Journal of Pulmonary & Respiratory Medicine

Pusztaszeri et al. J Pulmonar Respirat Med 2011, S:5
DOI: 10.4172/2161-105X.S5-002

Research Article Open Access

Cytopathological Diagnosis of Non Small Cell Lung Cancer: Recent Advances Including Rapid On-Site Evaluation, Novel Endoscopic Techniques and Molecular Tests

Marc Pusztaszeri[1]*, Paola M. Soccal[2,3], Nicolas Mach[4], Jean-Claude Pache[1] and Tom Mc Kee[1]

[1]Department of Clinical Pathology, Geneva University Hospital, Switzerland
[2]Service of Pulmonary Medicine, Geneva University Hospital, Switzerland
[3]Clinic of Thoracic Surgery, Geneva University Hospital, Switzerland
[4]Department of Oncology, Geneva University Hospital, Switzerland

Abstract

Important advances in non small cell lung cancer (NSCLC) diagnosis, staging and treatment have been made over the last decade. Minimally-invasive endoscopic techniques including endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) and navigational bronchoscopy have emerged as valid alternatives to transthoracic and/or surgical approaches, providing aspiration cytology material instead of histological material for diagnosis and staging of mediastinal and lung lesions. Several drugs designed to target molecular pathways involved in cancer-cell growth and survival have been shown to be effective in a selected fraction of NSCLC patients, mostly with adenocarcinoma (targeted therapy). Somatic activating mutations in several genes involved in those pathways (EGFR/KRAS) can predict patients' responses to targeted therapies (individualized therapy). Those mutations are commonly detected in histopathological samples (core-needle biopsy/surgical resection). However, when histological tissues are not available, molecular testing can be performed on cytological specimens. This scenario is increasing in frequency, due to the use of less invasive procedure for diagnosis and staging such EBUS-TBNA and/or patients in advanced stage of disease who are not candidates for surgery. Several strategies exist and may be combined to ensure that the less abundant material that results from minimally invasive techniques can be used efficiently for molecular analyses. These include Rapid On-Site Evaluation (ROSE) of EBUS-TBNA cytological material, to ensure optimal sampling and triage of the material (e.g. cell-block preparation), and microdissection techniques, to select an adequate population of tumor cells. Major issues raised by cytological diagnosis of NSCLC and molecular testing on cytological specimen are discussed in this article.

Keywords: On-site evaluation; EBUS; Endobronchial ultrasound-guided fine-needle aspiration; Non-small cell carcinoma; Cytology; EGFR; KRAS; BRAF; EML4-ALK; Molecular testing; Microdissection

Introduction

The diagnosis of lung cancer carries a particularly somber prognosis in both men and women. Non small cell lung carcinoma (NSCLC), represented mostly by adenocarcinoma, constitutes about 80% of lung cancers [1,2]. At diagnosis, most patients present with locally advanced and/or metastatic disease and are thus not candidates for surgery. Among those patients who are operable, the majority will have recurrence. Over the last decade, important advances have been made in lung cancer diagnosis, staging and treatment including palliation. Several drugs, designed to target molecular pathways involved in cancer-cell growth and survival, have been shown to be effective in a selected fraction (<20%) of patients with NSCLC, mostly with adenocarcinoma. Somatic mutations in several genes (EGFR and KRAS) can predict patients' responses to these targeted therapies. Cytologic diagnosis and staging of lung cancer has gained importance over histological diagnosis (tissue biopsy) due to the advent of novel less invasive diagnostic procedures such as endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) or navigational bronchoscopy. Therefore an increasing number of molecular tests that predict patients' responses to targeted therapies will be performed on cytological specimens rather than on tissue biopsies. This paradigm shift and the suitability of the cytological material for molecular tests are discussed in this article.

Materials and Methods

Sampling of Tissue

A pathological diagnosis remains crucial in the diagnosis, staging and planning of the appropriate treatment of lung and mediastinal lesions given their diversity and the low specificity of radiologic imaging. Traditional diagnostic modalities include noninvasive or minimally invasive approaches based on exfoliative cytology (sputum, bronchial brushing or washing, and pleural fluid) and fine needle aspiration cytology (transbronchial needle aspiration). The diagnostic yield of these techniques is highly variable but usually suboptimal (ranging from 20-90%) requiring often more invasive approaches. Transthoracic CT-guided fine needle aspiration is the most common approach for parenchymal lesion, with usually >90% reported diagnostic yield. However, complications including hemorrhage and pneumothorax are frequent, especially in high-risk patients. Finally, surgery, including mediastinoscopy and thoracoscopy/thoracotomy is the most invasive and costly approach, requiring general anesthesia and hospitalization [3].

Over the last decade, several endoscopic techniques, including EBUS-TBNA and navigational bronchoscopy have emerged as valid

***Corresponding author:** Dr. Marc Pusztaszeri, Service de Pathologie Clinique, Hôpitaux Universitaires de Genève, 1 rue Michel-Servet, 1211 Genève 14, Switzerland, Tel: +412 2372 8574; Fax: +412 2372 4906; E-mail: Marc.Pusztaszeri@hcuge.ch

Received Dececmber 01, 2011; **Accepted** January 01, 2012; **Published** January 03, 2012

Citation: Pusztaszeri M, Soccal PM, Mach N, Pache J, Mc Kee T (2012) Cytopathological Diagnosis of Non Small Cell Lung Cancer: Recent Advances Including Rapid On-Site Evaluation, Novel Endoscopic Techniques and Molecular Tests. J Pulmonar Respirat Med S5:002. doi:10.4172/2161-105X.S5-002

diagnostic alternatives [3,4]. Systematic reviews and meta-analyses have demonstrated a major impact of EBUS-TBNA on management of patients with NSCLC and mediastinal involvement, with a diagnostic yield comparable to mediastinoscopy [5]. EBUS-TBNA is particularly appealing as it is minimally invasive, safe, and can be targeted in real-time by ultrasound guidance. This image guidance distinguishes it from other traditional methods, such as regular (without ultrasound guidance) transbronchial TBNA, and increases the diagnostic yield of the procedure by allowing direct visualization of the target, while preventing extranodal needle insertion and thus decreasing the complication rate. Moreover, compared to mediastinoscopy, EBUS-TBNA has the ability to access more mediastinal lymph node stations.

Besides mediastinal adenopathies, lung parenchymal nodules sampling is also a frequently encountered clinical issue. Navigational bronchoscopy based on an electromagnetic guidance system offers the opportunity to maneuver the instruments into the desired direction and location. Once the target is reached, this technique allows the operator to perform cytological as well as histological sampling of the lesion.

Rapid On-Site Evaluation (ROSE) by a cytopathologist, although not available in every center, is an important complement for these new techniques, especially for EBUS-TBNA. ROSE warrants the accuracy of the procedure, yet it does not necessarily improve its yield [6]. Major expectations of the clinician are the confirmation by the cytopathologist that the specimen is adequate and representative of the targeted lesion (e.g. a significant number of lymphocytes should be found when a lymph node is targeted), and that there is sufficient material for a definitive final diagnosis. This is particularly important for the subclassification of malignant lesions (e.g. adenocarcinoma vs squamous cell carcinoma) and molecular analyses such as EGFR if indicated (Figure 1). Because the endoscopic procedure can be interrupted as soon as the cytopathologist confirms that the sampling is adequate, ROSE clearly minimizes the duration and thus the biopsy-associated risks. It optimizes also the handling and adequate allocation and processing of the specimens for additional specific analyses according to the diagnosis (e.g. flow cytometry in case of a lymphoma suspicion or the confection of a paraffin-embedded cell pellet (cell-block) which can be used for immunohistochemistry and/or molecular analyses).

Diagnosis and Subclassification of NSCLC on Cytological Specimens

With the advent of targeted therapies, a generic cytological (or histological) diagnosis of NSCLC has become insufficient for patient management [2,7-9]. The pathologist must, when feasible, discriminate between adenocarcinoma and squamous cell carcinoma or favor one over the other. While a minor fraction (<20%) of patients with pulmonary adenocarcinoma may harbor EGFR mutations which may confer them susceptibility to anti-EGFR targeted therapy with tyrosine kinase inhibitors (TKI) [10,11], almost all patients with squamous cell carcinoma will be EGFR wild-type and, therefore, are not candidate for anti-EGFR therapy. Moreover, fatal pulmonary hemorrhages have been reported in these patients after anti-VEGF targeted therapy (bevacizumab) [2,7-9].

When the morphology is ambiguous and there is no clear glandular or squamous differentiation, immunocytochemistry using a panel of antibodies (typically TTF-1, p63, cytokeratin 7 and cytokeratin 5/6) may be used on previously stained slides (smears or automated slides) and/or on cell-blocks. In general, the results of these tests allow one diagnosis to be favored. Pulmonary adenocarcinomas are typically

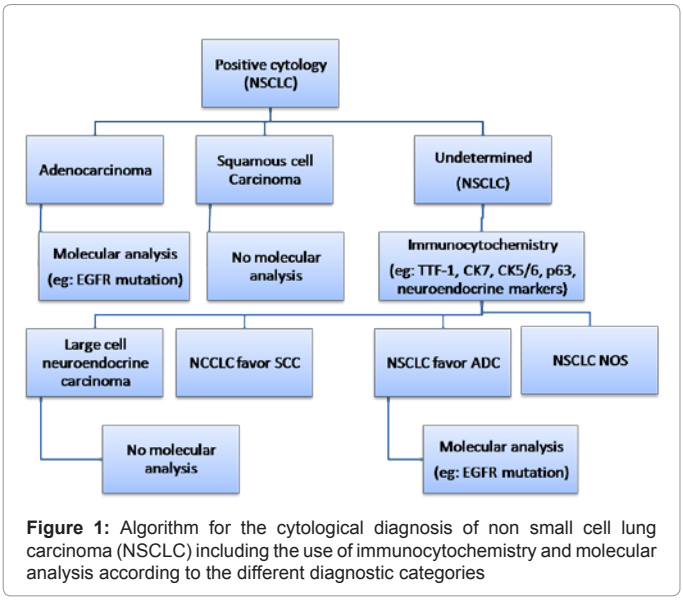


Figure 1: Algorithm for the cytological diagnosis of non small cell lung carcinoma (NSCLC) including the use of immunocytochemistry and molecular analysis according to the different diagnostic categories

immunoreactive for TTF-1 and cytokeratin 7 and non reactive for p63 and cytokeratin 5/6. Squamous cell carcinomas typically have the opposite immunoprofile [9]. Although this subclassification, based on a limited amount of tumor cells on cytological specimens, raises the issue about sample representativity and tumor heterogeneity (morphological and molecular), the concordance between needle aspirate and biopsy in NSCLC subtyping of is very high (96%), especially when analysis of material in cell-blocks can be performed [5]. Mixed tumors with a dual differentiation such as adenosquamous carcinoma (at least 10% of both components) do exist but are rare (<4%) and may harbor EGFR mutations, in both components of the tumor (as shown by microdissection), as frequently as adenocarcinoma [2,12]. Depending on the subcategory of NSCLC, molecular tests such as EGFR mutation may or may not be warranted (Figure 1) [13].

Molecular Tests for Targeted Therapy

An increasing number of drugs for targeted therapy have been approved by the FDA or are currently in clinical trials. They are mainly of two types: either small molecules acting at an intracellular level (e.g. TKI such as gefitinib, erlotinib and afatinib targeting EGFR and crizotinib targeting ALK-EML4), or monoclonal antibodies which are larger molecules acting at an extracellular level (e.g. cetuximab for EGFR and bevacizumab for VEGF).

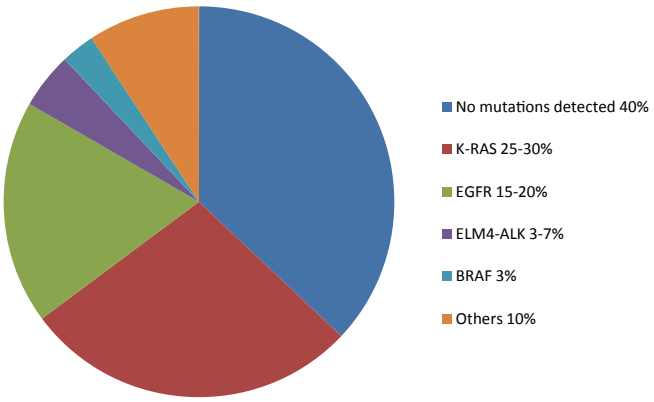
In a certain proportion of cases, tumor cell growth and survival depends upon the activation of specific signaling pathways, triggered by mutations of specific oncogenes (so called oncogene addiction). The determination of the mutational status is thus essential to predict the potential response to targeted therapy. Other mechanisms may also be responsible for the activation of oncogenic pathways (such as increased copy number of the gene and/or overexpression of oncogenes, similar to HER2-neu in breast cancer) but their clinical impact has been less well studied in NSCLC so we will focus here on the most common mutations studied so far with potential impact on therapy.

Most Significant Mutations in Pulmonary Adenocarcinoma

The most significant mutations that have been detected in pulmonary adenocarcinoma are shown in (Figure 2) [2,10,14]. These mutations are generally activating and mutually exclusive (e.g. mutation of EGFR is associated with wild-type K-RAS and B-RAF) [2].

EGFR gene, located on the short arm of chromosome 7 (7p11.2), consists of 28 exons and codes for a transmembrane protein with an extracellular domain (receptor) and an intracytoplasmic domain with tyrosine kinase activity [1,15]. EGFR belongs to the HER/ERBB family of tyrosine kinase receptors (EGFR/HER-1, ERBB2/HER-2/neu, ERBB3/HER-3 and ERBB4/HER-4) that have a similar molecular structure. The binding of the ligand with EGFR results in homo- or heterodimerization of the receptors and activation of the tyrosine kinase and downstream signaling that modulates cell proliferation, apoptosis, invasion and metastasis (Figure 3) [1,2,11]. EGFR mutations are present in about 10% of the NSCLC category and in about 15-20% of adenocarcinomas [10,11]. They are more frequent in female, non-smokers and Asiatic patients (40-50%) [10,11, 14,15]. The clinical response to TKI in patients with various mutations seems to depend on the specific EGFR mutation present. Most of them are associated with a better prognosis and a potential response to TKI [14,16]. They are most commonly found in exons 18-21 (90%), and consist of deletions found in exon 19 (associated with 70-100% response rate to TKI therapy) or point mutations found in exon 21 (associated with 20-70% response rate to TKI) [2,15].

Many new EGFR mutations are being discovered and their clinical significance remains to be clarified. Some EGFR mutations, such as T790M in exon 20 (which may be primary or secondary) are associated with poor response/resistance to TKI [2], possibly by decreasing the affinity of the drug with its target while still activating the signal pathway. Moreover, acquired resistance is seen in patients after TKI treatment, probably due to the selection of tumor clones with distinct mutations conferring resistance to treatment (e.g.: T790M) [1-2,17]. In order to overcome the issue of resistance, next-generation TKI are being developed, which in contrast to the first generation TKI (erlotinib and gefitinib), irreversibly block multiple EGFR family members (e.g. afatinib [BIBW 2992] and dacomitinib [PF-00299804]) and/or vascular endothelial growth factor receptor pathways (e.g. BMS-690514 and XL647) [17-19].



Others: CTNNB1 (Beta-catenin), PIK3CA, MEK, HER-2, PTEN

Figure 2: Mutation profile of pumonary adenocarcinomas

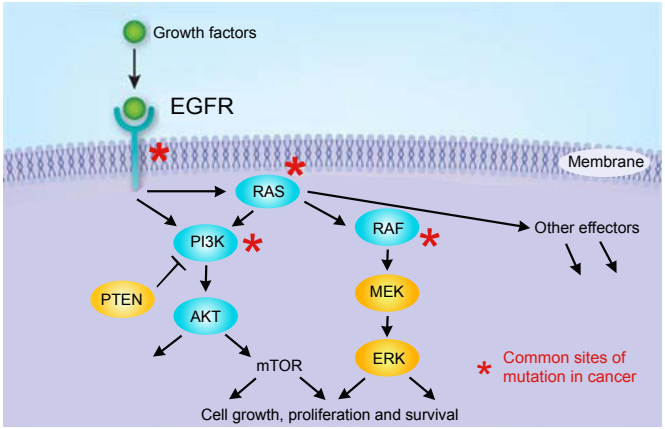


Figure 3: EGFR signaling pathway.

K-RAS is a GTPase located downstream in EGFR signaling pathway (Figure 3). Although mutations of K-RAS gene are more common than EGFR (25-30%) [10], there is no targeted therapy for K-RAS yet. They are typically found in smokers and associated with anti- EGFR TKI resistance and a poor prognosis [14,16].

BRAF gene codes for a kinase located immediately downstream of K-RAS in the EGFR signaling pathway. B-RAF mutations (e.g.: V600E) are found infrequently (3%) in lung adenocarcinomas, which are wild-type for EGFR and K-RAS, and are associated with resistance to anti-EGFR TKI [10]. Selective inhibitors of BRAF (e.g.: PLX4032) are currently under evaluation for NSCLC as well as for other cancers more often associated with BRAF mutations (melanoma, thyroid papillary carcinoma) [10,20].

The ALK-EML4 (« *anaplastic lymphoma receptor tyrosine kinase-Echinoderm microtubule-associated protein-like 4* ») fusion gene is generated by a rearrangement of chromosome 2 and occurs in a mutually exclusive fashion with EGFR or KRAS mutations almost exclusively in adenocarcinomas (3-7%) [10,21]. This alteration is also associated with anti-EGFR TKI resistance [16,22]. Crizotinib, a new and selective TKI targeting ALK, MET/HGF and their oncogenic variants has been approved on August 26, 2011, by the FDA to treat locally advanced or metastatic NSCLC that harbor the abnormal fusion gene [23]. In Phase I and Phase II trials, crizotinib was shown to be highly active in patients with advanced ALK-positive NSCLC, with overall response rates of 50–60% [24]. However, the FDA approval requires the use of a companion FDA-approved molecular test (Vysis ALK Break Apart FISH Probe Kit) for the detection of EML4-ALK fusion gene, validated on formalin-fixed paraffin-embedded tissue [23]. Alternative methods of testing for this fusion gene, including immunohistochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR) are under investigation [25]. Nevertheless, ALK testing by FISH, IHC and RT-PCR has been shown to be also feasible on cytological specimens obtained from EBUS-TBNA [26].

Some of the mutations mentioned above and others are more commonly seen with special histological subtypes of adenocarcinoma (Table 1) [7]. Therefore, the mutations likely to be found and to be looked for may be predicted based on the morphological characteristics of the adenocarcinoma.

Molecular Tests on Cytological Specimens

Histological samples, obtained by forceps/needle biopsy (endoscopic, transbronchial or CT guided biopsy) or by surgical resection when available, are generally used in order to test for the specific mutations discussed above to guide potential targeted therapy [8]. In a growing number of cases however, there is only cytological material available to diagnose, subclassify and genotype the tumor. Depending on the case, the cytological material may come from pleural/pericardic fluid, bronchial brushing or aspiration, bronchoalveolar washing or from needle aspiration of a pulmonary mass or lymph nodes.

Mutation type/alteration	Adenocarcinoma subtype
EGFR	Well differentiated, lepidic/in situ non mucinous, papillary and/or micropapillary
K-RAS	Mucinous (invasive or lepidic/in situ)
B-RAF	Papillary
ELM4-ALK	Solid or with isolated signet-ring cell component
CTNNB1 (Beta-catenin)	Clear cell or foetal

Table 1: Possible associations between genetic alterations and histological subtype of lung adenocarcinoma

The most common methods used to detect EGFR/K-RAS mutations are RT-PCR and direct sequencing [2]. The latter technique can detect any possible mutations, including some as yet undescribed and/or of unknown clinical significance, but it is less sensitive than RT-PCR and is limited by the quantity of non neoplastic cells as discussed below [1,11]. RT-PCR uses oligonucleotides (primers) which specifically bind to the mutated nucleotides. Although highly sensitive, this method only detects the mutations already characterized which are also the most frequent.

Several recent studies have shown that molecular analyses such as EGFR, K-RAS, BRAF, ALK and PIK3CA are feasible on cytological specimens and provide results in most cases (about 80%), which are equivalent to those obtained from histological specimens [1,11,15,26-28]. One study even showed using PCR for EGFR mutations that the material from standard cytological smears stained with Papanicolaou was more suitable than biopsy material. This may be due to the different fixative used for both techniques; the quality of the DNA being better when methanol (cytology) rather than formol (histology) is used [1]. Any type of cytological sample and preparation may be used, including stained or unstained smears, automated slides (Thin Prep) and cytospin. The most popular cytological preparation for molecular testing is the cell-block. Less than 25% of cytological specimens are unsuitable for molecular analyses but the proportion of unsatisfactory cases varies significantly with the type of cytological specimen; EBUS-TBNA and pleural fluid being much more suitable than bronchial aspiration or bronchoalveolar washing, due to the larger number of tumor cells and the possibility to make a cell-block [11,15,27].

Therefore, the suitability of molecular analyses on cytological samples must be evaluated for each individual case because it depends on both the absolute and relative amount of tumor cells present in the specimen. In contrast to histology where the tumor area/cells is usually separated from non tumoral area/cells and may therefore be easily selected, in cytological samples, tumor cells are mixed with other non tumoral benign and inflammatory cells, which dilutes the tumor cell population and diminish the sensitivity of molecular analyses. However, there is no need for a pure population of tumor cells in order to perform these molecular analyses. Studies have demonstrated that if a specimen contains at least 100 tumor cells with a tumor cells/non tumor cells ratio above 25%, it is suitable for EGFR/K-RAS mutation analyses. In some studies, the analyses have been successful in samples with only 30 tumor cells or with a tumor cell ratio of 1%, using more sensitive techniques such as « *High resolution melting analysis* (HRMA) » or PNA-LNA (« *peptide nucleic acid-locked nucleic acid* »- PCR) [1,29]. In general, the quality of DNA seems to be more important than its quantity (number of tumor cells) [1].

Simple manual microdissection may increase the sensitivity of the analyses by increasing the proportion of tumor cells. In addition, the PinPoint Slide DNA Isolation system (Zymo Research, Orange, Calif) can be used to further enhance the manual microdissection. The system utilizes tissue glue that is deposited on the slide in a small dot over the area to be microdissected, allowing multiple small areas of 1mm^2 or more to be selected form the slides [30-32]. The glue with the embedded cells is peeled away from the slide using a scalpel and the detached material is put into a tube and submitted to nucleic acid extraction. In our experience, this method is very simple, relatively fast and inexpensive. Its precision is usually largely sufficient for molecular tests such as EGFR mutation analysis which do not require a pure tumor cell population. Alternatively, laser capture microdissection may be used if a manual microdissection may not provide an adequate cellular sample for the analysis. Successful mutational analyses have been performed on cases with as few as 8 cells. However, laser microdissection machines and analyses are very expensive, more time-consuming than manual microdissection and not available in many centers. Therefore the use of laser microdissection is still marginal and limited in clinical practice [30].

In the limited number of cases where there is not enough material for the analyses and/or a failure of it, cytological or histological tissue sampling must be repeated in order to obtain molecular results.

Conclusions

Subclassification of NSCLC and molecular analyses are now essential in order to offer the optimal treatment to patients including individualized targeted therapies. The emergence of novel minimally invasive endoscopic techniques relying on cytological material for diagnosis and/or staging of lung cancer has significantly changed clinical practice. These techniques have also resulted in less material (tumor cells) being available not only for the diagnosis but also for subsequent analyses such as molecular tests. Therefore, it is of paramount importance to use adequate techniques in order to be able to use this material efficiently. ROSE optimizes the diagnostic accuracy of endoscopic sampling, ensuring that adequate material has been sampled and that material is appropriately selected for subsequent analyses. Molecular tests may be performed on cytological samples as well than on histological samples. However, cellular adequacy on cytological samples must be evaluated individually for each case. Use of cell-blocks and/or manual microdissection, which may be enhanced by the use of simple commercial kits, guarantees the selection of an adequate sample of tumor cells in most cases for mutation analyses. Laser microdissection may be reserved for the rare cases where those techniques failed to yield enough tumoral cells and/or ratio for molecular analyses.

Although EGFR mutations are now routinely performed for NSCLC, many other potential therapeutic targets are currently being investigated (e.g. BRAF, HER-2, c-KIT). Thus, it is likely that the number and range of molecular tests will increase in the future in general but also for each individual NSCLC patient. The identification of new prognostic and predictive markers for lung cancer and the development of new laboratory techniques in order to detect them are major challenges for the future and should allow for more patients to benefit from targeted therapy.

References

1. Da Cunha Santos G, Saieg MA, Geddie W, Leighl N (2011) EGFR gene status in cytological samples of non small cell lung carcinoma: Controversies and opportunities. Cancer Cytopathol 119: 80-91.

2. Ladanyi M, Pao W (2008) Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. Mod Pathol 21: S16-22.

3. Yasufuku K, Pierre A, Darling G, de Perrot M, Waddell T, et al. (2011) A Prospective controlled trial of endobronchial ultrasound-guided transbronchial needle aspiration compared with mediastinoscopy for mediastinal lymph node staging of lung cancer. J Thorac Cardiovasc Surg 142: 1393-1400.

4. De Leyn P, Lardinois D, Van Schil PE, Rami-Porta R, Passlick B, et al. (2007) ESTS guidelines for preoperative lymph node staging for non-small cell lung cancer. Eur J Cardiothorac Surg 32: 1-8.

5. Tournoy KG, Carprieaux M, Deschepper E, van Meerbeeck JP, Praet M (2011) Are EUS-FNA and EBUS-TBNA specimens reliable for subtyping non-small cell lung cancer? Lung Cancer [Epub ahead of print].

6. Griffin AC, Schwartz LE, Baloch ZW (2011) Utility of on-site evaluation of endobronchial ultrasound-guided transbronchial needle aspiration specimens. Cytojournal 8: 20.

7. Cagle PT, Allen TC, Dacic S, Beasley MB, Borczuk AC et al. (2011) Revolution in lung cancer: new challenges for the surgical pathologist. Arch Pathol Lab Med 135: 110-116.

8. Travis WD, Rekhtman N (2011) Pathological diagnosis and classification of lung cancer in small biopsies and cytology: strategic management of tissue for molecular testing. Semin Respir Crit Care Med 32: 22-31.

9. Khayyata S, Yun S, Pasha T, Jian B, McGrath C, et al. (2009) Value of P63 and CK5/6 in distinguishing squamous cell carcinoma from adenocarcinoma in lung fine-needle aspiration specimens. Diagn Cytopathol 37: 178-183.

10. Paik PK, Arcila ME, Fara M, Sima CS, Miller VA, et al. (2011) Clinical Characteristics of Patients with Lung Adenocarcinomas Harboring BRAF Mutations. J Clin Oncol 29: 2046-2051.

11. Smouse JH, Cibas ES, Janne PA, Joshi VA, Zou KH, et al. (2009) EGFR mutations are detected comparably in cytologic and surgical pathology specimens of non small cell lung cancer. Cancer 117: 67-72.

12. Jia XL, Chen G (2011) EGFR and KRAS mutations in Chinese patients with adenosquamous carcinoma of the lung. Lung Cancer 74: 396-400.

13. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, et al. (2011) International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma. J Thorac Oncol 6: 244-285.

14. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, et al. (2008) Somatic mutations affect key pathways in lung adenocarcinoma. Nature 455: 1069-1075.

15. Billah S, Stewart J, Staerkel G, Chen S, Gong Y, et al. (2011) EGFR and KRAS mutations in lung carcinoma: Molecular testing by using cytology specimens. Cancer Cytopathol 119: 111-117.

16. Ganti AK, Huang CH, Klein MA, Keefe S, Kelley MJ (2011) Lung cancer management in 2010. Oncology 25: 64-73.

17. Giaccone G, Wang Y (2011) Strategies for overcoming resistance to EGFR family tyrosine kinase inhibitors. Cancer Treat Rev 37: 456-464.

18. Metro G, Crinò L (2011) The LUX-Lung clinical trial program of afatinib for non-small-cell lung cancer. Expert Rev Anticancer Ther 11: 673-682.

19. Pietanza MC, Lynch TJ Jr, Lara PN Jr, Cho J, Yanagihara RH, et al. (2012) XL647-A Multitargeted Tyrosine Kinase Inhibitor: Results of a Phase II Study in Subjects with Non-small Cell Lung Cancer Who Have Progressed after Responding to Treatment with Either Gefitinib or Erlotinib. J Thorac Oncol 7: 219-226.

20. Pratilas CA, Solit DB (2010) Targeting the mitogen-activated protein kinase pathway: physiological feedback and drug response. Clin Cancer Res 16: 3329-3334.

21. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, et al. (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448: 561-566.

22. Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, et al. (2009) Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 27: 4247-4253.

23. Ou SH (2011) Crizotinib: a novel and first-in-class multitargeted tyrosine kinase inhibitor for the treatment of anaplastic lymphoma kinase rearranged non-small cell lung cancer and beyond. Drug Des Devel Ther 5: 471-485.

24. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, et al. (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 363: 1693–1703.

25. Shaw AT, Solomon B, Kenudson MM (2011) Crizotinib and Testing for ALK. J Natl Compr Canc Netw 9: 1335-1341.

26. Sakairi Y, Nakajima T, Yasufuku K, Ikebe D, Kageyama H, et al. (2010) EML4-ALK fusion gene assessment using metastatic lymph node samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration. Clin Cancer Res 16: 4938-4945.

27. Schuurbiers OC, Looijen-Salamon MG, Ligtenberg MJ, van der Heijden HF (2010) A brief retrospective report on the feasibility of epidermal growth factor receptor and KRAS mutation analysis in transesophageal ultrasound- and endobronchial ultrasound-guided fine needle cytological aspirates. J Thorac Oncol 5: 1664-1667.

28. van Eijk R, Licht J, Schrumpf M, Talebian Yazdi M, Ruano D, et al. (2011) Rapid KRAS, EGFR, BRAF and PIK3CA Mutation Analysis of Fine Needle Aspirates from Non-Small-Cell Lung Cancer Using Allele-Specific qPCR. PLoS One 6: e17791.

29. Tanaka T, Nagai Y, Miyazawa H, Koyama N, Matsuoka S, et al. (2007) Reliability of the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based test for epidermal growth factor receptor mutations integrated into the clinical practice for non-small cell lung cancers. Cancer Sci 98: 246-252.

30. Hunt JL, Finkelstein SD (2004) Microdissection techniques for molecular testing in surgical pathology. Arch Pathol Lab Med 128: 1372-1378.

31. Oshita F, Matsukuma S, Yoshihara M, Sakuma Y, Ohgane N, et al. (2006) Novel heteroduplex method using small cytology specimens with a remarkably high success rate for analysing EGFR gene mutations with a significant correlation to gefitinib efficacy in non-small-cell lung cancer. Br J Cancer 95: 1070-1075.

32. Turbett GR, Barnett TC, Dillon EK, Sellner LN (1996) Single-tube protocol for the extraction of DNA or RNA from paraffin-embedded tissues using a starch-based adhesive. Biotechniques 20: 846–850, 852–853.

This article was originally published in a special issue, **Lung Cancer Challenges to the future** handled by Editor(s). Dr. Ramon Andrade de Mello, University of Porto, Portugal