrangements[73,79] Very recently, a complete workflow for detection of low frequency somatic mutations from cell-free DNA was developed for a NGS platform. Basically, the workflow consists of cell-free DNA isolation, library preparation, sequencing and a user-friendly data analysis. This panel comprises >150 *hotspots* variants across 11 crucial genes for NSCLC, such as *EGFR*, *KRAS*, *TP53* and *MET*. The workflow was initially tested on samples from matched tumor FFPE and plasma collected from NSCLC patients. According to the manufacturer, results indicated high sensitivity and significant overlapping of variants detected in the FFPE and plasma samples with a limit of detection of 0.1% of somatic mutations.[80] Another platform has been developed for liquid biopsies, specially focused on *EGFR* mutation testing in plasma samples from NSCLC patients, although different biopsies specimens have also been used such as core-needle and cytology.[81] These approaches have emerged as useful for the monitoring of treatment and disease progression in body fluids from NSCLC patients. In this context, liquid cytology might also be potentially used as a specimen

for liquid biopsy, which is a less invasive method, easily obtained and likely to be better tolerated than rebiopsy for patients. Another possible use of cytology is the identification of causative agents and management of patients with infectious diseases.[8,74] Because the lung is the largest epithelial surface in the body, it constitutes the major portal for the entry of microorganisms such as viruses, bacteria and fungal organisms.[82] The diagnosis of microorganisms is based particularly on molecular methods of nucleic acid analysis and microarrays, and classified as amplified, unamplified.[83] In clinical practice, PCR is the most common method for the detection of microorganisms such as *Streptococcus pneumonia*, *Mycoplasma pneumonia* and *Staphylococcus aureus*, among others. Currently, real-time PCR has been widely used because it offers a lower risk of contamination, rapid turn-around and its sensitivity allows the detection of infectious agents found in low concentrations in cytological samples. More recently, studies have shown the utility of BAL for investigation of respiratory microbiota and metabolic profiles from HIV-infected individuals[84] and patients with cystic fibrosis,[85] these conditions being contributor conditions for lung cancer development. So far, cytological samples have not been employed for microbiota investigation in the pulmonary oncology field; however, there are predictable data for attracting the attention to a high-risk population for developing lung neoplasms. Thus, these findings reinforce the importance of cytological samples for the lung cancer field.

2 | CONCLUDING REMARKS

The current era of personalized medicine using mutational screening for clinical management of lung cancer patients has brought major advances in lung cancer therapy and it has allowed the identification of patients who could benefit from targeted therapies. Cytology samples have become an attractive tool to be employed in association with the histology approach.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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