THE ROLE OF ENDOSCOPIC ULTRASOUND AND ENDOBRONCHIAL ULTRASOUND ASSOCIATED WITH MOLECULAR TECHNIQUES IN DIAGNOSING AND STAGING LUNG CANCER

- ABSTRACT -

Scientific coordinator
Professor Adrian Săftoiu, PhD

PhD Student
Ana Maria Ioncică

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Key Words
Lung cancer, Endoscopic ultrasound (EUS), endoscopic ultrasound-guided transesophageal fine needle aspiration (EUS-PFA), Echobronchoscopy (EBUS), Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), Cytology, Immunocytochemistry exam, MMR Genes, Quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Background

Worldwide lung cancer is one of the most frequent cancers, having a very high rate of mortality. Most of the cases (55%) are recorded in the developing countries. Lung cancer takes first place in men, everywhere in the world, (1.1 million cases, 16.5% of the total neoplasias) as the rates are higher in Central and Southern Europe, Northern America and Eastern Asia and very low in Western Africa and Middle East Africa. In women, we record a lower incidence, but at global level it currently takes the fourth place (516 000 cases, 8.5% of the total) and the second place as a cancer death cause (427 000 deaths, 12.8% of the total deaths) after breast cancer. In Romania, lung cancer takes the first place in men, and the fifth place as incidence in women after breast cancer, cervical cancer, rectal cancer and colon cancer.

Approximately 50% of the cases are depicted in inoperable stages, and the prognosis is extremely severe, as the 5-year surviving rate is 15%. It is also characterized by high invasiveness degree because there is very strong sanguine and lymphatic vascularisation in the lungs, allowing cancer cells to migrate to the neighboured organs since the incipient stages.

Imaging methods have been making great progress in the recent years, allowing positive diagnosis the correct stadializing of bronchopulmonary cancer that is a space replacing lesion. Computed tomography (CT), magnetic resonance imaging (MRI) and most recently PET (positron emission tomography) and PET-CT association (positron emission tomography – computed tomography) offer valuable information both on localizing the primary lesion and on the possible involvement of the adjacent tissues. Also, PET-CT offers information not only on localizing and staging, but also on the metabolism of cancer cells because, in tumour diseases, metabolic changes are followed by morphologic changes.

The complete positive diagnosis of patients with lung cancer requires histological confirmation and the exact staging because the therapeutic approach depends on it. Histological confirmation is possible in over 70% of the patients by bronchoscopy with cytological or histopathologic exam. Almost a third of the patients need, for confirmation,
CT guided transthoracic needle aspiration, mediastinoscopy or thoracoscopy. These methods are invasive and they have a high degree of morbidity and mortality. Endoscopic ultrasound accompanied by endoscopic ultrasound-guided transesophageal fine needle aspiration and endobronchial ultrasound with ultrasound-guided transbronchial needle aspiration are minimum invasive complementary methods offering positive diagnosis and correct staging accomplishing a real “medical mediastinoscopy” and avoiding the practice of invasive procedures.

**Endoscopic ultrasound** is a method used for the assessment of the digestive tract (esophagus, stomach, duodenum, colon and rectum), as well as of the surrounding organs (mediastinum, pancreas, bile ducts, suprarenal ducts) by significantly extending the possibilities of early diagnosis, exact staging or minimum-invasive therapy. The introduction of endoscopic ultrasound-guided fine needle aspiration has determined the increase of the diagnostic possibilities of endoscopic ultrasound by obtaining an accurate diagnosis (through cytological or microhistological exam). Endoscopic ultrasound has proved to be an ideal method for diagnosing and staging lung cancer, but it cannot accomplish the complete evaluation of mediastinum, as it is not able to assess the region in front of the trachea and the lung hilum due to the limited contact and to the interposition of the respiratory ducts. These inconveniences are solved by **endobronchial ultrasound** which allows the assessment of the respiratory ducts, on their walls and on the surrounding structures: large veins, cava vein, pulmonary arteries, esophageal wall, superior anterior mediastinum.

**Material and method**

The study developed within the Research Centre of Gastroenterology and Hepatology of University of Medicine and Pharmacy from Craiova during October 2007 – October 2011, prospectively including consecutive patients.

The batches of study were structured as follows:

- **1st batch** consisting of 20 patients suspected of lung cancer, examined by bronchoscopy and with negative biopsies. The purpose was to evaluate the utility
of endoscopic ultrasound-guided transesophageal fine needle aspiration, associated with a cytological and immunocytochemistry examination in assessing the patients suspected of lung cancer

- **2\(^{nd}\) batch** consisting of 56 patients suspected of lung cancer examined by EUS and EBUS accompanied by fine needle aspiration with a cytological and immunocytochemistry examination in order to establish the role of this combined methods of evaluating lung cancers

- **3\(^{rd}\) batch** consisting of 48 samples from 45 patients where the cellular material extracted through fine needle aspiration underwent genetic tests by extracting RNAm and determining the expression of the genes involved in repairing the DNA (MMR): MSH2 and MSH6

All the included patients were suspected of lung cancer by at least of one the following: clinical emphasis of the tumour with neoplastic impregnation signs (weight loss, physical asthenia, dysphonia, dyspnoea, haemoptysis) or the abnormal results of the biological tests (leukocytosis, high ESR, anaemia, hypoxemia).

The information was collected through a structured form including:

- Personal data (surname, first name, age, birth date, personal number, diagnosis at hospitalization date)
- Medical history (smoking, drinking, toxic environment)
- Symptoms (pain, weight loss, dyspnoea, dysphonia, haemoptysis, etc)
- Objective exam (weight, pallor, adenopathies, vesicular breath sound, bronchial rales)
- Biological explorations (hemoleukogram, ESR, carcinoembryonic antigen, CYFRA 21-1, NSE – specific neuron enolase)
Results and discussions

The role of endoscopic ultrasound-guided transesophageal fine needle aspiration and immunocytochemistry exam in assessing the patients suspected of lung cancer and negative bronchoscopic biopsies

Of all 20 patients with negative bronchoscopic biopsies for malignancy, we performed endoscopic ultrasound-guided fine needle aspiration in 4 cases directly at the level of the primary mediastinal tumour, respectively in 4 cases at the level of lymph node station 5 (aorto-pulmonary window) and in 7 cases at the level of lymph node station 7 (subcarinal space). In a patient, we could not accomplish the endoscopic ultrasound-guided fine needle aspiration due to the impossibility of finding a trajectory that should not cross the large vessels, but the diagnosis was confirmed by the fine needle aspiration of the left adrenal gland. In other 4 patients, the endoscopic ultrasound-guided fine needle aspiration was negative, as the diagnosis was confirmed by EUS-guided trucut needle biopsy. The cytopathologic diagnosis was positive for malignity on the smears obtained through EUS-FNA in 15 patients. Therefore, in 11 patients, we obtained cellular material from mediastinal lymph nodes, and we spotlighted small lymphocytes on the smears (attesting a normal lymph node structure) and frequent placards of big cells with moderate nuclear atypical features in 4 cases (and severe ones in 7 cases, with big, unequal, pleomorphic (polylobated) nuclei, with intranuclear and anisochromia. In 4 cases, needle aspiration was performed straight in the mediastinal tumour, and on the smears we obtained abundant cellularity consisting of frequent placards of cells with certain malignity features: pleomorphic nuclei, with severe, tahi-chromatic anisocorias, with anisochromia and nuclear overlapping. In the 4 cases of mediastinal tumours where EUS-FNA was positive for malignancy, some of the cellular material was included to paraffin by obtaining cellular blocks. These presented a real advantage for patients whereas, on the sections obtained from cellular blocks, we could develop the immunocytochemical exam for two purposes: first of all for establishing the epithelial or mesenchymal phenotype of malignant cells and second of
all for establishing the membership to a certain organ of the identified atypical cells (the origin of the tumour). In these 8 cases, the material obtained by fine needle aspiration was reduced enough for certainly establishing the histopathologic type and this is why we developed the immunohistochemical exam complementarily. Thus, in 5 cases, the immunostaining of the tumour cells was intensely diffuse positive for cytokeratin 7 (CK 7) and low focal positive for cytokeratins AE1 / AE3, that certified the epithelial nature of the tumour. Also, in these cases, the cytoplasm positive immunoreactions for CEA and the nuclear intensely diffuse positive one for the thyroïdal transcription factor (TTF-1) certified the adenocarcinoma histologic type and the pulmonary origin of the malignant tumour cells. In the other 3 cases, tumour cells were diffusely positive for cytokeratins AE1/AE3, the immunoreaction was negative for CK 7 and CEA, and TTF-1 was focally positive in rare tumour cells, as these aspects certify the squamous type of carcinoma with pulmonary origin. Cytokeratin 20 was negative in all of these cases.

The current study has proved that fine needle aspiration allows to obtain enough material for accomplishing cellular blocks and an immunocytochemical exam, even with the complete determination of the tumour type, not only with the confirmation of a malignity diagnosis. Thus, we can curatively operate the patients in incipient stages, or we can guide the patients in advanced stages towards radio- and chemotherapy, avoiding thus useless surgeries and thoracotomies.

The importance of endoscopic ultrasound-guided transesophageal fine needle aspiration and ultrasound-guided transbronchial needle aspiration combined with cytological exam in diagnosing and staging bronchopulmonary cancer

This study included 59 patients aged between 32 and 83, being averagely 63 years old with a standard deviation of 10.4 years. Most of the patients included in the study came from the urban environment 81.36% compared to 18.64% of the rural environment. Of the 59 patients, 49 were men and 10 women and most of them are in the 5th and 6th life decade.
Of the 59 patients, 11 patients underwent fine needle aspiration only in the tumour mass displaying malignant cells on smears, allowing their framing as malignant smears. In one case, even if the needle aspiration in tumour was negative for malignancy, on the sections of the corresponding cellular block we spotlighted the placards of big cells, some of them round-oval (possibly epithelial), other fusiform with severe nuclear atypical features and atypical mitoses frequencies, sometimes having round, eosinophiles formations looking like cornous globes (keratosic pearls); this aspect was suggestive for a SCLC that led to the recommendation for investigating a squamo-cellular carcinoma. The certainty of this histological type on cellular blocks could not be confirmed because of little cellular material, but subsequently it was confirmed by the biopsy collected from bronchoscopy.

In case of the 11 patients undergoing fine needle aspiration, both in the lung tumour and in the regional lymph nodes, the cytological examination revealed the following:

- In 7 cases, on the smears collected from the tumor, malignant cells and pseudoglandular structures cells were revealed, suggesting an adenocarcinoma, examining the smears obtained from the 5th station ganglions did not reveal the presence of lymph nodes metastasis (negative smears);

- In one case, the smears come from the tumour were benign, while the ones from the lymph node needle aspiration (7th station) were diagnosed as positive for metastasis. Thus, on these smears, we revealed frequent placards of epithelial cells with severe nuclear atypical features, on a background with numerous lymphocytes come from the lymph node structure. In this case, we also accomplished the trucut needle biopsy which revealed a microscopic structure of averagely differentiated adenocarcinoma. For confirming the lung origin of the tumour, we developed an immunohistochemistry exam that revealed an intensely positive and diffuse immunostaining for TTF-1 and focal for cytokeratin 7 in tumour.

- In 3 cases we could not confirm the clinic suspicion of malignancy because both the smears come from the tumour and the ones come from lymph nodes (5th and
7th stations) were framed as benign. The histological exam on the sections come from the cellular blocks in the product of tumour needle aspiration also revealed cylindrical cells lambos and very inflammating exudate with polymorphonucleair leukocytes.

We calculated the values of sensibility, specificity, VPP, VPN in case of the fine needle aspiration, they are between 87.9%, 100%, 100% and respectively 84%. In conclusion, with such high rates EUS-FNA with EBUS-TBNA are excellent methods in diagnosing and staging lung cancer.

<table>
<thead>
<tr>
<th>Statistic Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Sensibility (%)</td>
<td>86.00%</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>100.00%</td>
</tr>
<tr>
<td>Positive Predictive Value (%)</td>
<td>100.00%</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>56.25%</td>
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</tbody>
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Table 1. Values of sensibility, specificity, VPP, VPN in diagnosis of CBP

**MMR genes expression in lung cancer**

For analysing the expression of the genes involved in repairing DNA errors in lung cancer, we studied 48 samples from 45 patients undergoing endoscopic ultrasound-guided transesophageal fine needle aspiration and of endobronchial ultrasound-guided transbronchial needle aspiration at the Research Centre in Gastroenterology and Hepatology. The samples were represented by mediastinal tumour masses (no.14), mediastinal lymph nodes (no.30) and adrenal metastatic masses (no.4).

In our study, we managed to extract RNA for all the 48 samples, as the smallest quantity is 0,7 μg/mL and the biggest one 73,7 μg/mL. Even if the quantities are very small by qRT-PCR we could accomplish the determination of gene expression of the two genes MSH2 and MSH6. In certain samples, their expression could not be
detected, either because of the heterogeneity of gene mutations, or because of the insufficient material. Thus, MSH2 gene was expressed in 61.02% of the cases, and MSH6 in 55.93% of the cases. Therefore, as a result of our studies, both genes can be assessed in order to establish the CBP diagnosis.

By using a cut-off value of 4900 copies of NRA/ml for MSH2 gene, the sensitiveness and the specificity of the method in CBP detection was, in our study 75% and 83% and for MSH6 gene, at the cut-off value of 9520 copies of NRA/ml the resulting sensitiveness and specificity were 81% and respectively 83%.

Also, we noticed that, in batch A, with negative smear samples (inflammatory smear) and a final benign diagnosis depending on the cut-off values established for the two genes, they were over-expressed, by revealing the capacity of repairing the possible errors produced during the DNA retort by the two genes. In batch C, samples with positive smear and lung cancer final diagnosis, we notice that the two genes MSH2 and MSH6 are under-expressed, suggesting thus the incapacity of repairing systems acting. In batch B, even if there are negative smear samples but having a lung cancer final diagnosis, the under-expression of the genes MSH2 and MSH6 can be already seen in 46.67% of the cases, by suggesting a change of the repairing systems, and therefore the possibility of a neoplasia process.
Conclusions

- Lung cancer is one of the most frequent cancers worldwide, having a very high rate of mortality being the first cause of death cancer. Considering the worst prognosis, there is a special interest in developing new techniques for diagnosing and staging of lung cancer.

- Fine needle aspiration allows to obtain enough material for accomplishing cellular blocks and an immunocytochemical exam, even by completely establishing the tumour type, not only by confirming a malign diagnosis.

- Endoscopic ultrasound-guided transesophageal fine needle aspiration and endoscopic ultrasound-guided transbronchial needle aspiration are minimum invasive complementary methods offering positive diagnosis and correct staging, accomplishing a real "medical mediastinoscopy" and avoiding the practice of invasive procedures.

- Our study certifies the possibility to accomplish molecular analyses even from small product quantities, which require a deep analysis of the possible existent mutations at the level of the genes MSH2 and MSH6, explaining thus the non-decisive values appeared during the study.
References