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INVITED REVIEW

EBUS and EUS guided fine needle aspirations for molecular diagnostic analysis in lung cancerHenricus F.M. van der Heijden¹, Monika G. Looijen-Salamon², Olga C.J. Schuurbijs¹, Johan Bussink³ & Marjolijn J.L. Ligtenberg^{2,4}

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Abstract

In daily clinical practice the diagnosis of lung cancer is often based on cytological specimens. These cytological samples are increasingly obtained by ultrasound-guided techniques with fine needle aspirations. Recent developments have shown that transesophageal ultrasound guided fine needle aspiration (EUS-FNA) and endobronchial ultrasound guided transbronchial fine needle aspiration (EBUS-TBNA) are minimally invasive diagnostic and staging procedures that have shown to be highly sensitive and accurate. Although several studies have shown that these cytological samples allow for reliable diagnosis and sub classification of non-small cell lung cancer, cytological samples for molecular analysis are not yet routinely used. In this paper we review the current literature regarding the results of molecular analysis of samples obtained by EUS-FNA and/or EBUS-TBNA, focusing on the targets for currently available treatments of non-small cell lung cancer like epidermal growth factor receptor (EGFR), Kirsten rat sarcoma oncogene (KRAS) and Echinoderm microtubule-associated protein-like 4 gene anaplastic lymphoma kinase gene translocation (EML4-ALK). We conclude that the cytological samples obtained by endosonography guided fine needle aspirations (EUS and EBUS) are highly accurate for molecular analysis. This analysis can be performed reliably in the vast majority of patients in daily practice.

Introduction

On the global scale lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer death in males.^{1,2} Among females, it is the fourth most commonly diagnosed cancer and the second leading cause of cancer death.^{1,2} The five year survival rate of the various types of lung cancer for both Europe and the United States is approximately 16% and did not significantly improve in the last decade, despite the emergence of new diagnostic and therapeutic developments. Following the changes in tobacco use, the incidence of lung cancer is ever increasing in a number of countries, including China.^{2,3} This trend is attributable to an increase in the incidence of lung cancer in women. Furthermore, adenocarcinoma has become the most frequent histological subtype of non-small cell lung cancer (NSCLC).⁴ In

general, these adenocarcinomas arise in more peripherally located parts of the lung that are more difficult to reach for diagnosis. As a result, in clinical practice the diagnosis of lung cancer is often based on cytological specimens. Several studies have shown that these cytological samples allow for reliable diagnosis and sub classification of NSCLC.⁵⁻⁹

Cytological samples are increasingly obtained by ultrasound-guided techniques with fine needle aspirations. Recent developments have shown that transesophageal ultrasound guided fine needle aspiration (EUS-FNA) and endobronchial ultrasound guided transbronchial fine needle aspiration (EBUS-TBNA) are minimally invasive diagnostic and staging procedures which have shown to be highly sensitive and accurate. Indications, technique and diagnostic results have been extensively reviewed in this journal.¹⁰⁻¹⁶ These ultrasound-guided techniques allow safe cytological

sampling of mediastinal lymph nodes and centrally located intrapulmonary tumors or metastases in the upper abdomen, including the adrenal gland.^{17,18} EUS-FNA and EBUS have therefore been incorporated in the guidelines of the European Society of Thoracic Surgeons (ESTS) and the American College of Chest Physicians (ACCP)^{19,20} and a combination of these techniques has been shown to have a better sensitivity and negative predictive value than cervical mediastinoscopy.^{21–24}

However, as opposed to cervical mediastinoscopy and other surgical procedures, EUS-FNA and EBUS result in cytological specimens with a limited amount of cancer cells available for the increasing number of analytical tests. These fine needle aspirates are preferably processed on slides for rapid onsite evaluation (ROSE) and vials or tissue coagulum clots for analysis of cell-blocks. Since these samples are often the only available proof of lung cancer, molecular and genetic analysis performed on cytological specimens is of increasing interest, as it will allow for optimal and personalized therapy choice.^{6,25–28}

In this paper we review the current literature regarding the results of molecular analysis of samples obtained by EUS-FNA and/or EBUS-TBNA, focusing on targets for currently available treatments of lung cancer. Furthermore we have added practical information on the tissue handling procedures we use in our endosonography center and pathology department for cytology and tumor genetics.

EGFR

Determining the mutation status of the epidermal growth factor receptor (EGFR) gene is of crucial importance to adequately select patients with both early and advanced non-small cell lung cancer (NSCLC) for targeted treatment with tyrosine kinase inhibitors (TKI's). EGFR mutation status is also important to predict prognosis and response to EGFR targeted treatment, possibly combined with radiotherapy,^{29,30} as well as systemic chemotherapy.^{31,32} Recently, a number of studies reported on EGFR analysis in cellblock based cytological specimens from EBUS.^{33–35} In our study, both molecular analysis of EGFR and KRAS mutations were performed on cytological material obtained by EUS or EBUS. We showed that this was feasible and applicable in daily practice. Molecular analysis could be performed in 77% of the adenocarcinoma samples, which was in agreement with the study by Garcia-Olive *et al.*^{33,35} They showed that EGFR gene analysis of the EBUS-TBNA sample was feasible in 26 (72.2%) out of the 36 patients with lymph node metastasis using a similar method.^{33,35} Nakajima *et al.* performed molecular analysis in histological core biopsies obtained by EBUS in 43 of 46 lung adenocarcinoma patients (94%).³⁴ The percentage of EGFR mutations found in the Spanish and Japanese studies mentioned above was 10% and 26%, as compared to 7.4% found

in our cohort. This might reflect a high percentage of cigarette smoking patients in our predominantly male, Caucasian group of patients. Recently, several studies have confirmed these results in EBUS TBNA samples. In a study using co-amplification at lower denaturation temperature-polymerase chain reaction (COLD-PCR) technique Santis *et al.* showed that molecular analysis of all EGFR target sequences could be achieved in 126 of 132 (95.5%) of the cases, with a prevalence of EGFR mutations of 10.5%.³⁶ Billah *et al.* analyzed 99 cases of EBUS-FNA specimens and found 96% to be adequate for analysis with a prevalence of EGFR mutations of 29% in lung adenocarcinomas and 7% in non-adenocarcinomas.³⁷ And finally, in a large multigene analysis, Nakajima *et al.* showed a high correlation between the EBUS based analysis of EGFR, KRAS and tumor protein 53 (*p53*) mutations and clinical outcome of treatment in a group of 153 patients.³⁸

In conclusion, these studies uniformly show that EGFR mutation status analysis can be performed adequately and reliably in the vast majority of samples obtained by EUS or EBUS guided fine needle aspirations. For this analysis obtaining additional histological core samples is redundant and will only increase the risk of complications.⁸

KRAS

An increasing number of studies report combined molecular testing for both Kirsten rat sarcoma oncogene (KRAS) and EGFR mutations applied in all available cytological specimens (both cell blocks and direct smears) as we reported first in our study.³⁵ In these specimens, the applicability in daily routine analysis of EUS-FNA or EBUS guided fine needle aspirations was demonstrated. The KRAS mutational status is of importance as KRAS is an important signaling step downstream of the EGF-receptor and mutations in KRAS relate to resistance to EGFR targeted therapy, adjuvant chemotherapy.^{39,40} KRAS mutations may also be of relevance to other downstream pathways like the PI3K/AKT pathway.^{30,41} The yield of KRAS mutation testing is generally equal to or better than the results stated above for EGFR analysis. For example, Santis *et al.* reported that KRAS status could be obtained in 130 of 132 (98.4%) patients and found mutations in 19% of lung adenocarcinomas and 28% of NSCLC not otherwise specified using a COLD-PCR technique for cytological material obtained by EBUS.³⁶ Other studies have reported approximately similar prevalence results ranging from 3.5 to 38% KRAS mutation positive cases in different study populations – in terms of race, gender and smoking habits – and using DNA sequencing techniques.^{38,42} Because KRAS and EGFR mutations can in general be regarded as mutually exclusive, KRAS mutation analysis might be used to predict the absence of an EGFR mutation in samples in which the EGFR analysis fails.^{43,44}

EML4-ALK

In NSCLC, EML4-ALK gene fusions occur as a result of small inversions within the short arm of chromosome 2 by which the Echinoderm microtubule-associated protein-like 4 (EML4) gene and the anaplastic lymphoma kinase (ALK) are juxtaposed. The constitutively active EML4-ALK fusion protein can be inhibited by the protein kinase inhibitor crizotinib.^{45–48} EML4-ALK rearrangements have been identified in 2 to 7% of tumors using fluorescence in situ hybridization (FISH). Kwak *et al.* published the first clinical study with ALK inhibition by using the dual ALK and MET inhibitor crizotinib in patients with ALK-fusion-positive advanced lung carcinoma.⁴⁷ It is important that adenocarcinomas that are negative for EGFR and KRAS mutations can be screened for the presence of chromosomal translocation, as the FDA has approved Crizotinib as the first drug successfully targeting this mechanism.^{46,48} Two studies have shown that this analysis can be performed on cytological material obtained by EBUS.^{49,50} Nakajima reported EML4-ALK fusion in a case study with both FISH and reverse transcriptase-polymerase chain reaction (rt-PCR) in an EBUS-TBNA obtained cytological sample.⁴⁹ Sakairi screened EBUS samples of 109 patients for ALK positivity using immunohistochemical staining. In the ALK positive cases (6%) subsequent FISH analysis was performed to detect ALK-rearrangements, which were found in all ALK-positive cases.⁵⁰ Sequential testing for the different clinically relevant molecular tests is reasonable and it is likely that the most cost-effective strategy is to start with either KRAS and/or EGFR analysis and reserve ALK analysis for KRAS- and EGFR-negative specimens as, until now, only five patients have been diagnosed with both EGFR mutation and loss of ALK translocation.⁵¹ However, when treatment delay is of concern these analyses can be performed simultaneously, provided that sufficient material for molecular testing is obtained by the EUS and/or EBUS.

Other targets, indications and limitations

It is likely that in the near future new drugs targeting proliferation mechanisms or pathways in lung cancer will become available.^{26,43} Likely candidates are BRAF mutations, MET amplification, PIK3CA mutations, HER2 mutations, AKT mutations, MAP2K1 mutations and epithelial mesenchymal transition (EMT) pathway transitions.^{43,52,53} Research activities are focusing on these candidate targets, but also on genotyping or sequencing multiple targets.⁴⁴ The challenge will be to refine the techniques to allow multiple testing in small amounts of tumor tissue. In cytological specimens analysis of total RNA, DNA and protein analysis have been reported.⁵⁴

The abovementioned analytical procedures can also be applied for restaging procedures in patients to detect acquired resistance after treatment.⁵⁵ However, in line with the findings

obtained in patients with stage III disease eligible for surgery after induction (concomitant) chemoradiation therapy, one must be aware of a lower negative predictive value and diagnostic accuracy of EUS and EBUS.⁵⁶ Besides mediastinal lymph node staging, EBUS and EUS guided analysis can be used for accurate diagnosis of metastatic sites of non-pulmonary primary tumors^{57,58} rendering the same opportunities for molecular analysis of the obtained cytological samples.

However, we need to stay aware of potential false positive and negative results caused by regional differences and different clonal or genetic tumor profiles, as has been reported in studies targeting multiple sites of metastatic lung cancer.^{42,59}

ROSE and tissue handling

Rapid On-Site cytological Evaluation (ROSE) of the EUS and EBUS guided fine needle aspirations is effective in optimizing the yield and efficiency of the EBUS-TBNA procedure and increases the sensitivity of EUS-FNA from 80% to 88% without increasing procedure length.⁶⁰ Indeed, the additional staff will increase the operational costs but recent data convincingly demonstrates that aspirates performed with ROSE optimize the utility of specimens obtained.^{61–63} Onsite feedback from the cytopathologist or cytology technician will not only guide the endoscopist to repeat aspirations in order to determine the correct diagnosis and stage, but also to obtain sufficient material for cellblocks (or tissue clot coagulum) in order to maximize the yield for a complete cytological, immunohistochemical and molecular analysis in that patient.^{6,9,28,64}

In our endosonography center we have performed over 1200 procedures. From each fine needle aspiration, direct smears are made for Giemsa and Papanicolaou staining (Fig 1). Giemsa stained smears are processed and analyzed onsite for rapid onsite evaluation (ROSE) by a cytotechnician. The remaining material is flushed from the needle into fixative solution and is processed in cellblocks. Aspirations are repeated until adequate and sufficient material is obtained for a diagnosis. When tumor tissue is found in ROSE, we usually take one or two additional needle aspirations to allow for a full immunohistochemical and molecular analysis from cellblocks. From these cellblocks, 4 μ m slides are cut for Hematoxyline-eosine staining and immunocytochemistry. For the molecular analysis of the tumor tissue and to minimize the chance of false-negative results, both cellblocks and smears are considered suitable for molecular analysis of EGFR and KRAS when DNA can be isolated from regions with >40% and >10% tumor cells, respectively.^{35,65} More sensitive next-generation-techniques interrogating multiple targets on minimal amounts of cells are currently being validated. Other studies have also used 40%³⁷ or 70% tumor cells as a cut-off value.⁵³

For DNA isolation from the cellblocks, the relevant regions are manually micro dissected from two to three 20 μ m sections

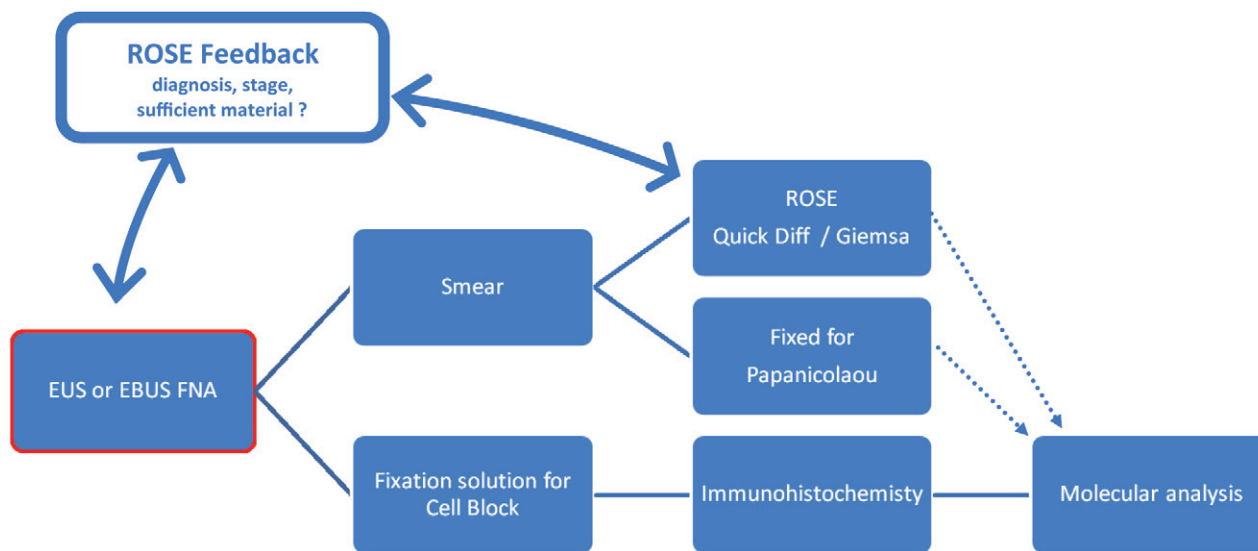


Figure 1 Schematic representation of the tissue handling procedures at the Pulmonary Endosonography Center of the Radboud University Medical Center Nijmegen, the Netherlands. Fine needle aspirations (FNA) are collected by transesophageal ultrasound (EUS) or endobronchial ultrasound (EBUS) guidance. The samples are used for multiple analysis. Onsite two cytologic smears are prepared for each aspiration; one smear is air-dried (for Giemsa/quick diff staining) and the second smear is fixed with 95% ethanol-fixed for Papanicolaou stain. The remainder of the aspirate is washed from the needle with into a liquid fixation used for cellblock and molecular testing. Occasionally, tumor cells from smears are also used for molecular analysis. Onsite feedback with the cytologist is of great importance to maximize the yield and quality of the samples. ROSE, Rapid On-Site cytological Evaluation.

using flanking hematoxyline-eosine stained slides as a reference. For DNA isolation from the smears, regions with sufficient amount of tumor cells are scraped from the glass slides. Relevant mutation analysis can subsequently be performed using standard polymerase chain reaction (PCR) sequencing techniques. In our institute, the average processing time for EGFR sequencing is three days after arrival of the material.

Conclusion

Endosonography guided fine needle aspirations (EUS and EBUS) and molecular analysis of the cytological material obtained from these procedures have become an imperative part of the diagnosis of lung cancer and choice of treatment in daily practice. These diagnostic procedures are minimally invasive and can be performed on an outpatient basis. The cytological samples obtained by these procedures are highly accurate for diagnostic purposes and sub classification of NSCLC as well as sub typing of adenocarcinomas by using additional immunohistochemical staining. This review also shows that relevant molecular analysis can be performed reliably in the vast majority of patients. Although a recent consensus meeting regarding EGFR testing still advocates the preferential use of histological samples,⁶⁶ the evidence supporting the use of cytological samples for molecular analyses is increasing.

We strongly advocate ROSE and incorporation of trained cytotechnicians or cytopathologists in the team as this allows for immediate feedback on the amount and quality of the

obtained tissue sample. This team effort will further increase the diagnostic yield and will help the endosonographer in effectively sampling sufficient tumor cells in order to allow for multiple molecular analyses.

Disclosure

No authors report any conflict of interest.

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