FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

LUNG ADENOCARCINOMA: SUBTYPING AND EGFR, HER2 AND KRAS MUTATIONAL STATUS

Trabalho Final do 6º Ano Médico com vista à obtenção do grau de Mestre no âmbito do Ciclo de Estudos de Mestrado Integrado em Medicina, realizado sob a orientação da Professora Doutora Lina Carvalho (Faculdade de Medicina da Universidade de Coimbra) e do Dr. Víctor Sousa (Faculdade de Medicina da Universidade de Coimbra).

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor and co-supervisor, Professora Doutora Lina Carvalho and Dr. Víctor Sousa, respectively, for their expertise and constant incentive. I would also like to thank them for all the time invested on this project.

I would like to thank to Dr.ª Ana Alarcão, Dr.ª Patrícia Couceiro and Dr.ª Maria Silva for all the assistance on the immunohistochemical, PCR and FISH procedures, for their gentleness and constant availability to help whenever I needed.

A very special thanks goes out to my family and closest friends for all the support, incentive and strength given in the hardest moments, which supported me in achieving the best.

This research would not have been possible without the finantial assistance of Instituto de Anatomia Patológica - Faculdade de Medicina Universidade de Coimbra.

TABLE OF CONTENTS

LIST OF ABREVIATIONS 4

RESUMO 5

ABSTRACT 7

INTRODUCTION 9

MATERIAL AND METHODS 13

RESULTS 19

DISCUSSION 30

REFERENCES 36

LIST OF ABREVIATIONS

CK7-Cytokeratin 7

CK20-Cytokeratin 20

TTF1 - Thyroid Transcription Factor-1

LP34 – Cytokeratin 5,6 And 18

EGFR-Epidermal Growth Factor

FISH-Fluorescent In Situ Hybridization

IHC-Immunohistochemistry

SCLC-Small Cell Lung Cancer

NSCLC-Non Small Cell Lung Cancer

ADC-Adenocarcinoma

TKIs-Tirosine Kinase Inhibitors

BAC-Bronchiolo-Alveolar Carcinoma

RESUMO

Os adenocarcinomas bronco-pulmonares continuam sob estandardização morfológica, imunohistoquímica e genética por forma a atingir parâmetros que possam ser preditivos. As classificações actualmente propostas para os adenocarcinomas do pulmão necessitam de esclarecer determinados pontos, nomeadamente qual o valor da interpretação histológica dos diferentes padrões observados em pequenas biópsias. Um conjunto de 31 adenocarcinomas do pulmão foi submetido a estudos imunohistoquímicos, a FISH e a PCR, seguido de sequenciação e análise de fragmentos dos genes *EGFR, HER2* e *KRAS* após a sua subdivisão baseada na classificação de 2004 da WHO.

Foi aplicado o seguinte painel imunohistoquímico: CK7, CK5,6,18, CK20, TTF1, Cromogranina A, HER2, EGFR e Ki-67. O estado mutacional do *EGFR* e *KRAS* foi pesquisado simultaneamente com o número de cópias por FISH para o *EGFR* e *HER2*. Aplicaram-se os parâmetros explicados aos padrões histológicos encontrados em cada adenocarcinoma, quando pertencentes ao tipo misto da classificação de 2004 da WHO: acinar, papilar e carcinoma bronquíolo-alveolar não mucinoso; os padrões micropapilar e mucinoso (incluindo os padrões bronquíolo-alveolar mucinoso e glandular produtor de mucina) também foram isolados por microdissecção digital e manual.

Não foi identificada expressão para CK5,6,18 e Cromogranina A; o padrão sólido não expressou TTF1 e expressou um índice de proliferação do Ki-67 mais alto. O HER2 apenas revelou positividade citoplasmática e a expressão membranar de EGFR foi aumentando desde

o carcinoma bronquíolo-alveolar não mucinoso até ao sólido e do micropapilar até ao padrão acinar. Para as mutações do *EGFR*, principalmente a do exão 19, a presença de tumor nas mulheres e nos não fumadores, apresentou uma significância estatística elevada (p=0,000) enquanto que as mutações do exão 2 do *KRAS* estavam predominantemente presentes nos casos do sexo masculino (p=0,000), com relevância para o padrão sólido. O número de cópias do *EGFR* detectado por FISH estava fortemente relacionado com a existência de mutação no *EGFR* e o número de cópias do *HER2* estava aumentado, principalmente nos tumores presentes no sexo feminino.

Esta pequena amostra é suficientemente representativa para permitir a eliminação definitiva da designação de NSCLC e considerar o padrão sólido como aquele TTF1 negativo, CK5,6,18 e CK7 positivo, como um adenocarcinoma com elevado índice de proliferação, principalmente nos doentes do sexo masculino, onde os tumores com mutações do exão 2 do *KRAS* poderão ser um factor de exclusão para prescrição de TKIs. O padrão carcinoma bronquíolo-alveolar não mucinoso, não deverá ser eliminado visto que ele apresenta determinadas características ausentes nos restantes carcinomas bronquíolo-alveolares mucinosos que podem ser incluídas no tipo adenocarcinoma mucinoso. Permanece para ser explorada, a designação de carcinoma pulmonar pleomórfico, quando células largas, gigantes e fusiformes estão presentes em pequenas biópsias, por forma a terminar com esta designação pobremente diferenciada, visto que tais características histológicas não foram observadas neste conjunto de adenocarcinomas.

ABSTRACT

Bronchial-pulmonary adenocarcinomas are still under morphological, immunohistochemical and genetic standardization to reach predictive parameters. The recent proposed classification for lung adenocarcinomas needs to be clarified at certain points, namely the value of histological patterns to be interpreted in small biopsies. A series of 31 pulmonary adenocarcinomas was submitted to immunohistochemical study, FISH and PCR followed by sequencing and fragment analysis of the genes *EGFR, HER2* and *KRAS* after classification based in 2004 WHO guidelines.

The following immunohistochemical panel was applied: CK7, CK5,6,18, CK20, TTF1, Chromogranin A, HER2, EGFR and Ki-67. *EGFR* and *KRAS* mutational status was searched together with copy number by FISH for *HER2* and *EGFR.* The explained parameters were applied to each histological pattern found in all adenocarcinomas as belonging to the mixed type of WHO 2004 classification: acinar, papillary, solid and non-mucinous BAC; the micropapillary pattern and mucinous pattern (gathering mucinous BAC and glandular mucin producing pattern) were also isolated, all by digital and manual microdissection.

Expression for CK5,6,18 and Chromogranin A was not verified; solid pattern failed to express TTF1 and expressed higher Ki-67 proliferation index. HER2 revealed only cytoplasmatic positivity and EGFR membranous staining had increasing expression from non-mucinous BAC to solid pattern and micropapillary till acinar pattern. For *EGFR* mutations, mainly in exon 19, female tumors had higher statistical significance (p=0,000) together with non-

smoking status, while *KRAS* exon 2 mutation was present in male cases (*p*=0,000), with relevance to solid pattern. FISH *EGFR* copy number related grossly with *EGFR* mutations and *HER2* copy number was raised, mainly in female tumors.

This small series is representative to allow definite elimination of the NSCLC designation from pathology language and consider a solid pattern TTF1 negative, CK5,6,18 negative, CK7 positive as an adenocarcinoma with high proliferation index, mainly in male patients where *KRAS* exon 2 mutation may be eliminatory to TKIs prescription. BAC non-mucinous pattern should not be abolished as it presents with particular characteristics other than the former mucinous BAC that can be gathered under mucinous adenocarcinoma typing. Still to be explored, the pleomorphic carcinoma of the lung designation, when large cells, giant cells and fusiform cells are present in small biopsies, to cut the poorly differentiated designation, as those histological characteristics have not been observed in this series of adenocarcinomas.

KEY WORDS: Lung, *EGFR*, *KRAS*,*HER2*, Immunohistochemistry, adenocarcinomas.

INTRODUCTION

Lung cancer totalize 1,3 million deaths annually worldwide and is the leading cause of cancer-related mortality in Japan and Western-countries[1-2]. Tobacco smoking can be the main etiology in 85-90% of the cases. It has a poor prognosis, with a 5-years survival rate of 15% roughly for all stages and, more than half of the patients at time of first diagnosis, has metastatic disease[3-5].

Lung cancer had been classified as Small Cell Lung Cancer (SCLC) and Non Small Cell Lung Cancer (NSCLC), representing 13% and 85% of lung cancers respectively and NSCLC is a malignant epithelial tumour that includes different carcinoma subtypes like squamous cell carcinoma, adenocarcinoma, large cell carcinomas and pleomorphic carcinomas[5-6].

The subdivision of NSCLC into squamous cell carcinoma (SQCC) and adenocarcinoma (ADC) has recently become mandatory due to personalized therapy and NSCLC designation should not be reported.

Bronchial-pulmonary adenocarcinomas are malignant epithelial tumours with glandular differentiation, and/or mucin production; acinar, papillary, micropapillary, bronchiolo-alveolar or solid with mucin growth patterns, complement the morphological spectrum of mixed adenocarcinoma. The incidence differs between gender and population, being roughly 28% in men and 42% in women. In men, the incidence exceeds for the squamous cell carcinoma in certain Asian populations (Chinese, Japanese) and in North America (USA, Canada). However, in women, excepting Poland and England, adenocarcinoma is the dominant histological type. The incidence in Asian females is significant: 72% of cancer in Japan, 65% in Korea, 61% in Singapore Chinese[5].

It is preponderant in East Asia and USA and is becoming more frequent than squamous cell carcinoma in some European countries[7].

Several described molecular abnormalities are related to patients clinical history and histologic subtypes. Mutations in non-smokers are different from those occurring in smokers, suggesting different molecular etiology, pathogenesis and prognosis. Some data showed that *KRAS* mutations, detected in 10-30% of NSCLC cases, were strongly associated with smoking history and have been related to poor prognosis[8].

Epidermal Growth Factor Receptor (*EGFR*) is a transmembrane tyrosine kinase receptor expressed in the majority of NSCLCs. It belongs to *EGFR* family of receptor tyrosine kinases (TKs) called the HER or ErbB family consisting in four members – *EGFR* (*HER1*/*ErbB1*), *HER2* (*ErbB2*), *HER3* (*ErbB3*) and *HER4* (*ErbB4*) [9-10]. When *EGFR* is over-expressed it correlates with poor prognosis and with aggressive disease and decreased survival [3, 11-13].

RAS/MAPK and *PI3K/AKT* pathways are signalling networks that connect *EGFR* activation with cell proliferation and survival. Activation of *EGFR* allows cell proliferation, invasion, angiogenesis and metastatic disease[11, 14-16]. The deregulation of tyrosine kinase (TK) activity of *EGFR* can be due to various oncogenic mechanisms as *EGFR* gene mutation, increased gene copy number and EGFR protein overexpression[16]. EGFR overexpression is frequently correlated with poor prognosis or resistance to chemotherapy activating mutations of the *EGFR*[2, 6, 17-18].

Mutations are found in the first four exons of the TK domain of the *EGFR* gene. Short-mutations, in-frame deletions in exon 19 or point mutations resulting in a substitution of arginine for leucine at amino acid 858 (L858R), constitute roughly 90% of *EGFR* mutations[2, 19]; 45% are exon 19 mutations, 41% are exon 21 mutations and the remaining 10% are exon 18 and 20 mutations (approximately 4% of exon 18 and 5% of exon 20) - firstly described as

more frequent in females, non-smokers, adenocarcinomas and Japanese patients[3, 6, 19]. More than 70% of NSCLC with *EGFR* mutations are responsive to EGFR-TKIs while only 10% of tumours without *EGFR* mutations are responders [2].

The certainty that this mutations occurs in a subset of lung carcinomas and predict for sensitivity to TK inhibitors (TKIs) has generated a huge interest. Their determination, according to Shigematsu, can identify subpouplations that will demonstrate the optimal response to targeted therapies[3].

HER2 also belongs to *EGFR* family[3, 11-13] and has strong kinase activity. Mutations also occur in the same region (3’ of the αC-helix) in exon 20 just as *EGFR* in-frame duplications/insertions, present in a very small fraction of adenocarcinomas of non-smokers and females, (the same as *EGFR* mutations)[10, 20]; the majority are insertion mutations in exon 20 and the existence of *HER2* mutations, confers resistant to *EGFR*-TKIs but make tumours sensitive to *HER2* target therapies[3, 10, 20-23].

KRAS mutations are associated with poor prognosis, reported since 1990, occurring in codon 12, occasionally at codon 13 and rarely at codon 61[10, 24-25]. According to the current data, *EGFR* and *KRAS* mutations are mutually exclusive[10, 26-27]. The explanation found was that *KRAS-MAPK* pathway inserts in the downstream signalling pathway of *EGFR*[10]. This mutation appears in 30% of Caucasian patients with lung cancer and 10% of East Asian adenocarcinomas[20, 25, 27]. Higher incidence is seen in patients with smoking history and is considered a marker of poor prognosis in lung adenocarcinomas[10, 25-26]. Lung cancers with *KRAS* mutations are resistant to EGFR-TKIs and Yatabe, confirmed in his study that none of the lung cancers with *KRAS* mutation achieved clinical response[10, 28].

Treatment of lung cancer depends on TNM staging, consisting in surgery, chemotherapy and radiotherapy to improve clinical management together with personalized therapy.

Preventing ligand binding with monoclonal antibodies or blockade of intracellular receptor phosphorylation by TKIs, signal transduction through pathways *RAS/RAF/MAPK* and *PI3K/AKT* cascades can prevent tumoural progression. Five anti-EGFR drugs have been approved for the treatment of solid tumours, including two monoclonal antibodies (Cetuximab and Panitumab), two small molecules that bind to the intracellular kinase domains of EGFR (Erlotinib and Gefitinib) and one that binds to both EGFR and HER-2 (Lapatinib). In NSCLC with activated *EGFR* mutations (in exon 19 and 21), Gefitinib and Erlotinib are the TKIs recommended.

Due to what has been said, the *EGFR-MAPK* signal transduction pathway is important to understand the role of individual somatic changes in tumour predicting the response to *EGFR* TKIs and *EGFR* status is a favourable prognostic factor. Despite the positive response observed in 10-30% of patients, different data concluded that not every patient had benefits from treatment with TKIs, probably due to mutations in the downstream effectors of *EGFR* signalling, more frequently *KRAS* gene. Mutations in this intermediate transduction pathway may also select patients: as *KRAS* acts downstream of *EGFR* receptor, its somatic changes can lead to a non response to *EGFR* TKIs as response rate to anti-*EGFR* therapy is less than 3% in patients with *KRAS* mutant tumours in opposite to 20% in NSCLC with wild-type *KRAS*.

Characterization of gene copy number of *HER2* and *EGFR* and mutations of *EGFR* and *KRAS* genes, in different patterns of pulmonary adenocarcinomas expecting to determine the value of pattern subtyping to understand its potential role in deciding anti-*EGFR* therapy was searched.

MATERIAL AND METHODS

Subtyping Adenocarcinomas

Surgical specimens of 31 consecutive bronchial-pulmonary adenocarcinomas were classified according with WHO 2004 histological classification and each pattern was submitted to independent validation.

In this collection, only case 29 expressed a unique histologic pattern (solid adenocarcinoma pattern).

In Table 1 the 30 cases of mixed type adenocarcinomas have a representation of decrescent percentage of the corresponding patterns as identified: 29 (96,67%) cases with acinar pattern, 18 (60%) with non-mucinous bronchiolo-alveolar pattern (BAC), 13 (43,33%) solid pattern, 9 (30%) micropapillary pattern, 3 (10%) papillary pattern and 3 (10%) with mucinous pattern.

At least 2 patterns were represented in 18 cases in which 6 cases had acinar and BAC non-mucinous patterns, 6 cases had acinar and solid patterns, 1 case had acinar and papillary patterns, 3 tumors had acinar and micropapillary patterns, 1 had acinar and mucinous glandular patterns and 1 had solid and BAC non-mucinous patterns.

The most frequent patterns association was acinar, BAC and solid (n=4) followed by acinar, BAC and micropapillary (n=3).

In this series 19 (61,29%) cases belonged to females, 12 (38,71%) to masculine gender and smoking habits (18 (58,06%) non-smokers, 9 (29,03%) smokers and 4 (12,9%) ex-smokers) are also registered in Table 1; of 18 non-smokers patients (admitting passive smoking), 13 were females. It is important to notice that histological patterns present in lymph node metastasis belonged to acinar, solid and micropapillary morphology (pN1/2-8 cases).

Table 1 - Clinical and pathological characteristics of adenocarcinomas

CASE	GENDER	AGE	SMOKING HABITS			TNM CLASSIFICATION			STAGE	HISTOLOGIC PATTERNS	METASTASIS
1	M	73	Non-smoker			T1a	N0	Mx	IA	Acinar, Solid	
2	F	71	Non-smoker			T1a	N0	Mx	IA	BAC, Acinar	
3	M	69		Smoker		T4	N0	MX	IIIA	BAC, Acinar, Papillary	
4	M	75	Non-smoker			T1a	N2	Mx	IIIA	Acinar, Solid	Acinar, Solid
5	F	53	Non-smoker			T1a	N0	Mx	IA	Acinar, BAC	
6	M	76			Ex-Smoker	T2b	N0	Mx	IIA	Acinar, Solid, BAC	
7	F	62			Ex-Smoker	T1a	N2	Mx	IIIA	Acinar, Solid	Acinar, Solid
8	F	74	Non-smoker			T1a	N0	Mx	IB	Acinar, Solid, BAC	
9	F	68	Non-smoker			T2a	N0	Mx	IB	Acinar, Solid, Micropapillary BAC, Mucinous	
10	F	50	Non-smoker			T1b	N2	Mx	IIIA	Acinar, Solid	Acinar
11	F	57		Smoker		T2a	N0	Mx	IB	Acinar, Solid	
12	M	51	Non-smoker			T1a	N1	Mx	IIA	Acinar, BAC	Acinar. Solid
13	F	75	Non-smoker			T2a	N0	Mx	IB	Acinar, Solid, Micropapillary	
14	M	85			Ex-Smoker	T1a	N0	Mx	IA	Acinar, BAC	
15	F	63		Smoker		T1a	N2	Mx	IB	Acinar, Micropapillary BAC	Acinar, Solid
16	F	55		Smoker		T1a	N0	Mx	IA	Acinar, solid	
17	F	67		Smoker		T1a	N0	Mx	IA	BAC, Acinar, Micropapillary	
18	M	48		Smoker		T1a	N0	Mx	IA	Acinar, Solid, BAC	
19	M	71	Non-smoker			T2a	N1	Mx	IIA	Acinar, Micropapillary	Acinar
20	F	80	Non-smoker			T1b	N0	Mx	IA	Acinar, Micropapillary	
21	F	68	Non-smoker			T2a	N1	Mx	IIA	Acinar, Micropapillary	Acinar, Micropapillary
22	M	48	Non-smoker			T2b	N1	Mx	IIA	BAC, Acinar, Micropapillary	Acinar, Micropapillary
23	F	67	Non-smoker			T2a	N0	Mx	IB	Acinar, Mucinous	
24	M	76			Ex-Smoker	T1b	N0	Mx	IA	BAC, Acinar, Mucinous	
25	F	56	Non-smoker			T1a	N0	Mx	IA	BAC, Acinar,	

								Micropapillary, Papillary
26	M	64	Smoker	T2b	N0	Mx	IIA	Acinar, Papillary
27	F	71	Non-smoker	T2a	N0	Mx	IB	BAC, Acinar, Solid
28	F	50	Smoker	T2a	N0	Mx	IB	BAC, Solid
29	F	80	Non-smoker	T1a	N0	Mx	IA	Solid
30	M	56	Smoker	T1a	N0	Mx	IA	BAC, Acinar
31	F	56	Non-smoker	T1a	N0	Mx	IA	BAC, Acinar

Immunohistochemistry

Beyond histological classification all tumors had representative sections submitted to IHC to reinforce the pure condition of adenocarcinomas mainly to validate solid pattern and establish differential diagnosis, as well as characterize proliferation index (Ki67-MIB1 antibody). A single panel of five antibodies: CK7, TTF1, CK20, CK5,6,18 and Chromogranin A was applied, according with the manufacture recommendations. Proliferative index was characterized using Ki-67 antibody and antibodies against c-erbB1 and c-erbB2 were specifically applied: clone 31G7-Invitrogen Corporation and clone CB11-Novocastra Laboratories, respectively, after using positive and negative controls recommended.

The intensity of the staining was graded semi-quantitatively on a four point scale (0;1+,2+,3+). The percentage of immunostained cells was also registered. A final score was obtained multiplying the intensity by the percentage of cells with immunohistochemical expression and the cut off considered was 10% positive cells.

Fluorescent In Situ Hybridization -FISH

The Vysis LSI EGFR/CEP7 probe assay (*Vysis*; Abbott Molecular, USA) was applied to sections of 4µm thickness of the tumoral sections, baked overnight at 56ºC, deparaffinized in xylol, rehydrated in 100%, 70% ethanol and bidestilled water. A pressure cooker with 10mM

citric acid-trisodium salt buffer pH 6, for 4 minutes, was used to submit slides to a pre-treatment. Washed in 2x SSC salts (sodium chloride and sodium citrate) pH 7 for 5 minutes at room temperature. During 15 minutes slides were immersed in proteinase K solution at 37ºC and then, they were rinsed in 2x SSC pH 7 for 5 minutes at room temperature. The slides were then dehydrated in 70%, 90% and 100% ethanol, then air dried. Ten microliters of probe mixture were applied on the target areas and a 22mmx22mm glass coverslip was placed over probe.

After sealed with rubber cement and codenaturation at 83ºC for 5 minutes, coverslips were incubated overnight at 37ºC in a humidity chamber. Post-hybridization washes in buffer (50% formamide 2x SCC pH 7) at 46ºC and washed with 2x SCC pH 7. Slides were air-dried in the dark and counterstained with DAPI.

Overlaping cells were excluded from analysis. Two signals were counted as adjacent or fused only if they were separated by less than one domain. Two different individuals examined one hundred spindle cells interphase nuclei with strong and well delineated signals.

FISH was used to analyse the cromossome 7 and *EGFR* gene, and they were scored according to Cappuzzo's (2005) method. Positive FISH cases show high polysomy or amplification. Positive and negative FISH cases were according to Varella-Garcia, et al. [(29)]

The microscopical analyses was realized in a Nikon Eclipse 80i of brilliant field and epifluorescence microscopeusing (LUCIA cytogenetics software).

Fluorescente signals were observed and quantified with a score previously difined using DAPI, FITC, Texas Red (unique band) and triple band (DAPI, FITC e Texas Red) filters.

Images were captured and registered with a digital camera (Nikon DXM 1220F), in monochromatic images/layers posteriorly joint in one single image. This process was assisted by Nikon ACT-1 capture software.

The same procedure was followed to *HER-2* probe (HER-2/Neu (17q12)/SE17; Kreatech diagnostics; Amsterdam).

PCR, Sequencing and Fragment Analysis

Genomic DNA was extracted from 5 µm section of paraffin-embebed tissue. For that, the QIAmp DNA Mini Kit (Qiagen, IZAZA, Germany), was used. One hundred nanograms (ng) of DNA was amplified in a 50 µl reaction solution containing 5 µl of 10x buffer (Roche, Germany), 2,5 mM $MgCl_2$, 0,2 µM of each complementary primer, 200 µM deoxynucleoside triphosphate and one unit of DNA polymerase (Roche, Germany). A 5 minutes initial denaturation at 95ºC was used to perform the amplifications; this was followed by 40 cycles 30 seconds at 95ºC, 1 minute at 60ºC (for exon 19) or 57ºC (for exon 21), 1 minute at 72ºC and 10 minutes of final extension at 72ºC. The *EGFR* gene mutations located at exons 19 and 21, were determined using the intron-based primers according to the published method.[(30)] Mutational analysis of exon 19 deletion L858R point mutation of the *EGFR* gene was explored, as described.[(31)] The determination of exon 19 deletion was made by common fragment analysis using PCR with an FAM-labeled primer set, and the products were electrophoresed on ABI PRISM 3100 (Applied Biosystems®) and all eletropherograms were reanalyzed by visual inspection in order to check for mutations. To evaluate the L858R mutation, was also used MyCcycler (Bio-Rad), and its products were then studied by direct sequencing.

The same procedure was applied to *KRAS* except for amplification which we performed using a 5 minutes initial denaturation at 95ºC; followed by 40 cycles 30 seconds at 95ºC, 1 minute at 53ºC, 1 minute at 72ºC and a 10 minutes of final extension at 72ºC.

The same procedure was applied to *HER2* except for amplification which we performed using a 1 minutes initial denaturation at 95ºC; followed by 35 cycles 30 seconds at 95ºC, 1 minute at 64ºC, 1 minute at 72ºC and a 10 minutes of final extension at 72ºC.

Statistical Analyses

PASW *Statistics*, version 18 to do the statistical analysis of the information was applied.

To do the characterization of the sample we calculated mean and standart deviation for quantitative variables and we determined the absolute and relative frequencies for qualitative variables.

We made several comparisons between nominal variables (gender, smoking habits, mutations) using Chi-square test, Fisher's Exact test and ANOVA.

Between FISH and Mutational (PCR) status we calculated kappa coefficient in order to determine their concordance. The comparison between the existence of different types of mutation and age was realized with Mann-Whitney's test.

A significance level of 5% ($p \geq 0{,}005$) was considered.

RESULTS

Immunohistochemistry

All histological types and patterns identified were CK 5,6,18 and Chromagranin A negative without statistical significant differences (p>0,05). Normal epithelial basal cells were all positive for CK5,6,18 as internal control; lymph node metastasis did not express these high weight molecular cytokeratins (HWMC) as well.

As normal parenchyma, equivalent results were seen after the application of CK20: in two cases of mixed type adenocarcinoma, acinar and BAC non-mucinous patterns were identified with 2+ expression (moderate intensity) in 50% of the cells; in another case, mucinous glandular pattern had CK20 weak expression (1+) in 10% of the neoplasic cells. These three cases expressed CK7 3+ and TTF1 3+ (Fig.1). Lymph node metastasis of the other cases were all negative for CK20.

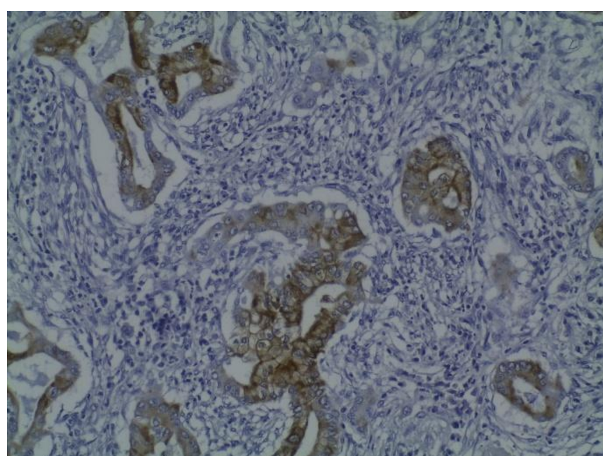

Figure 1- Case 23: 2+ CK20 expression; acinar pattern. 200x

Mucinous pattern showed lower expression of CK7 than normal pulmonary epithelial cells and respiratory cylindrical epithelial cells (p=0,09). Also, acinar pattern (p=0,0584), BAC non-mucinous pattern (p=0,0822), micropapillary pattern (p=0,086) and solid pattern (p=0,0661) had higher expression of CK7 than mucinous glandular pattern (Fig. 2).

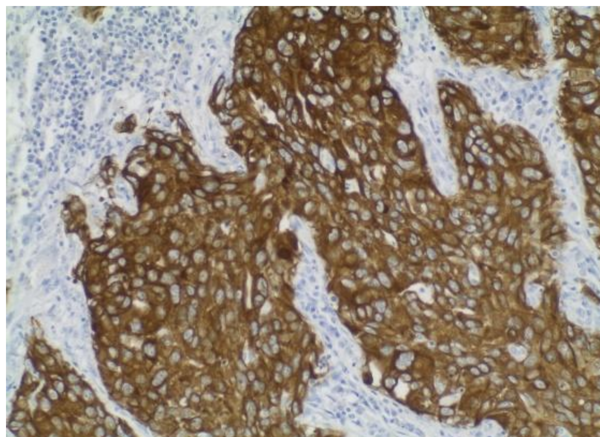

Figure 2- Case1: CK7 intense expression (3+) in solid pattern. 200x.

In one case of mucinous pattern there was no CK7 expression; at another case, the CK7 expression was moderately intense (2+) in 50% of the cells and a third case had intense (3+) in 80% of the cells.

TTF1 expression was discretely positive in normal alveolar septae as expected; it was negative in 6 of acinar pattern cases, 2 of BAC pattern cases, 1 papillary pattern and 2 solid pattern cases. Overall expression, taking into account the intensity and the percentage of positive cells, TTF1 higher expression was seen in acinar pattern (*p*=0,002) and in micropapillary pattern (*p*=0,005), when compared to normal alveolar septae. In mucinous patterns, expression was lower when compared to normal tissues (*p*=0,0192). Adenocarcinomas showed higher proliferative index, validated by Ki67 expression, than normal tissues (p<0,05). Ki67 expression was also higher in BAC pattern when compared to mucinous pattern (*p*=0,0845) and in solid compared to mucinous patterns (*p*=0,0817). Basal cells in respiratory epithelium expressed Ki67 in 50% of cells.

We did not find membrane staining/expression for CerbB2 in any case of the studied adenocarcinomas. Citoplasmatic expression was seen in all patterns and in normal tissues adjacent to the neoplasias, with significant statistical differences between alveolar septae and: acinar patterns (*p*<0,0001), BAC pattern (*p*<0,0001), papillary pattern (*p*=0,004), micropapillary pattern (*p*<0,0001), solid pattern (*p*=0,0006) and mucinous pattern (*p*=0,0073).

Solid pattern had lower expression than acinar (p=0,0034), BAC (p=0,004), papillary patterns (p=0,0334) and micropapillary patterns (p=0,0089) (Fig. 3 and 4).

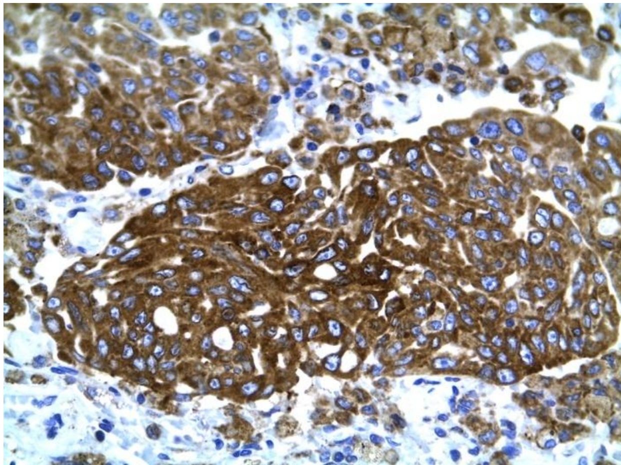

Figure 3- Case9:ErbB-2 3+ cytoplasmatic expression; solid pattern. ErbB-2, 400x

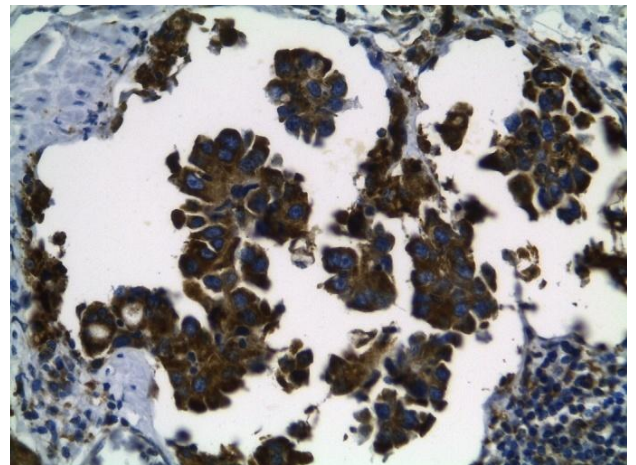

Figure 4- Case9:ErbB-2 3+ cytoplasmatic expression; micropapillary pattern. ErbB-2, 400x

EGFR membrane immunostaining was revealed in acinar pattern of 5 adenocarcinomas as moderate (2+) and in 4 cases, as intense (3+). We did not find membrane expression in papillary pattern. In micropapillary pattern, one case showed 2+ membrane expression and 3 cases 3+. BAC pattern showed 2+ membrane expression in 2 cases and 3+ in one case. Solid pattern showed 2 cases with 2+ and 3 cases with 3+ membrane expression. Mucinous pattern was negative in all studied adenocarcinomas.

Pulmonary parenchyma also expressed membranous EGFR in epithelial cells but at a very low rate when compared to adenocarcinomas (p=0,0029). Higher expression was verified in solid pattern (p=0,097) and in micropapillary pattern (p=0,0457) when compared to BAC pattern (Fig. 5).

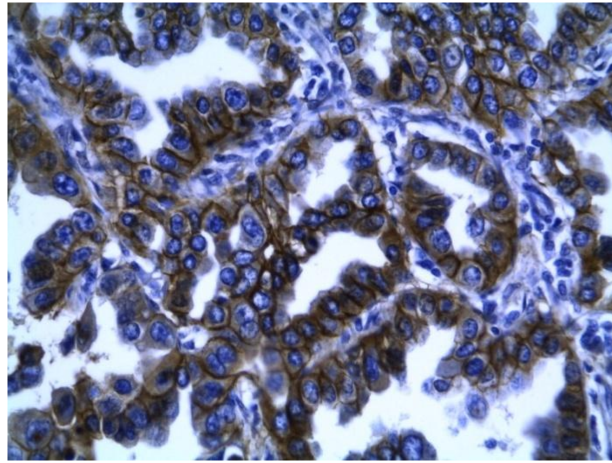

Figure 3-Case13: Intense (3+) EGFR expression; acinar pattern. EGFR, 200x

Considering immunohistochemical expression between primary adenocarcinomas patterns and lymph nodes metastasis there was not statistical significance for CK7, CK20, CK 5,6,18, Chromogranin A, TTF1 and Ki67 proliferative index.

CerbB2 was not expressed as membrane staining in the metastatic (some cytoplasmatic expression was seen). There was no statistical significant differences between primitive patterns in adenocarcinomas and respective metastasis (p=0,3711) for cytoplasmatic stain previously described.

Taking into account membrane expression of EGFR there were no differences between the adenocarcinomas patterns and the lymph node metastasis (p=0,108).

HER2, EGFR and *KRAS* Mutational Status

HER2 mutations

HER2 exon 20 mutation in 31 cases of adenocarcinomas was absent. In all cases, all patterns were wild-type (WT) as the search was made as rigorous as possible after manual microdissection.

EGFR mutations

EGFR exon 21 point mutation occurred in 8 cases of the 31 adenocarcinomas studied: in 6 cases, the mutation was present in all the patterns of the adenocarcinoma, in 2 cases the mutation was present in the acinar pattern and the other patterns were WT (BAC and solid and BAC pattern respectively).

A group of 10 adenocarcinomas showed exon 19 deletions: 7 with mutations in all patterns; one case with mutation in BAC pattern and acinar pattern WT; 1 mixed type adenocarcinoma with BAC pattern mutated and acinar pattern WT.

Delection of 9pb (DEL 9pb) was present in 3 cases, deletion 15pb (DEL 15pb) in 4 cases, DEL 18pb in 1 case and DEL 12pb in one case.

One singular case showed 3 types of deletion, mainly DEL 19pb, 15 and 18pb in the acinar pattern and DEL of 15pb in solid and micropapillary.

In 4 adenocarcinomas, coexisting L858R exon 21 point mutations and exon 19 deletion (DEL 9pb) were seen and in two of these cases, the mutation was present in all patterns (acinar, BAC and solid in one case and acinar in another case). In one case L858R exon 21 point

mutation in acinar and exon 19 deletion (DEL 18pb) in BAC patterns. In another case exon 19 deletion (DEL 9pb) in BAC and L858R exon 21 point mutation in acinar.

Tables 2 and 3 explain the obtained mutational status.

The obtained results showed that for *EGFR* exon 19 mutations were present in the different histological patterns of the same tumor. In cases 9 and 31, BAC, acinar, solid and micropapillary patterns expressed different mutations: in case 9, solid and micropapillary patterns only expressed DEL 15pb, acinar pattern also expressed DEL 9pb and DEL 18pb simultaneously and case 31 expressed differences between BAC and acinar patterns as the first one expressed DEL 9pb and the second one was WT.

In case 27 and again in case 31, BAC pattern was DEL 9pb while the acinar pattern was not determined in case 27 and was WT in case 31.

Table 2 - *EGFR* exon 19 mutations (n.p. – not present this pattern; n.d. – not determined)

CASE	BAC	ACINAR	SOLID	MICROPAPILLARY
8	n.p.	DEL 9 pb	n.p.	n.p.
9	n.p.	DEL 9,15,18 pb	DEL 15 pb	DEL 15 pb
10	n.p.	DEL 9 pb	n.p.	n.p.
13	n.p.	DEL 15 pb	DEL 15 pb	n.p.
17	DEL 12 pb	DEL 12 pb	n.p.	DEL 12 pb
20	n.p.	DEL 15 pb	n.p.	DEL 15 pb
21	DEL 15 pb	DEL 15 pb	n.p.	n.p.
25	DEL 9 pb	DEL 9 pb	n.p.	DEL 9 pb
27	DEL 18 pb	n.d.	n.p.	n.p.
31	DEL 9 pb	WT	n.p.	n.p.

Table 3 - *EGFR* exon 21 mutations (n.p. – not present this pattern)

CASE	BAC	ACINAR	PAPILLARY	SOLID	MICROPAPILLARY
1	L858R	L858R	n.p.	n.p.	n.p.
8	n.p.	L858R	n.p.	n.p.	n.p.
15	n.p.	L858R	L858R	n.p.	n.p.
19	n.p.	L858R	L858R	n.p.	n.p.
24	L858R	L858R	n.p.	n.p.	n.p.
25	L858R	L858R	n.p.	n.p.	L858R
27	n.p.	L858R	n.p.	WT	n.p.
31	n.p.	L858R	n.p.	n.p.	n.p.

After applying The Mann-Whitney Test, we found that patient's age was not statistically related with the existence of *EGFR* mutations (neither *EGFR*-Exon 19 nor *EGFR*-Exon 21 mutations) with p=0,156.

There was statistically relationship between *EGFR* mutations and gender (p=0,001) female respondance (Chi-Square Tests). The estimated risk obtained *Odds ratio for sex*=6,286 (95% Confidence Interval between 1,918 and 20,603) for females.

KRAS Mutations

Of the 31 adenocarcinomas studied 5 cases had codon 2 point mutations, 3 of them of the type G12V (Valine) and 2 of the type G12C (Cystein).

The cases with *KRAS* mutation, 4 had no *EGFR* mutations with the exception of one case that had also exon 21 *EGFR* mutation and these mutations were present in the two patterns (BAC and acinar) of this mixed type adenocarcinoma.

In what concerns to *KRAS* exon 2 wild type, the mean age was 66,35 years with a 95% Confidence Interval for mean between 64,12 and 68,59 years, with a minimum of 50 and a maximum of 80 years for *KRAS* exon 2 mutated, the mean of ages was 62,60 years with a 95% Confidence Interval for mean between 51,16 and 74,04 years and with a minimum of 48 and a maximum of 85 years. After applying the Man Whitney Test, no statistical relevance was found between the relation of *KRAS*-exon 2 and the age of the individuals (for WT and mutated) as *p*=0,401 (Table 4).

Table 4 - *KRAS* exon 2 mutations (n.p.-not present this pattern)

CASE	BAC	ACINAR	PAPILLARY	SOLID
14	n.p.	G12C	G12C	n.p.
18	n.p.	G12V	n.p.	G12V
22	G12V	G12V	n.p.	n.p.
24	G12V (*EGFR* L858R)	G12V (*EGFR* L858R)	n.p.	n.p.
30	G12C	G12C	n.p.	n.p.

Mutations of *KRAS* were statistically related with male gender (*p*=0,000). (Fisher's Exact Test).

The relationship between smoking habits and *EGFR* and *KRAS* mutations was statiscally relevant (*p*=0,004 and *p*=0,014, respectively) with incidence in smokers.

EGFR And *HER2* Copy Number - FISH

For EGFR copy number, 13 FISH positive cases (either high polisomy and amplification Fig.6-7) and 18 FISH negative cases were demonstrated. In the 13 FISH positive cases, 3 were *EGFR* WT and 10 had *EGFR* mutations. On the other hand, of the 18 FISH negative cases, 14 were *EGFR* WT and 4 had *EGFR* mutations with concordance K Test of *k*=0,5412 (moderate agreement).

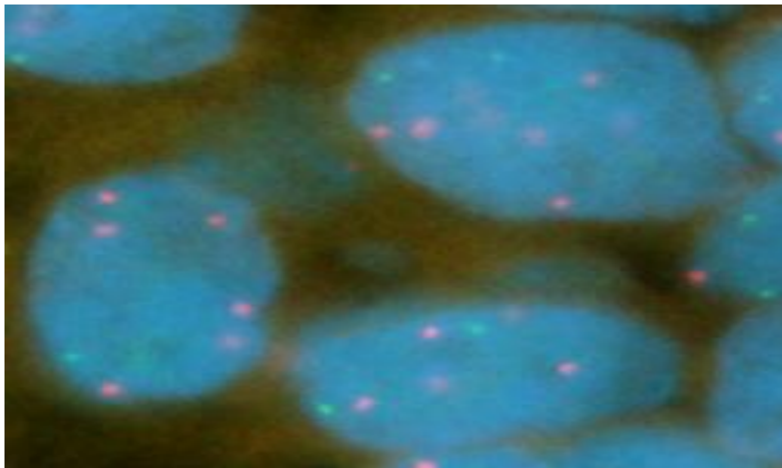

Figure 4- Case 9: FISH positive for EGFR/chromosome 7 centromer. Gene high polisomy; *EGFR* – red signal, chromosome 7-green. 1000x

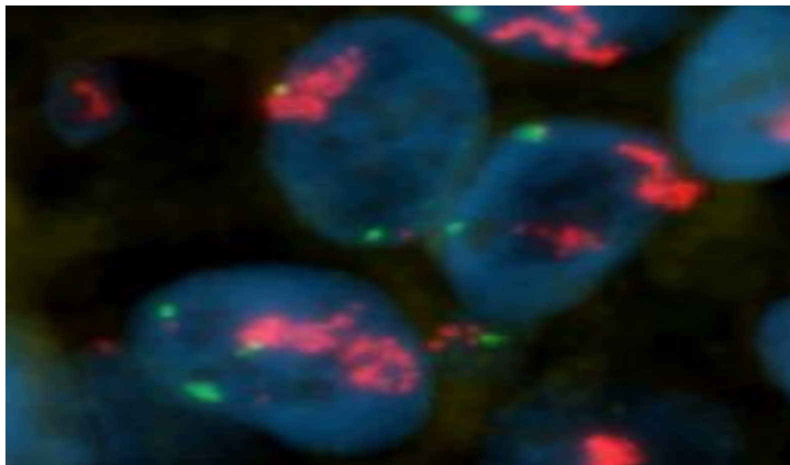

Figure 5- Case 13: EGFR/chromosome 7.Gene amplification; FISH positive . EGFR/chromosome 7 centromer. *EGFR* – red signal, chromosome 7-green. 1000x

When considering the K Test for concordance between FISH results an each mutational type (exons 21 and 19) the concordance was only considered as fair (*k*=0,3702 and *k*=0,3841 respectively).

The concordance (K Test) between mutational status and EGFR immunohistochemical expression (positive/negative) was considered fair (*k*=0,2556). The concordance (K Test) between FISH status and IHC results were also considered as fair (*k*=0,2635).

HER-2 FISH positive (polisomy) cases were identified in 10 cases corresponding mainly to women and 21 cases were *HER-2* FISH negative (Table 5 concises *HER2* and *EGFR* positive cases). It was relevant that FISH negative adenocarcinomas had also FISH negative lymph node metastasis. In FISH positive adenocarcinomas, where lymph nodes metastasis were present, they were FISH negative.

Table 5- *EGFR* and *HER2* FISH Positive Results

CASE	PATTERN	EGFR	RATIO	ESTRAT	HER2	RATIO	ESTRAT
1	BAC	Pos	1,4	HP	Neg	1	Dis
1	Acinar	Pos	1,4	HP	Neg	1	Dis
3	Papillary	Pos	1,3	HP	Pos	1	HP
3	BAC	Neg	1,3	LP	Pos	1,3	HP
8	Solid	Pos	1	HP	Pos	1,2	HP
8	Acinar	Pos	1	HP	Pos	1,2	HP
9	Acinar	Pos	1	HP	Pos	1	HP
9	Solid	Pos	1	HP	Neg	1	BP
9	Micropapillary	Pos	1,6	HP	Pos	1	HP
12	Acinar	Neg	1	Tris	Pos	2	Amp

13	Solid	Pos	2	Amp	Neg	1	Tris
13	Acinar	Pos	2	Amp	Neg	1	Tris
13	Clear Cells	Pos	2	Amp	Neg	1	Tris
15	Papillary	Pos	2	Amp	Pos	1,3	HP
15	Acinar	Pos	2	Amp	Pos	1,3	HP
20	Micropapillary	Pos	1,2	HP	Neg	1	Tris
21	BAC Muc	Pos	1,2	HP	Neg	1	Tris
24	BAC	Pos	1,2	HP	Pos	1,2	HP
24	Acinar	Pos	1,2	HP	Pos	1,3	HP
27	Acinar	Neg	1	Tris	Pos	1,1	HP
27	Clear Cells	Neg	1	Dis	Pos	1,1	HP
28	Clear Cells	Pos	1,4	HP	Pos	1,4	HP
31	BAC	Pos	1,1	HP	Pos	1	HP
31	Acinar	Pos	1,1	HP	Pos	1	HP

Estrat.- Estratification for FISH results; **DIS**- Disomy; **LP**- Low polisomy; **Tris**- Trisomy; **HP**- High polisomy; **AMP**- Amplification

DISCUSSION

The predictive importance of making accurate and more specific diagnosis of bronchial-pulmonary carcinoma, especially differentiating adenocarcinomas from squamous cell carcinoma and other histological types was searched by applying a panel of antibodies (CK7, TTF1, CK 5,6,18, CK20 and Chromogranin A) to establish the diagnose of primary adenocarcinomas towards other pulmonary histological types and metastatic adenocarcinomas, following the still confusing proposed classification for lung adenocarcinoma[32].

All morphological patterns were registered for each adenocarcinoma and revealed negativity for CK 5,6,18 and for Chromogranin A, excluding squamous and neuroendocrine differentiations, as showed by other authors[33-35].

High importance is given to CKs family, formed by more than 30 polypeptides, distributed by tissue and differentiation-specific weights, allowing phenotyping by combining particularly CK7, CK20 and epidermoid/basal cells high weight CKs to determine origin of cell types or tissue[34-40].

All morphological registered patterns comprising this series of lung adenocarcinomas expressed CK7 positivity, without differences ($p>0,05$), and predominant negativity for CK20, like other series published in the literature[32, 42-43]. CK20 was expressed in 3 cases of primary lung adenocarcinoma, with lower expression of CK7 and TTF1[35, 40-41].

Mucinous glandular pattern showed lower expression of CK7 than the other cases and one mucinous pattern had CK20 1+ expression in 10% of the cells and 2 mixed adenocarcinomas showed 2+ expression in 50% of the cells in both acinar and BAC patterns[5, 36]. At this point

it is relevant to make a comment about the proposed classification for lung adenocarcinomas(32). This classification proposes the abolition of the use of BAC – bronchiolo-alveolar carcinoma designation. It is a huge mistake when dealing with classical non-mucinous BAC, because this histological type concerns TRU (terminal respiratory unit) cells, pool of adult stem cells in the lung where pneumocytes type II and Clara cells malignant counterparts are recognized; the proposed lepidic pattern means going back to obscurity as a morphologic description substitutes the meaning of carcinogenesis.

Mucinous pulmonary adenocarcinoma cells exhibiting positivity for intestinal immunohistochemical markers like CK20, MUC2 and COX2, fail of express TTF1(44) and with bronchial origin may comprise either bronchial adenocarcinomas or intestinal type bronchial adenocarcinomas. At this point, the new proposed lung adenocarcinoma classification may contain a future relevant importance by gathering together all mucinous types of bronchial-pulmonary adenocarcinomas under the mucinous adenocarcinoma umbrella, eliminating the BAC mucinous type and again, give life to BAC simply referring to the non-mucinous type. Predictive subtypes will be described to clarify origin and prognosis, between glandular and non-glandular mucinous bronchial-pulmonary adenocarcinomas(45).

Thyroid transcription factor-1 (TTF-1), tissue-specific transcriptional factor, identifies epithelial respiratory cells involved in the regulation of surfactant as pneumocytes type II and Clara cell secretory protein gene expression and distinguishes primary lung adenocarcinoma from metastasis of colorectal cancer. With exception for papillary and solid patterns, all the other patterns had intensive expression suggesting that some patterns showing decreased expression of TTF1 can explain a small number of cases developing in pure CK7 positive (bronchial) cells(36-37).

It is a classical concern of pathologists to report a parameter related with tumoral proliferation index and Ki67-MIB1 as a nuclear proliferation associated antigen expressed in cell cycle (G1,S,G2 and M) but not in the resting phase, G0, provides information about the abortion of active cells in the cell cycle[(46)]. The detection of the expression of Ki-67 by immunohistochemistry can be a prognostic marker allowing the prediction of post operative survival in different types of cancer. High expression of Ki-67 (cut off above 10) is associated with worse survival in adenocarcinomas[(39)]. Solid pattern expressed higher levels of Ki-67 when compared to micropapillary (p=0,0219), acinar (p=0,0731) and mucinous (p=0,068) patterns and we can hypothesize that solid pattern can have a worse biological behavior, either reflecting a particular differentiational origin or as already referred, bronchial development when without expression of either TTF1 and high weight molecular cytokeratines (CK 5,6/ CK5,6,18).

Membrane expression of C-erbB-2 as observed in breast carcinomas is absent in lung adenocarcinomas and has been reported as cytoplasmatic since early 90s, in all morphological patterns. Solid pattern showed lower expression than acinar, papillary and micropapillary patterns raising again a question of predictivity and specific genetic pathways worth to explore.

In the literature, there are numerous references concerning immunohistochemistry and genetic studies in lung adenocarcinomas and specially under the old nomenclature of NSCLC. The importance of this work lies in the fact that we compared IHC between the different patterns of lung adenocarcinomas to identify differences and relevance between them, pionnering the searching *EGFR* and *KRAS* mutations together with *EGFR* and *HER2* copy number also in between the different morphological patterns of lung adenocarcinomas.

In the submitted population we did not find correlations between age and mutation status, namely *EGFR* and *KRAS* mutations. However *EGFR* mutations were more frequent in women and *KRAS* mutations in men. Also, gender and smoking habits were significantly related with *EGFR* mutations[1, 3, 9, 39].

The commonly reported *EGFR* mutation rate by PCR sequencing and fragment analyses in lung adenocarcinomas is around 20% [47]. In our study, we had a rate of *EGFR* mutations of 38,6% gathering all patterns. Female patients had a 61,1% rate and male patients had a 20% rate (5 cases in 25) (*p*=0,001). Female lung cancer patients had a 6 (Odds Ratio=6,286) higher risk of having *EGFR* mutation than male patients Shigematsu and Gazdar, defined an *EGFR* superior mutational rate in women (49%) than in male patients (19%). A reference in this study is made to two another studies, based on Japanese subjects, where over 50% of female patients with lung cancer had higher expression of *EGFR* mutations.

We also tried to find the same relationship for the isolated mutations in exons 19 and 21. For exon 19, female patients had a rate of 44,1% (15 in a total of 28 females) while male patients had a rate of 0% (0 in a total of 16 males), *p*=0,000. For *EGFR* exon 21, we found no significant differences (*p*=0,835) between female and male patients as female lung cancer had a mutation rate of 22,2% while male patients had a rate of 20%. Also, *EGFR* exon 19 mutations were more frequent in women than in men.

EGFR mutations were described as more frequent in never smokers (*p*=0,047) and the same was true in our study where *p*=0,004 (*EGFR* mutation was present in 60% of non-smokers and only in 23,1% of smokers). So, *EGFR* mutations are still associated with female gender and never smoking status. Despite *EGFR* mutations being more prevalent in non-smokers we can not assure that smoking can prevent *EGFR* mutations. These findings only suggest that carcinogens contained in tobacco smoke probably are not correlated with the presence of

mutations in *EGFR* and that it might be caused by another type of carcinogens than higher numbers *EGFR* copies[10].

When comparing *KRAS* exon 2 mutations and gender, we found that male lung cancer had significantly more incidence of mutation than female cases (*p*=0,000). Male patients had a rate of 31% (5 in 16 male patients) while female had a rate of 0% (0 in 28 female patients). Our findings are similar to Kim and al. study, also releasing that *KRAS* mutations are significantly related with smoking habits (*p*=0,014) in a total rate of 30,8% in smokers while in non-smokers it was only 5,7%[10, 39].

FISH *EGFR* and *HER2* copy numbers may be used when DNA quantity is not enough for *EGFR* mutation search and to future use of TKIs for HER2 in women, respectively[3, 9-10, 20, 48-49].

EGFR mutations and *KRAS* mutations have been described as mutually exclusive but we found 1 case of *KRAS* mutations simultaneously with *EGFR* mutations. So we conclude that although *EGFR* and *KRAS* mutations are generally mutually exclusive, in some cases they can coexist. The clinical, therapeutical and prognostic issues concerning this coexistence need to be understood.

Although many studies demonstrated that *EGFR* mutations are usually related with an amplification in *EGFR* locus, the relationship between *EGFR* mutations, gene copy number, and IHC expression is still unclear and without relationship in our study[10, 47-50].

Increased copy number of *EGFR* was present in 40,7% of our cases which is comparable to the 36% already demonstrated. In our study, *EGFR* mutations had 7 times more (Odds Ratio=7,016) probability of having *EGFR* FISH positive (*p*=0,001) than *EGFR* WT. We conclude that *EGFR* mutations are concordant with an increased gene copy number by FISH

(*k*=0,001) and this determination can substitute *EGFR* mutation searching in small biopsies[48-49].

Concordance of the *EGFR* gene copy number by FISH and protein expression by IHC was seen in 13 (37%) of 15 IHC-positive cases[49].

BAC non-mucinous pattern has been described as having mutations of *EGFR* more frequently (*p*=0,008). Comparing our five different patterns (BAC, acinar, papillary, solid and micropapillary), despite not having statistical significance, *EGFR* and *KRAS* mutations were higher for the acinar pattern with a rate of 42,9% and 17,9% respectively with no statistical significance between them, followed by the BAC pattern. If we artificially aggregate BAC and acinar patterns, *EGFR* mutations are more frequent in these two patterns when compared to the other patterns all together[17].

The above referred conclusions are predictive and again raising the controversies explored in the proposed lung adenocarcinomas classification: a small biopsy with a carcinoma CK7 and TTF1 positive is still an adenocarcinoma (CK 5,6 negative) and has to be submitted to *KRAS* mutation search in a male patient, when a solid pattern or BAC (non-mucinous type) pattern are present and *EGFR* copy number may be as suitable as *EGFR* mutational status for TKIs prescription.

Also to summarize, some adenocarcinomas show CK20 positivity in mucinous patterns/subtypes and solid patterns show lower expression of nuclear TTF1 and higher expression of Ki67, reflecting a particular cell origin and more aggressive biological behaviour and have to be reported as lung adenocarcinomas, avoiding poorly differentiated carcinoma reporting.

REFERENCES

1. Kim YT, Kim TY, Lee DS, Park SJ, Park JY, Seo SJ, Choi HS, Kang HJ, Hahn S, Kang CH, Sung SW, Kim JH. *Molecular changes of epidermal growth factor receptor (EGFR) and KRAS and their impact on the clinical outcomes in surgically resected adenocarcinoma of the lung*. Lung Cancer 2008; 59(1): 111-118.
2. Uramoto H, Mitsudomi T. *Which biomarker predicts benefit from EGFR-TKI treatment for patients with lung cancer?* Br J Cancer 2007; 96: 857-863.
3. Shigematsu H, Gazdar AF. *Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers.* Int J Cancer 2006; 118(2): 257-262.
4. Jemal A, Clegg LX, Ward E, Ries LA, Wu X, Jamison PM, Wingo PA, Howe HL, Anderson RN, Edwards BK. *Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival.* Cancer 2004; 101(1): 3-27.
5. Travis WD, Brambillia E, Müller-Hermelink HK, Harris CC, *Pathology Genetics of Tumours of the Lung, Pleura, Thymus and Heart*, ed. W.C.o. Tumours. 2004, Lyon: IARC Press.
6. Sharma SV, Bell DW, Settleman J, Haber DA. *Epidermal growth factor receptor mutations in lung cancer.* Nat Rev Cancer 2007; 7(3): 169-181.
7. Stahel RA. *Adenocarcinoma, a molecular perspective.* Ann Oncol 2007; 18 (9): 147-149.
8. Eberhard DA, Johnson BE, Amler LC, Goddard AD, Heldens SL, Herbst RS, Ince WL, Jänne PA, Januario T, Johnson DH, Klein P, Miller VA, Ostland MA, Ramies DA, Sebisanovic D, Stinson JA, Zhang YR, Seshagiri S, Hillan KJ. *Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib.* J Clin Oncol 2005; 23(25): 5900-5909.

9. Gazdar AF. *Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors.* Oncogene 2009; 28(1): 24-31.

10. Mitsudomi T, Yatabe Y. *Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensivity in lung cancer.* Cancer Sci. 2007; 98(12): 1817-1824.

11. Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA, Murray S. *Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer.* Lancet Oncol 2008: 962-972.

12. Herbst RS, Fukuoka M, Baselga J. *Gefitinib-a novel targeted approach to treating cancer.* Nat Rev Cancer 2004; 4: 956-965.

13. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. *Epidermal growth factor receptor (EGFR) signaling in cancer.* Gene 2006; 366(1): 2-16.

14. Mendelsohn J, Baselga J. *Epidermal growth factor receptor targeting in cancer.* Semin Oncol 2006; 33(4): 369-385.

15. Hynes NE , Lane HA. *ERBB receptors and cancer: the complexity of targeted inhibitors.* Nat Rev Cancer 2005; 5(5): 341-354.

16. Ciardiello F, Tortora G. *EGFR antagonists in cancer treatment.* N Engl J Med 2008; 358(11): 1160-1174.

17. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. *Activating mutations in the epidermal growth factor receptor underlying*

responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350(21): 2129-2139.

18. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. *EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy.* Science 2004; 304(5676): 1497-1500.
19. Mitsudomi T, Kosaka T, Yatabe Y. *Biological and clinical implications of EGFR mutations in lung cancer.* Int J Clin Oncol 2006; 11(3): 190-198.
20. Shigematsu H, Takahashi T, Nomura M, Majmudar K, Suzuki M, Lee H, Wistuba, II, Fong KM, Toyooka S, Shimizu N, Fujisawa T, Minna JD, Gazdar AF. *Somatic mutations of the HER2 kinase domain in lung adenocarcinomas.* Cancer Res 2005; 65(5): 1642-1646.
21. Han SW, Kim TY, Jeon YK, Hwang PG, Im SA, Lee KH, Kim JH, Kim DW, Heo DS, Kim NK, Chung DH, Bang YJ. *Optimization of patient selection for gefitinib in non-small cell lung cancer by combined analysis of epidermal growth factor receptor mutation, K-ras mutation, and Akt phosphorylation.* Clin Cancer Res 2006; 12(8): 2538-2544.
22. Wang SE, Narasanna A, Perez-Torres M, Xiang B, Wu FY, Yang S, Carpenter G, Gazdar AF, Muthuswamy SK, Arteaga CL. *HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors.* Cancer Cell 2006; 10(1): 25-38.
23. Cappuzzo F, Bemis L, Varella-Garcia M. *HER2 mutation and response to trastuzumab therapy in non-small-cell lung cancer.* N Engl J Med 2006; 354(24): 2619-2621.

24. Rodenhuis S, van de Wetering ML, Mooi WJ, Evers SG, van Zandwijk N, Bos JL. *Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung.* N Engl J Med 1987; 317(15): 929-935.

25. Suda K, Tomizawa K, Mitsudomi T. *Biological and clinical significance of KRAS mutations in lung cancer: an oncogenic driver that contrasts with EGFR mutation.* Cancer Metastasis Rev 2010; 29: 49-60.

26. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. *Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications.* Cancer Res 2004; 64(24): 8919-8923.

27. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba, II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF. *Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers.* J Natl Cancer Inst 2005; 97(5): 339-346.

28. Endoh H, Yatabe Y, Kosaka T, Kuwano H, Mitsudomi T. *PTEN and PIK3CA expression is associated with prolonged survival after gefitinib treatment in EGFR-mutated lung cancer patients.* J Thorac Oncol 2006; 7: 629-634.

29. Varella-Garcia M, Diebold J, Eberhard DA, Geenen K, Hirschmann A, Kockx M, Nagelmeier I, Ruschoff J, Schmitt M, Arbogast S, Cappuzzo F. *EGFR fluorescence in situ hybridisation assay: guidelines for application to non-small-cell lung cancer.* J Clin Pathol 2009; 62(11): 970-977.

30. Inoue A, Suzuki T, Fukuhara T, Maemondo M, Kimura Y, Morikawa N, Watanabe H, Saijo Y, Nukiwa T. *Prospective phase II study of gefitinib for chemotherapy-naive patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations.* J Clin Oncol 2006; 24(21): 3340-3346.

31. Yoshida K, Yatabe Y, Park JY, Shimizu J, Horio Y, Matsuo K, Kosaka T, Mitsudomi T, Hida T. *Prospective validation for prediction of gefitinib sensitivity by epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer.* J Thorac Oncol 2007; 2(1): 22-28.

32. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, Beer DG, Powell CA, Riely GJ, Schil PE, Garg K, Austin JH, Asamura H, Rusch VW, Hirsch FR, Scagliotti G, Mitsudomi T, Huber RM, Ishikawa Y, Jett J, Sanchez-Cespedes M, Sculier JP, Takahashi T, Tsuboi M, Vansteenkiste J, Wistuba I, Yang PC, Aberle D, Brambilla C, Flieder D, Franklin W, Gazdar A, Gould M, Hasleton P, Henderson D, Johnson B, Johnson D, Kerr K, Kuriyama K, Lee JS, Miller VA, Petersen I, Roggli V, Rosell R, Saijo N, Thunnissen E, Tsao M, Yankelewitz D. *International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma.* J Thorac Oncol 2011; 6(2): 244-285.

33. Heatley M, Maxwell P, Whiteside C, Toner P. *Cytokeratin intermediate filament expression in benign and malignant breast disease.* J Clin Pathol 1995; 48(1): 26-32.

34. Obermuller S, Calegari F, King A, Lindqvist A, Lundquist I, Salehi A, Francolini M, Rosa P, Rorsman P, Huttner WB, Barg S. *Defective secretion of islet hormones in chromogranin-B deficient mice.* PLoS One 2010; 5(1): e8936.

35. Pelosi G, Pasini F, Sonzogni A, Maffini F, Maisonneuve P, Iannucci A, Terzi A, De Manzoni G, Bresaola E, Viale G. *Prognostic implications of neuroendocrine differentiation and hormone production in patients with Stage I nonsmall cell lung carcinoma.* Cancer 2003; 97(10): 2487-2497.

36. Ikeda S, Fujimori M, Shibata S, Okajima M, Ishizaki Y, Kurihara T, Miyata Y, Iseki M, Shimizu Y, Tokumoto N, Ozaki S, Asahara T. *Combined immunohistochemistry of*

beta-catenin, cytokeratin 7, and cytokeratin 20 is useful in discriminating primary lung adenocarcinomas from metastatic colorectal cancer. BMC Cancer 2006; 6: 31.

37. Kubba LA, McCluggage WG, Liu J, Malpica A, Euscher ED, Silva EG, Deavers MT. *Thyroid transcription factor-1 expression in ovarian epithelial neoplasms.* Mod Pathol 2008; 21(4): 485-490.

38. Leech SN, Kolar AJ, Barrett PD, Sinclair SA, Leonard N. *Merkel cell carcinoma can be distinguished from metastatic small cell carcinoma using antibodies to cytokeratin 20 and thyroid transcription factor 1.* J Clin Pathol 2001; 54(9): 727-729.

39. Woo T, Okudela K, Yazawa T, Wada N, Ogawa N, Ishiwa N, Tajiri M, Rino Y, Kitamura H, Masuda M. *Prognostic value of KRAS mutations and Ki-67 expression in stage I lung adenocarcinomas.* Lung Cancer 2009; 65(3): 355-362.

40. Ikeda S, Fujimori M, Shibata S, Okajima M, Ishizaki Y, Kurihara T, Miyata Y, Iseki M, Shimizu Y, Tokumoto N, Ozaki S, Asahara T. *Combined immunohistochemistry of beta-catenin, cytokeratin 7, and cytokeratin 20 is useful in discriminating primary lung adenocarcinomas from metastatic colorectal cancer.* BMC Cancer 2006; 2(31): 6.

41. Scholzen T, Gerdes J. *The Ki-67 protein: from the known and the unknown.* J Cell Physiol 2000; 182(3): 311-322.

42. Tsao SC, Su YC, Wang SL, Chai CY. *Use of caveolin-1, thyroid transcription factor-1, and cytokeratins 7 and 20 in discriminating between primary and secondary pulmonary adenocarcinoma from breast or colonic origin.* Kaohsiung J Med Sci 2007; 23(7): 325-331.

43. Su YC, Hsu YC, Chai CY. *Role of TTF-1, CK20, and CK7 immunohistochemistry for diagnosis of primary and secondary lung adenocarcinoma.* Kaohsiung J Med Sci 2006; 22(1): 14-19.

44. Tsuta K, Ishii G, Yoh K, Nitadori J, Hasebe T, Nishiwaki Y, Endoh Y, Kodama T, Nagai K, Ochiai A. *Primary lung carcinoma with signet-ring cell carcinoma components: clinicopathological analysis of 39 cases.* Am J Surg Pathol 2004; 28(7): 868-874.

45. Rossi G, Murer B, Cavazza A, Losi L, Natali P, Marchioni A, Migaldi M, Capitanio G, Brambilla E. *Primary mucinous (so-called colloid) carcinomas of the lung: a clinicopathologic and immunohistochemical study with special reference to CDX-2 homeobox gene and MUC2 expression.* Am J Surg Pathol 2004; 28(4): 442-452.

46. Neves LR, Oshima CT, Artigiani-Neto R, Yanaguibashi G, Lourenco LG, Forones NM. *Ki67 and p53 in gastrointestinal stromal tumors-GIST.* Arq Gastroenterol 2009; 46(2): 116-120.

47. El-Zammar OA, Zhang S, Katzenstein AL. *Comparison of FISH, PCR, and immunohistochemistry in assessing EGFR status in lung adenocarcinoma and correlation with clinicopathologic features.* Diagn Mol Pathol 2009; 18(3): 133-137.

48. Amann J, Kalyankrishna S, Massion PP, Ohm JE, Girard L, Shigematsu H, Peyton M, Juroske D, Huang Y, Stuart Salmon J, Kim YH, Pollack JR, Yanagisawa K, Gazdar A, Minna JD, Kurie JM, Carbone DP. *Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer.* Cancer Res 2005; 65(1): 226-235.

49. Takano T, Ohe Y, Sakamoto H, Tsuta K, Matsuno Y, Tateishi U, Yamamoto S, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Shibata T, Sakiyama T, Yoshida T, Tamura T. *Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer.* J Clin Oncol 2005; 23(28): 6829-6837.

50. Li AR, Chitale D, Riely GJ, Pao W, Miller VA, Zakowski MF, Rusch V, Kris MG, Ladanyi M. *EGFR mutations in lung adenocarcinomas: clinical testing experience and relationship to EGFR gene copy number and immunohistochemical expression.* J Mol Diagn 2008; 10(3): 242-248.