Molecular diagnostics on tissue samples obtained through EBUS-TBNA: review on practice guidelines

C. Dooms, MD, PhD[1,4], B. Weynand, MD, PhD[2,4], S. Vander Borght, PhD[2,4], L. Vliegen, MSc[3,4], E. Verbeken, MD, PhD[2,4], J. Vansteenkiste, MD, PhD[1,4], P. Vandenberghe, MD, PhD[3,4]

Endobronchial ultrasonography is a minimally invasive endoscopic technique that enables a real-time transbronchial needle aspiration. Endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) specimens have a high diagnostic accuracy in the detection of intrathoracic lymph node metastasis for a variety of malignancies. Predictive biomarker testing is gaining wide importance to tailor the treatment with the largest benefit to the patient. Endobronchial ultrasound guided transbronchial needle aspiration also results in an accurate analysis of molecular alterations (by ImmunoHistoChemistry, Fluorescence In Situ Hybridisation, or gene sequencing) provided that the endoscopist takes sufficient tumour samples and a dedicated cytopathologist is involved in the mastery of the specimens.

Endobronchial ultrasound guided transbronchial needle aspiration samples can be handled in different ways. Liquid-based cytology and smears are mostly used. The choice of the testing method should be based primarily on the nature of the sample to be tested, testing laboratory's expertise, and available equipment. ImmunoHistoChemistry, Fluorescence In Situ Hybridisation and targeted polymerase chain reaction-based sequencing can be performed on > 80% of the endobronchial ultrasound guided transbronchial needle aspiration specimens, as the latter is more sensitive in terms of limit of detection than Sanger sequencing. The next step are the next-generation sequencing assays, with only 10-20 ng of DNA sample input per gene mutation, which will minimise rejected samples due to insufficient sample quantity.

(Belg J Med Oncol 2016;10(1):15-20)

Introduction

Over the past two decades, endobronchial ultrasound (EBUS) guided transbronchial needle aspiration (TBNA), or EBUS-TBNA, has emerged as a highly effective minimally invasive endoscopic technique to sample peribronchial hilar or mediastinal lymph nodes for pathologic examination. Subtyping and genotyping on EBUS-TBNA specimens has long been considered limited by the lack of tissue architecture in these small tissue samples, but their performance in tumour sub- and genotyping has been proven accurate in modern pathology practice.

Several groups have reported on EBUS-TBNA for the diagnosis of intrathoracic lymph node metastases in patients with an extrathoracic malignancy.[1-3] A recent meta-analysis calculated the diagnostic accuracy of EBUS-TBNA in the detection of intrathoracic lymph node metastases for a variety of extrathoracic malignancies as 86%, with a negative likelihood ratio and diagnostic sensitivity of 16% and 85%, respectively.[4] Furthermore, the high degree of diagnostic accuracy

[1]Department of Respiratory Diseases and Respiratory Oncology Unit, [2]Department of Pathology, [3]Center for Human Genetics, [4]Translational Research Unit, University Hospitals KU Leuven, Leuven, Belgium.

Please send all correspondence to: C. Dooms, MD, PhD, University Hospitals KU Leuven, Department of Respiratory Diseases and Respiratory Oncology Unit, Herestraat 49, 3000 Leuven, Belgium, tel: +32 1 634 68 01, email: christophe.dooms@uzleuven.be.

Conflict of interest: The authors have nothing to disclose and indicate no potential conflict of interest.

Keywords: carcinoma, EBUS-TBNA, FISH, gene sequencing, IHC.

Table 1. Thoracic and extrathoracic malignancies referred for an EBUS-TBNA investigation of suspected hilar and/or mediastinal lymph node metastases.*

Malignancy	Diagnosis	Predictive for Treatment		
	Morphology & IHC	IHC	FISH	PCR/NGS
NSCLC	+	ALK	ALK ; ROS1	EGFR
Breast Ca	+	ER ; PR ; HER2	HER2-Neu	-
Melanoma	+	-	-	BRAF ; (NRAS); KIT
RCC	+	-	-	-
Colorectal Ca	+	MSI	-	RAS ; BRAF
Prostate Ca	+	-	-	-
Head + Neck Ca	+	(p16)	-	-
GEJ + gastric Ca	+	HER2	HER2	-

**Others (< 5% of all extrathoracic malignancies) referred for EBUS-TBNA: bladder, ovarian, cervix, pancreatic, thyroid, ampulloma, GIST, testis, hepatocellular carcinoma.*

for tissue specimens from EBUS-TBNA also implies that these specimens can provide adequate material for predictive biomarker testing (either by IHC, FISH or mutation analysis). In our routine practice, cellblocks prepared from EBUS-TBNA derived material are used for predictive biomarker testing in a variety of extrathoracic malignancies (*Table 1*). Similarly, the use of EBUS-TBNA to successfully acquire adequate cellular material for molecular subtyping in non-small cell lung cancer (NSCLC) has been demonstrated. Successful testing of some targets (such as EGFR mutation and/or ALK translocation) was observed in 72-98% of the samples in several studies.[5-8]

EBUS-TBNA specimen collection and characteristics

A dedicated EBUS-scope has a diameter of around 6 mm and can visualise mediastinal and/or hilar lymph nodes in contact with the central airway as distal as the lower lobe bronchus (*Figure 1*). EBUS allows the exploration of the same paratracheal and subcarinal mediastinal lymph nodes as a cervical mediastinoscopy. In addition, EBUS allows exploration of hilar lymph nodes. It must be stressed that EBUS cannot access the para-oesophageal and pulmonary ligament nodes in the lower mediastinum. Several operators have therefore extended the use of the EBUS scope into the

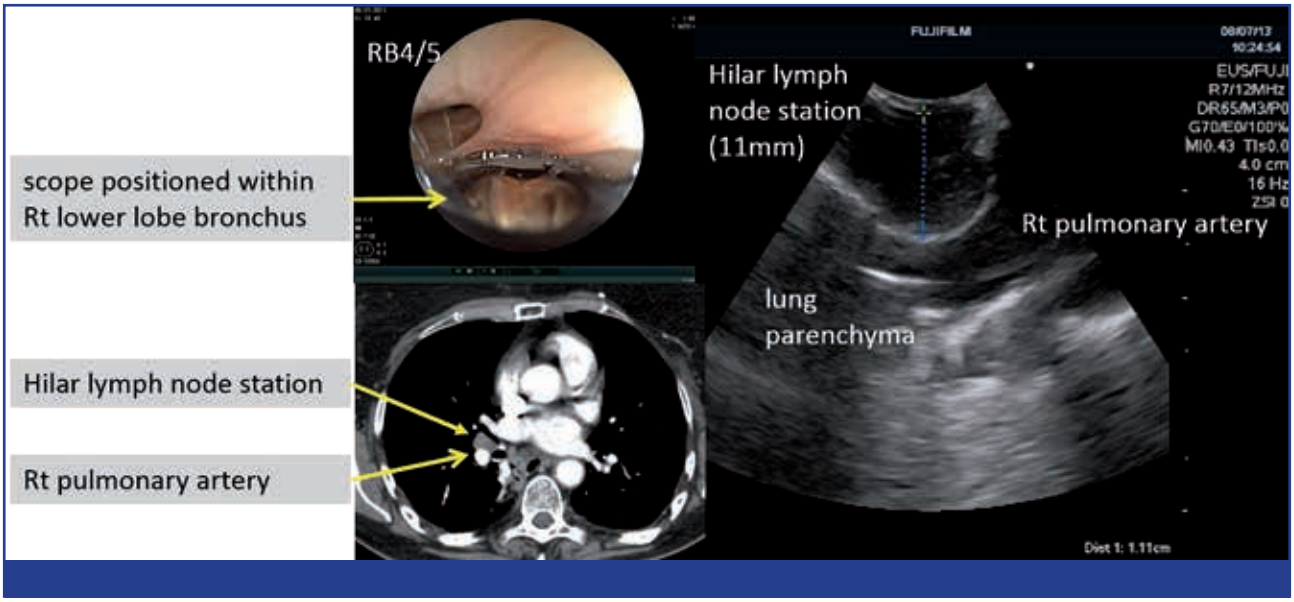

Figure 1. Case study of a patient with an enlarged hilar lymph node on spiral CT scan and previous history of malignant melanoma in whom an EBUS-TBNA investigation was performed; the specimen characteristics are discussed in *Figures 3* and *4*.

Figure 2. Dedicated flexible bronchoscope with ultrasound transducer at its tip to perform real-time endosonography and a fine needle aspiration.

oesophagus to perform an oesophageal ultrasound (EUS) exploration with the EBUS-scope, which can reach the para-oesophageal and pulmonary ligament nodes in the lower mediastinum, as well as the left paratracheal and subcarinal mediastinal nodes similar to the dedicated EUS scope that has a diameter of around 12 mm. The inner diameter of the working channel in the dedicated flexible bronchoscope is 2.0-2.2 mm and a 21, 22 or 25 gauge needle can be used to perform TBNA (*Figure 2*). Immediately after puncturing a lymph node, the stylet is used to clear any bronchial or cartilage debris and then the stylet is partially or completely removed and suction can be connected in the latter. At this time, the needle undergoes excursions inside the lymph node. There is no general consensus on the number of excursions or the exact location within the lymph node that should be biopsied. The number of needle passes needed to provide a significant sample for molecular analysis remains unknown, even though it has been reported in a recent practice guideline for NSCLC that a total of four punctures per lymph node provide diagnostic material in > 90% of patients.[9] After each pass, the needle is withdrawn, and a small amount of material can be applied to a slide for preparation of smears. Alternatively, the aspirate can be collected directly into a preservative solution (such as CytoLyt). The experience and skills of the bronchoscopist performing EBUS-TBNA directly impacts the cytopathologist as they interpret the cytologic materials obtained from the TBNA. Furthermore, a similar learning curve for the cytopathologist in the evaluation and mastery of the EBUS-TBNA specimens can be concluded. However, scant data exist on the number of cases necessary to achieve and maintain competency. In terms of safety, EBUS (within a recent prospective registry) has a low complication or serious adverse event rate of 1.4%.

Needle aspirations generate in general lower amounts of DNA compared to bronchial biopsies, but they result in an equally high success rate for mutation testing.[10] In one study, DNA extracted from formalin fixed paraffin embedded (FFPE) small bronchial biopsies (10 unstained slides, 4 mm thick) yielded an average 1,690 ng (range 250-3,600 ng) of DNA, while DNA extracted from needle aspirations generated lower amounts of DNA (average 230 ng; range 120-400 ng).[10] In our experience, bronchoscopic forceps biopsy samples and EBUS-TBNA cytology specimens exhibited a median tumour cell proportion of 30 versus 20% and DNA quantity of 1,610 versus 1,440 ng, respectively.[7] This observation is most likely related to the number of EBUS-TBNA needle passages performed by the endoscopist, which was 5±1 needle aspirations per patient in our cohort.[7]

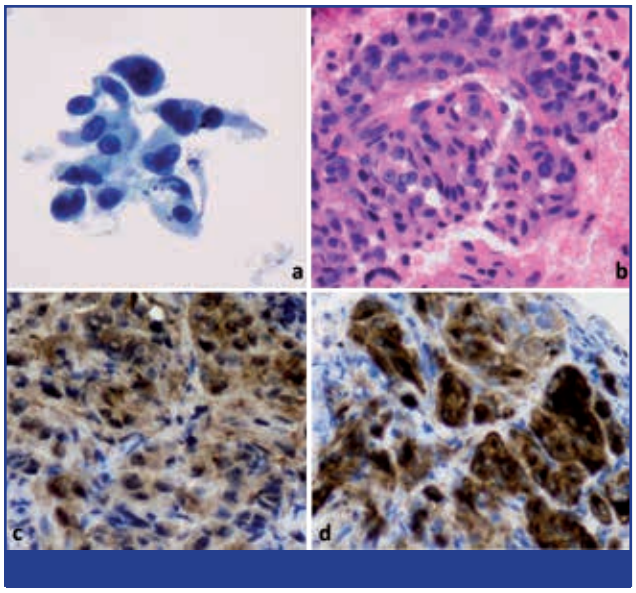

Figure 3. Representative image of the monolayer and cellblock used for diagnosis and molecular diagnostics of a malignant melanoma. (a) Papanicolaou stained monolayer showing a group of loosely cohesive neoplastic cells with an abundant cytoplasm and polymorph nuclei with a nucleolus. (b) corresponding HE stained cellblock. (c-d) immunohistochemical expression in tumour cells of respectively S100 protein and Melan A confirming the diagnosis of metastatic melanoma (magnification 400x).

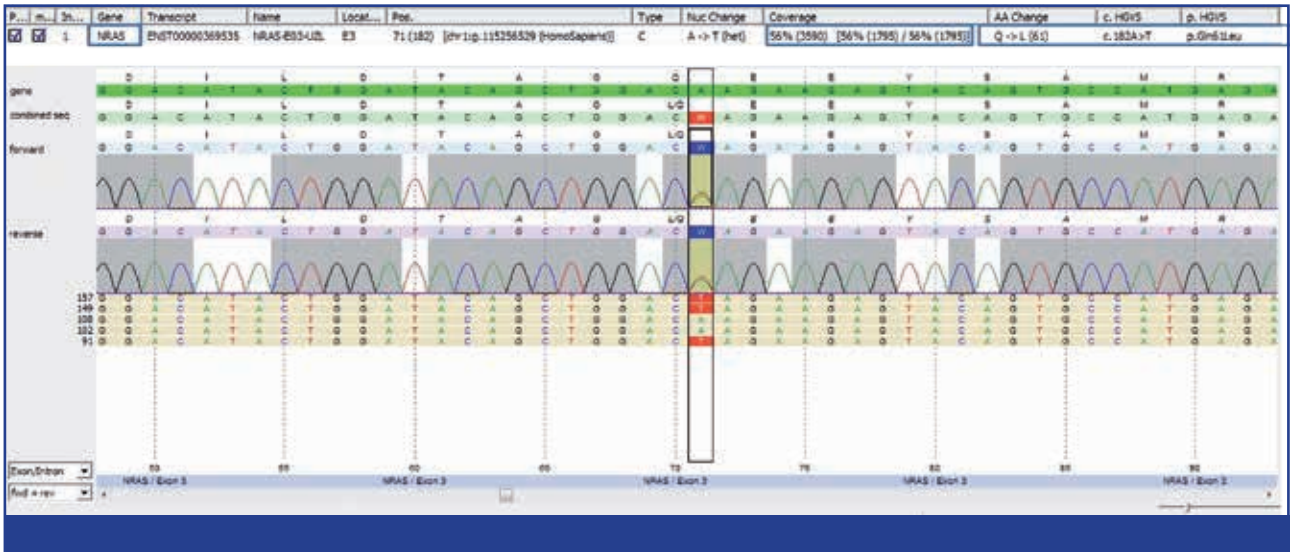

Figure 4. In the malignant melanoma (*Figure 3:* morphology) we found a NRAS p.Gln61Leu (p.Q61L), c.182A > T mutation (NM_002524.4) in 56% of the reads (SeqNext JSI version 4.2.2) with next-generation-sequencing (Somatic 1 Multiplicom MASTRv2) on a Illumina platform.

Analytical phase cytopathology on smears or cellblocks

Cytologic preparation, IHC and FISH (Figure 3)

Material obtained by EBUS-TBNA can be handled in different ways depending on local preferences. Smears can immediately be prepared and stained if rapid on-site evaluation (ROSE) is available. Several passes to ensure adequate material for further cytological diagnosis should always follow this. Liquid-based cytology with subsequent cellblock preparation is a valid alternative for diagnosis and molecular testing. In a retrospective study on extrathoracic malignancies diagnosed by EBUS-TBNA including 117 patients, a diagnostic accuracy of more than 90% was shown. Cellblock material was available in 92% of the malignant cases. Immunohistochemistry could be performed in 80%, including hormonal receptor status and HER2 FISH in cases of metastatic breast carcinoma.[2] For many years now, HER2 FISH has been performed on paraffin-embedded tissue material for breast tumours but FISH interpretation might be difficult because of signal loss by section artefacts, target DNA integrity or incomplete penetration of probes. Several advantages (e.g. assessment of the entire cell nucleus) can be envisioned for performing FISH directly on ThinPrep slides as compared to slides derived from FFPE cellblocks. A large NSCLC cohort with available liquid-based cytology material (majority TBNA specimen) for ALK status testing demonstrated that the routine use of ThinPrep-FISH is feasible and can reliably detect ALK gene rearrangements.[8]

DNA extraction/quantitation

The best results can be obtained if some technical issues and procedures are optimised. The first issue regards the use of a traditional cytologic smear or a cytoblock. Although it has been shown that smears can be used for mutation assays, we preferred to use cellblocks according to the guidelines from the College of American Pathologists.[11] Cellblocks are recommended over smears because of the ability to correlate with malignant cell content, the possible retention of more material for additional studies, and the proper fixation of the material from cytological cell block preparations. Alcohol-based fixatives as the starting material for gene mutation testing are associated with better preservation of DNA than formalin fixation.[12] Molecular analysis may be performed on samples fixed in alcohol, but the laboratory needs to extensively validate the tests to avoid false negative or false positive results.[13] Successful sequencing is also reported on smears irrespective of type of fixation or staining.[14]

Depending on the amount of cells in the block, up to ten consecutive 4 μm sections are prepared, of which the first and last are stained with hematoxylin and eosin (H&E) and evaluated for the presence and amount of tumour cells by an experienced pathologist. The proportion of tumour cells is estimated semi-quantitatively

Key messages for clinical practice

1. EBUS-TBNA is a minimally invasive endoscopic technique to sample peribronchial hilar/mediastinal lymph nodes under local anaesthesia.
2. EBUS-TBNA specimens yield a high diagnostic accuracy in the detection of intrathoracic lymph node metastases for a variety of malignancies.
3. EBUS-TBNA specimens can provide adequate material for predictive biomarker testing either by IHC, FISH or mutation analysis.
4. EBUS-TBNA specimens can be handled in different ways depending on local preferences.
5. For IHC and FISH, either smears or liquid-based cytology with subsequent ThinPrep and cellblock preparation can be used.
6. For mutation analysis, cellblocks are recommended over smears, in line with the guidelines from the College of American Pathologists.

and the representative area is marked on the H&E slide. For EBUS-TBNA, macro-dissection is often impossible due to spreading of the tumour cells. For most next-generation sequencing (NGS) methods, a 10% malignant cell fraction or a 5% mutant allele frequency is warranted for a correct interpretation. NGS technologies rely on high quality double stranded DNA that is suitable for library preparation followed by sequencing. For low concentrations, any fluorophore-based method, for example Qubit 2.0, is well suited due to higher sensitivity. Poor sample quality limits the amount of DNA that can be amplified. DNA of high molecular weight with little evidence of band shearing, containing no evidence of contamination from protein and RNA and a 260/280nm absorbance ratio of approximately 1.8-2.0 is therefore required.[15] Also recommended, is to test the global DNA quality based on delta crossing point (ΔCp) values. Specifying the relation between sample quality and input requirement will help in selecting the correct range of input DNA.

Sequencing (Figure 4)

NGS has emerged as a powerful tool for identifying genetic variants in a clinical laboratory setting. Defining the optimal workflow and use of the available cytological tumour material is a huge challenge for the pathologist. Recent, but still limited studies, stated that NGS could also be performed on EBUS-TBNA specimens as reliable and robust as on surgical specimens.[14,16-18] The error rate, library complexity, enrichment performance and depth of coverage does not seem to be significantly different between both sample types.[14,16,18] However, the amount of input DNA needed for performing NGS is quite variable, ranging from ten to > 250 ng, depending of the target capture and sequencing platform used. Irrespective of this feature, a lot of cytology samples yielded suboptimal and insufficient DNA and could not be successful tested by NGS.[14] False negative and false positive events can be generated by preferential amplification of non-tumour DNA or by amplification of a homologous internal region of the desired amplicon by multiplex PCR in tumour samples with low DNA amounts and/or low tumour content.[19,20] A minimum read depth of 500x is required to reliably detect minor allele frequencies of 5-10%.[14]

A great advantage of performing NGS on alcohol fixed cellblocks or a smear is that no sequence artefacts associated with formalin fixation are observed and this does not hamper the variant calling. We believe that all platforms will generate equal results but cost-efficiency, DNA input requirements, technical feasibility, and turn-around time will be critical subjects in choosing a good and reliable platform for the diagnostic service, especially for those samples with a low tumour content. NGS assays should have a reasonable turn-around time of ten working days from receipt of suitable material in the testing laboratory to reporting the results, consistent with the length of time previously accepted for a single gene testing. NGS testing is only feasible in centres that have sufficient case throughput, appropriate equipment and technical/pathological expertise.

Conclusion

EBUS-TBNA specimens have a high diagnostic accuracy in the detection of intrathoracic lymph node metastasis for a variety of malignancies, and can result in an accurate analysis of their molecular alterations (by IHC, FISH, or gene sequencing) provided that the endoscopist takes sufficient tumour samples and a dedicated cytopathologist is involved in the mastery of the specimens. The choice of testing method should be based primarily on the nature of the sample to be tested (certainly for cytology material), testing laboratory's expertise, and available equipment. Targeted methods based on real-time PCR can detect only specific mutations but are more sensitive in terms of limit of detection than Sanger sequencing. Probably a definitive answer will be given by NGS assays, with only 10-20 ng of DNA sample input per gene mutation, which will minimise rejected samples due to insufficient sample quantity.

References

1. Tournoy K, Govaerts E, Malfait T, et al. Endobronchial ultrasound-guided transbronchial needle biopsy for M1 staging of extrathoracic malignancies. Ann Oncol. 2011;22:127-31.
2. Sanz-Santos J, Cirauqui B, Sanchez E, et al. Endobronchial ultrasound-guided transbronchial needle aspiration in the diagnosis of intrathoracic lymph node metastases from extrathoracic malignancies. Clin Exp Metastasis. 2013;30:521-8.
3. Navani N, Nankivell M, Woolhouse I, et al. Endobronchial ultrasound-guided transbronchial needle aspiration for the diagnosis of intrathoracic lymphadenopathy in patients with extrathoracic malignancy: A multicentre study. J Thorac Oncol. 2011;6:1505-9.
4. Yang B, Li F, Shi W, et al. Endobronchial ultrasound-guided transbronchial needle biopsy for the diagnosis of intrathoracic lymph node metastases from extrathoracic malignancies: A meta-analysis and systematic review. Respirology. 2014;19:834-41.
5. Garcia-Olive I, Monso E, Andreo F, et al. Endobronchial ultrasound-guided transbronchial needle aspiration for identifying EGFR mutations. Eur Respir J. 2010;35:391-5.
6. Navani N, Brown JM, Nankivell M, et al. Suitability of endobronchial ultrasound-guided transbronchial needle aspiration specimens for subtyping and genotyping of non-small cell lung cancer: a multicentre study of 774 patients. Am J Respir Crit Care Med. 2012;185:1316-22.
7. Dooms C, Vliegen L, Vander Borght S, et al. Suitability of small bronchoscopic tumour specimens for lung cancer genotyping. Respiration. 2014;88:371-7.
8. Minca E, Lanigan C, Reynolds J, et al. ALK status testing in non-small cell lung carcinoma by FISH on ThinPrep slides with cytology material. J Thorac Oncol. 2014;9:464-8.
9. van der Heijden EH, Casal RF, Trisolini R, et al. World Association for Bronchology and Interventional Pulmonology, Task Force on Specimen Guidelines. Guideline for the acquisition and preparation of conventional and endobronchial ultrasound-guided transbronchial needle aspiration specimens for the diagnosis and molecular testing of patients with known or suspected lung cancer. Respiration. 2014;88:500-17.
10. Arcila M, Oxnard G, Nafa K, et al. Rebiopsy of lung cancer patients with acquired resistance to EGFR inhibitors and enhanced detection of the T790M mutation using a locked nucleic acid-based assay. Clin Cancer Res. 2011;17:1169-80.
11. Lindeman N, Cagle P, Beasley M, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the study of Lung Cancer, and Association for Molecular Pathology. J Mol Diagn. 2013;15:415-53.
12. Travis WD, Rekhtman N, Riley G, et al. Pathologic diagnosis of advanced lung cancer based on small biopsies and cytology: a paradigm shift. J Thorac Oncol. 2010;5:411–4.
13. Jain D, Mathur S, Iyer V. Cell blocks in cytopathology: a review of preparative methods, utility in diagnosis and role in ancillary studies. Cytopathology. 2014;35:356-71.
14. Kanagal-Shamanna R, Portier B, Singh R, et al. Next-generation sequencing-based multi-gene mutation profiling of solid tumours using fine needle aspiration samples: promises and challenges for routine clinical diagnostics. Mod Pathol. 2014;27:314-27.
15. Simbolo M, Gottardi M, Corbo V, et al. DNA Qualification Workflow for Next Generation Sequencing of Histopathological Samples. PLoS One. 2013;8:e62692.
16. Karnes HE, Duncavage EJ, Bernadt CT. Targeted next-generation sequencing using fine-needle aspirates from adenocarcinomas of the lung. Cancer Cytopathol. 2014;122:104-13.
17. Qiu T, Guo H, Zhao H, et al. Next-generation sequencing for molecular diagnosis of lung adenocarcinoma specimens obtained by fine needle aspiration cytology. Sci Rep. 2015;5:11317.
18. Young G, Wang K, He J, et al. Clinical next-generation sequencing successfully applied to fine-needle aspirations of pulmonary and pancreatic neoplasms. Cancer Cytopathol. 2013;121:688-94.
19. Vigliar E, Malapelle U, Belevicine C, et al. Outsourcing cytological samples to a referral laboratory for EGFR testing in non-small cell lung cancer: does theory meet practice? Cytopathology. 2015;26:312-7.
20. Vigliar E, Malapelle U, de Luca C, et al. Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective. Cytopathology. 2015;26:271-83.