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UTILITY OF LASER MICRODISSECTION AND PRESSURE CATAPULTING IN THE DIAGNOSIS OF NON SMALL CELL LUNG CANCER: PRELIMINARY DATA

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ABSTRACT

***Background**: There are controversies about the adequacy of tumor tissue sample on which the sequencing of molecular diagnosis could be performed to achieve the targeted-therapy on lung cancer. The aim of this study is to demonstrate the role of the Laser Microdissection Pressure Catapulting (LMPC) technique to obtain adequate tumor tissue sample for the molecular analysis of gene mutations in the targeted therapy of lung cancer.*

***Findings**: From a consecutive series of 24 patients with a diagnosis of locally-advanced or metastatic Non Small Cell Lung Cancer (NSCLC), we performed 29 diagnostic procedures using the system of LMPC, to obtain an homogeneous samples where it was possible to run the sequencing of the 4 most frequently mutated exons of Epidermal Growth Factor Receptor (EGFR) (exon 18, 19, 20, 21).*

***Results**: There were 14 males (58.3%) and 10 females (41.7%), with a mean age of 61 years old. Twenty one patients were affected by adenocarcinomas, 2 by squamous cell carcinomas and 1 by large cell carcinoma. We were able to obtain the sequencing on 26 out 29 samples (89,6%) for EGFR mutation. EGFR mutation rate in our population was 7,7%. In 5 samples, we found a polymorphism in exon 20 and one of them carried a mutation on exon 18 as well. In another sample we found the deletion of exon 19. On the other 20 samples we did not find any mutation.*

***Conclusions**: Our preliminary data suggest that the LMPC technique permits to obtain the tumor cells sample more homogeneous facilitating the application of biological molecular analysis for EGFR-gene mutation in a larger number of patients with NSCLC.*

***Key words**: Lung cancer, bronchoscopy, Laser Microdissection Pressure Catapulting, molecular analysis, NSCLC, EGFR.*

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Introduction

Lung cancer is the most diagnosed malignant tumor (1.61million, 12.7% of the total) and the leading cause of worldwide cancer death (1.38 million, 18.2% of the total)[1]. Despite the strong correlation with smoking (about 85% of U.S. lung cancer deaths occur among smokers)[2] an increasing proportion of lung cancers (at least 10%) occurs in people who never smoked[3,4]. These people are mostly women, usually with Non-Small Cell Lung Cancer (NSCLC) histology and good response to treatment with specific Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor (erlotinib and gefitinib)[5]. The susceptibility to targeted-therapy is related to the mutations of EGFR[6,7,8,9,10]. For this reason, in recent years, molecular investigations may be considered complementary diagnostic techniques to instrumental and pathological standard procedures. The main limit to the use of simple and diffuse molecular methods of investigation (such as direct sequencing) on pathological specimens is the inadequacy of the sample. The technique of Laser Microdissection together with laser Pressure Catapulting (LMPC) system could be an efficient method, to select tissues areas, individual cells, fragments of cells and chromosomes to analyze status of EGFR mutation gene.

Procedures were performed in accordance with the ethical standards of the institutional committee on human experimentation. Informed consent was obtained from all patients.

Material and methods

The only inclusion criteria was the diagnosis of NSCLC. From January 2010 to February 2011 we observed a consecutive series of 24 patients with diagnosis of local advanced or metastatic NSCLC. The patients were 15 males (58.3%) and 9 females (41.7%). The mean age was 61.3 years, and it was 63.6 years for males (range 40-78) and 58.1 years for females (range 47-80). Data are summarized in Table 1. Diagnostic work-up consisted of accurate medical history, physical examination, chest CT-scan, laboratory blood tests, pulmonary function tests and 18F-fluoro-deoxy-D-glucose positron emission tomography (18 FDG-PET). Comorbidities were hypertension in 14 patients, type-2 diabetes in 10 patients and ischemic heart disease in 6 patients. Seven patients (29.1%) had no comorbidities.

We performed 29 sampling on 24 patients: 12 surgical biopsies, 11 video fiberoptic bronchoscopy (video-FBS) biopsies, 4 samples of pleural fluid, 1 lymph node fine needle aspiration and 1 cytological sample (Table 2). The histology of 24 patients, result from a standard pathologic analysis, was: 21 adenocarcinomas (including 3 Bronchioloalveolar carcinoma), 2 squamous cell carcinomas, 1 large cell carcinoma. (Table 1). Then we submitted the samples to LMPC.

PATIENTS FEATURES (overall = 24)					
Sex	***N.***	***Mean Age***	***Median***	***Range***	***%***
M	14	63,6	64	40-78	58,3
F	10	58,1	57	40-80	41,7
Histology					
Adenocarcinoma	21				
Adenocarcinoma	2				
Large Cell	1				

Table 1: Patient and histological features from our analysis.

DIAGNOSTIC PROCEDURES AND SAMPLES (overall = 29)	
Surgical biopsies	12
Video-FBS-guided biopsies	11
Pleural fluid samples	4
Lymph Node FNA	1
Cytological specimen	1

Table 2: Diagnostic procedures performed and samples obtained.

Laser Microdissection and Pressure Catapulting Technique

The system consisted of an inverted light Zeiss microscope connected to a computer equipped with special software and a UV-A stationary laser source of 337 nm. The sample set could be observed both by light microscopy and computer monitor. Microdissection could be performed at different magnifications, from 5x to 100x. The support of capture consisted of the cap of an Eppendorf tube placed a few millimeters above the preparation.

Before performing the microdissection 1μl of mineral oil was circulated over the sample and a membrane was placed on the slide under the tissue. The areas that needed to be dissected were selected from these instruments that could make high-precision micro-cuts from 1 to 1000 microns in diameter, calibrated to the specific tissue. After cutting, the strength of the photonic high pressure of the laser beam was used to give a certain amount of kinetic energy to the isolated material, the laser beam hit it from under the slide and catapulted it up at high speed against the gravity of the slide, directly into the slot catch (the cap of the Eppendorf tube), and was then removed and sent for further analysis.

On the samples obtained we have performed the sequencing of 4 exons of EGFR carrying more frequently mutations, giving preference, if the material was poor, to the exons 19 and 21 whose mutations positive predictive (response to therapy with TKI) are present in 90% of NSCLC EGFR+, [11] and secondly, to exon 20, which may carry the mutation negative predictive (resistance to treatment with TKI), and finally to exon 18.

Results

Concerning the 29 samples submitted to the LMPC system and sent to the laboratory for mutation analysis, 26 were considered suitable for

sequencing (90%), and 3 were not eligible (10%) for further analysis (Table 3). The exon 19 was sequenced in 18 samples (62%), exon 21 in 13 samples (45%), exon 20 in 11 samples (38%) and exon 18 in 8 samples (31%). In most cases the 4 exons showed wild type genotype. In 5 male samples we could highlight a polymorphism of exon 20 (17%), characterized by the replacement of the third base of the triplet CAG →CAA at position 787 which does not involve a change of aminoacid. In one of these 5 samples, in which there was evidence of that polymorphism, it has been also identified a mutation (3%) of exon 18 (V717G). A deletion in exon 19 (3%) was found in a female. Data are summarized in Table 3.

RESULTS		
Samples suitable for sequencing	26	89,6%
Samples non-eligible	3	10,4%
In suitable samples (overall = 26)		
Polymorphism of exon 20 (CAG → CAA at 787)	5	19,2%
Deletion at exon 19	1	7,7%
Mutation at exon 18 (V717G)	1	

Table 3: Results from our analysis.

Discussion

It is well known the utility of molecular diagnosis to select the patients with EGFR-gene mutations in NSCLC for the targeted therapy with TKI[10]. The IPASS trial[11] and INTEREST trial[12]) have demonstrated an improvement in terms of probability of progression-free survival in patients with NSCLC and EGFR-gene mutations.

The most frequent mutations responsive to the EGFR-TKI (gefitinib and erlotinib) are deletion in exon 19 or a missense mutation in exon 21 (L858R) and 18; mutation in other exons (such as T790M in exon 20) provide resistance to TKI[7,13].

An adequate sample is needed to obtain molecular diagnosis with at least 30-50% of tumor cells in the sample, corresponding at least to 10% of mutated DNA in order to prevent false negatives[14,15,16] with the possibility of direct sequencing.

However, in our clinical practice we obtained cytological and histological samples by Fine Needle Aspiration (FNA) of primary or metastatic lesions, bronchial or transbronchial biopsies obtained by video-FBS or core-biopsies of primary or metastatic lesions. These samples often do not allow to conduct molecular analysis of gene mutations because the cells and nuclear material from tumor is less than 30-50%[16].

Although Video Assisted Thoracic Surgery can increase the amount of tumor tissue sample, we don't perform invasive surgical procedures in patients with poor performance status and stage IV disease[17,18,19].

By using Laser microdissection combined with pressure catapulting, It is possible to submit different types of sample such as frozen or fixed in paraffin tissue section, cytospın preparations, smear cells, mucus, saliva or blood and living cell lines. Tumor cells can be isolated and a new pure and homogeneous sample can be established, extracting fragments or chromosomes in order to amplify DNA for gene sequencing.

Our preliminary data show that in 26 (89.6%) of 29 samples molecular biological analysis was performed, although a small sample was obtained by LMPC technique. We identified a mutation (V717G) of exon 18 in 3% of cases, not usually observed in the EGFR gene lung cancer as reported in literature but in other tumors. The significance is still uncertain. EGFR gene mutation was found in 8% of our series and this percentage is similar to that reported for the European population[8,10].

We want to emphasize that the use of oil and the insertion of a membrane between the glass slides and the tissue can improve the visibility and implementation of the method.

It is possible to transfer large selected areas and thus eliminating the disadvantages of oil alone or membrane placed over the substance as suggested by Bazan et al in the use of LPMC technique in colo-rectal cancer[20]. Moreover this technique can be suggested to be included in randomized controlled trial of patients with pulmonary metastasis in colo-rectal cancer[21]. We also note that the LMPC system is safe for biological samples because the wave of length of UVA is outside the range of highest absorption (260-280 nm) of nucleic acids and proteins, it permits that the light does not interfere with organic material and allows further analysis on the sample. It is also safe because the use of the laser beam as a real "optical scalpel" (catapulting) eliminates the contact between the operator and the sample, thus reducing the risk of errors associated with manual and the damage resulting from possible contamination or infection.

The LMPC system is characterized by precision because it allows to obtain pure cell lines from heterogeneous samples and easy running and appropriate in the approach of molecular analysis in patients affected by NSCLC.

Our preliminary data have to be confirmed by further clinical studies involving a larger number of patients as the LMPC technique could be used in routine clinical practice rather than more expensive methods such as the SSCP/SEQUENCING[20].

In conclusion, the Laser Microdissection and Pressure Catapulting (LMPC) technique permits the application of biological molecular analysis for EGFR-gene mutation in a larger number of patients with NSCLC.

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