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ORIGINAL ARTICLE

Lung adenocarcinoma: Sustained subtyping with immunohistochemistry and EGFR, HER2 and KRAS mutational status



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Abstract Pulmonary adenocarcinomas are still in the process of achieving morphological, immunohistochemical and genetic standardization. The ATS/ERS/IASLC proposed classification for lung adenocarcinomas supports the value of the identification of histological patterns, specifically in biopsies.

Thirty pulmonary adenocarcinomas were subjected to immunohistochemical study (CK7, CK5, 6, 18, CK20, TTF1, CD56, HER2, EGFR and Ki-67), FISH and PCR followed by sequencing and fragment analysis for *EGFR*, *HER2* and *KRAS*.

Solid pattern showed lower TTF1 and higher Ki-67 expression. TTF1 expression was higher in non-mucinous lepidic and micropapillary patterns when compared to acinar and solid and acinar, solid and mucinous respectively. Higher Ki67 expression was present in lepidic and solid patterns compared to mucinous. EGFR membranous staining had increasing expression from non-mucinous lepidic/BA pattern to solid pattern and micropapillary until acinar pattern. *EGFR* mutations, mainly in exon 19, were more frequent in females, together with non-smoking status, while *KRAS* exon 2 mutations were statistically more frequent in males, especially in solid pattern. FISH *EGFR* copy was correlated gross, with mutations. *HER2* copy number was raised in female tumours without mutations, in all cases. Although *EGFR* and *KRAS* mutations are generally considered mutually exclusive, in rare cases they can coexist as it happened in one of this series, and was represented in acinar pattern with rates of 42.9% and 17.9%, respectively. *EGFR* mutations were more frequent in lepidic/BA and acinar patterns. Some cases showed different *EGFR* mutations.

The differences identified between the adenocarcinoma patterns reinforce the need to carefully identify the patterns present, with implications in diagnosis and in pathogenic understanding. *EGFR* and *KRAS* mutational status can be determined in biopsies representing bronchial

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pulmonary carcinomas because when a mutation is present it is generally present in all the histological patterns.

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Introduction

There are 1.3 million deaths from lung cancer annually worldwide and it is the leading cause of cancer-related mortality in USA, Japan and Western-countries.^{1–4} Bronchial-pulmonary carcinomas were classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), representing 13% and 85% of lung cancers respectively. Squamous cell carcinoma (SQCC) and adenocarcinoma (ADC) subdivision become mandatory due to personalized therapy and NSCLC designation should not be reported.^{5,6}

Bronchial-pulmonary adenocarcinomas are malignant epithelial tumours with glandular differentiation, and/or mucin production, acinar, papillary, micropapillary, bronchioloalveolar (BA)/lepidic, or solid with mucin growth patterns, complementing the morphological spectrum of mixed-type adenocarcinoma. The incidence differs with gender and population, being roughly 28% in men and 42% in women. Recently a new classification was proposed by ATS/ERS/IASLC. This new classification recognizes several patterns such as lepidic instead of BA, acinar, papillary, solid, and micropapillary. It also recognizes mucinous adenocarcinomas including the former mucinous BA carcinoma and colloid carcinoma. It also highlighted the need for an assertive diagnosis specially in biopsy material, supported by immunohistochemical study, with clinical, prognostic and therapeutic implications.^{5–8}

The transmembrane tyrosine kinase epidermal growth factor receptor (*EGFR*) belonging to the *EGFR* family of receptor tyrosine kinases (TKs) called the *HER* or *ErbB* family (consisting of four members – *EGFR* (*HER1/ErbB1*), *HER2* (*ErbB2*), *HER3* (*ErbB3*) and *HER4* (*ErbB4*))^{9,10} may be over-expressed and this correlates with poor prognosis, with aggressive disease and decreased survival.^{3,11–14}

KRAS mutations are associated with poor prognosis, reported since 1990, occurring in codon 12, occasionally at codon 13 and rarely at codon 61.^{10,15,16} According to the current data, *EGFR* and *KRAS* mutations are mutually exclusive^{10,17,18}; the explanation is related to *KRAS*-*MAPK* pathway inserts in the downstream signalling pathway of *EGF*.¹⁰ This mutation appears in 30% of Caucasian patients with lung cancer and 10% of East Asian adenocarcinomas.^{16,18,19} *KRAS* mutations are more frequent in smokers and are related to poor prognosis.^{10,16,17} 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.^{10,20}

Due to what has been said, the *EGFR*-*MAPK* signal transduction pathway is important to understand the role of individual somatic changes in tumours, predicting the response to *EGFR*-TKIs. *EGFR* status is then a favourable

predictive factor in the case of sensitizing mutations. Despite the positive response observed in up to 70% of patients, different data concluded that not every patient benefitted 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 as opposed to 20% in NSCLC with wild-type *KRAS*.

The objectives of this work are to evaluate *EGFR* and *KRAS* mutational status, *EGFR* and *HER2* gene copy number and immunohistochemical *EGFR* and *HER2* expression in lung adenocarcinomas according to the patterns/subtypes present, in order to understand the value of pattern recognition, supported by an immunohistochemical set used in routine, in adenocarcinoma diagnosis and anti-*EGFR* therapy decision.

Materials and methods

Materials

A series of 30 bronchial-pulmonary adenocarcinomas classified according with WHO 2004 histological classification and the new ATS/ERS/IASLC classification were selected from the archive of the Pathology Service of Coimbra University Hospital.

The patterns present were registered (namely lepidic/BA, acinar, papillary, micropapillary, solid and mucinous). Metastases were also registered by patterns. Clinical data like age, gender, smoking habits and stage were also registered.

Ethical standards

The principles of Helsinki Declaration were respected and the study was developed according to the Faculty of Medicine of the University of Coimbra.

Ethical Committee rules for PhD theses were followed.

Immunohistochemistry (IHC)

Representative sections of the adenocarcinomas and their patterns were submitted to IHC (CK7, TTF1, CK5/6, CD56 and CK20) to validate the pure condition of bronchial-pulmonary adenocarcinomas mainly to validate solid pattern. Ki67-MIB1 antibody was used to characterize proliferation index. C-erbB-1/*EGFR* and c-erbB-2/*HER2* were applied to evaluate protein expression of these

molecules. Endogenous peroxidase activity was quenched using 15 min incubation in 3% diluted hydrogen peroxide (H_2O_2). For blocking nonspecific binding with primary antibodies we used Ultra V Block (Ultra Vision Kit; TP-125-UB; Lab Vision Corporation; Fremont CA; USA). Primary antibodies against CK7 (clone OV-TL12/30; DakoCytomation, Glostrup, Denmark) at a dilution of 1/50 for 30 min, Cytokeratin 5,6,18 (clone LP34; Novocastra Laboratories Ltd, Newcastle, United Kingdom) at a dilution of 1/100 for 60 min, TTF1 (clone 8G7G3/1; DakoCytomation, Glostrup, Denmark) at a dilution of 1/100 for 60 min, CD56 (clone CD564; Novocastra Laboratories Ltd, Newcastle, United Kingdom) at a dilution of 1:75 for 60 min, CK20 (clone KS20.8; DakoCytomation, Glostrup, Denmark) at a dilution of 1/50 for 30 min, Ki67 (clone MIB-1; DakoCytomation, Glostrup, Denmark) at a dilution of 1/50 for 30 min, c-erbB-2 (Polyclonal; DakoCytomation, Glostrup, Denmark) at a dilution of 1/200 for 30 min, and c-erbB-1 (clone 31G7; Invitrogen, Camarillo, California, USA) at a dilution of 1:20 for 30 min were applied to the cells and incubated at room temperature. They were washed with phosphate-buffered saline (PBS) (Ultra Vision; TP-125-PB; Lab Vision Corporation; Fremont CA; USA); and after this, for 15 min, slides were incubated with biotin-labelled secondary antibody (Ultra Vision Kit; TP-125-BN; Lab Vision Corporation; Fremont CA; USA). Primary antibody binding was localized in tissues using peroxidase-conjugated streptavidin (Ultra Vision Kit; TP-125-HR; Lab Vision Corporation; Fremont CA; USA) and 3,3-diaminobenzidine tetrahydrochloride (DAB) (RE7190-K; Novocastra Laboratories Ltd, Newcastle, United Kingdom) was used as chromogen, according to manufacturer's instructions. Pretreatment was done with Pronase, 10' for CK7, CK5,6,18, CK20 and c-erbB-1, with MW -micro wave, PH6, 20' for Ki67 and c-erbB-2 and with MW, EDTA, 40' for TTF1 and CD56. Haematoxylin was used to counterstain the slides which were then dehydrated and mounted. In parallel, known positive and negative controls were used.

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 tumour sections of 4 μ m thickness, baked overnight at 56 °C, deparaffinized in xylol, rehydrated in 100%, 70% ethanol and bidistilled water. A pressure cooker with 10 mM citric acid-trisodium salt buffer pH 6, for 4 min, was used to submit slides to a pre-treatment. They were washed in 2 \times SSC salts (sodium chloride and sodium citrate) pH 7 for 5 min at room temperature. At 15 min slides were immersed in proteinase K solution at 37 °C and then, they were rinsed in 2 \times SSC pH 7 for 5 min at room temperature. The slides were then dehydrated in 70%, 90% and 100% ethanol, and then air dried. Ten microliters of probe mixture were applied on the target

areas and a 22 mm \times 22 mm glass coverslip was placed over probe.

After being sealed with rubber cement and codenaturation at 83 °C for 5 min, coverslips were incubated overnight at 37 °C in a humidity chamber. Post-hybridization they had washes in buffer (50% formamide 2 \times SCC pH 7) at 46 °C and were also washed with 2 \times SCC pH 7. Slides were air-dried in the dark and counterstained with DAPI.

FISH was used to analyze the chromosome 7 and *EGFR* gene, and they were scored according to Cappuzzo's (2005) method. Positive FISH cases showed high polysomy or amplification and the same procedure was followed to *HER-2* probe (*HER-2/Neu* (17q12)/SE17; Kreatech diagnostics; Amsterdam). Positive and negative FISH cases were according to Varella-Garcia et al.²¹

The microscopic analyses were done in a Nikon Eclipse 80i of brilliant field and epifluorescent microscope (LUCIA cytogenetics software). Images were captured and registered with a digital camera (Nikon DXM 1220F), in monochromatic images/layers posterior joint in one single image. This process was assisted by Nikon ACT-1 capture software.

Overlapping 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.

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

PCR, sequencing and fragment analysis

Genomic DNA was extracted from 5 μ m section of paraffin-embedded tissue after manual microdissection of all independent patterns separation as supported in Table 1. For that, the QIAmp DNA Mini Kit (Qiagen, IZAZA, Germany), was used. One hundred nanograms (ng) of DNA were amplified in a 50 μ l reaction solution containing 5 μ l of 10 \times buffer (Roche, Germany), 2.5 mM $MgCl_2$, 0.2 μ M of each complementary primer, 200 μ M deoxynucleoside triphosphate and one unit of DNA polymerase (Roche, Germany). A 5 min initial denaturation at 95 °C was used to perform the amplifications; this was followed by 40 cycles, 30 s at 95 °C, 1 min at 60 °C (for exon 19) or 57 °C (for exon 21), 1 min at 72 °C and 10 min of final extension at 72 °C.

The *EGFR* gene mutations located at exons 19 and 21 were determined using the intron-based primers according to the published method.²² Mutational analysis of exon 19 deletion L858R point mutation of the *EGFR* gene was explored, as described.²³ The determination of exon 19 deletion was made by common fragment analysis using PCR with an FAM-labelled primer set, and the products were electrophoresed on ABI PRISM 3100 (Applied Biosystems®) and all eletrograms were reanalyzed by visual inspection in order to check for mutations. To evaluate the L858R mutation MyCycler (Bio-Rad) was also used 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 min initial denaturation at 95 °C, followed by 40 cycles, 30 s at 95 °C,

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, Lepidic/BA	
2	F	71	Non-smoker	T1a	N0	Mx	IA	Lepidic/BA, Acinar	
3	M	69	Smoker	T4	N0	MX	IIIA	Lepidic/BA, 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, Lepidic/BA	
6	F	62	Ex-smoker	T1a	N2	Mx	IIIA	Acinar, Solid	Acinar, Solid
7	F	74	Non-smoker	T1a	N0	Mx	IB	Acinar, Solid, Lepidic/BA	
8	F	68	Non-smoker	T2a	N0	Mx	IB	Acinar, Solid, Micropapillary Mucinous	
9	F	50	Non-smoker	T1b	N2	Mx	IIIA	Acinar, Solid	Acinar
10	F	57	Smoker	T2a	N0	Mx	IB	Acinar, Solid	
11	M	51	Non-smoker	T1a	N1	Mx	IIA	Acinar, Lepidic/BA	Acinar. Solid
12	F	75	Non-smoker	T2a	N0	Mx	IB	Acinar, Solid, Micropapillary	
13	M	85	Ex-smoker	T1a	N0	Mx	IA	Acinar, Lepidic/BA Papillary	
14	F	63	Smoker	T1a	N2	Mx	IB	Acinar, Lepidic/BA Micropapillary, Papillary	Acinar, Solid
15	F	55	Smoker	T1a	N0	Mx	IA	Acinar, solid	
16	F	67	Smoker	T1a	N0	Mx	IA	Lepidic/BA, Acinar, Micropapillary	
17	M	48	Smoker	T1a	N0	Mx	IA	Acinar, Solid, Lepidic/BA	
18	M	71	Non-smoker	T2a	N1	Mx	IIA	Acinar, Papillary, Micropapillary	Acinar
19	F	80	Non-smoker	T1b	N0	Mx	IA	Acinar, Micropapillary	
20	F	68	Non-smoker	T2a	N1	Mx	IIA	Acinar, Lepidic/BA Micropapillary	Acinar, Micropapillary
21	M	48	Non-smoker	T2b	N1	MX	IIA	Lepidic/BA, Acinar,	Acinar
22	F	67	Non-smoker	T2a	N0	Mx	IB	Acinar, Mucinous	
23	M	76	Ex-smoker	T1b	N0	Mx	IA	Lepidic/BA, Acinar, Mucinous	
24	F	56	Non-smoker	T1a	N0	MX	IA	Lepidic/BA, Acinar, Micropapillary, Papillary	
25	M	64	Smoker	T2b	N0	Mx	IIA	Acinar, Papillary	
26	F	71	Non-smoker	T2a	N0	MX	IB	Lepidic/BA, Acinar, Solid	
27	F	50	Smoker	T2a	N0	MX	IB	Lepidic/BA, Solid	
28	F	80	Non-smoker	T1a	N0	Mx	IA	Solid	
29	M	56	Smoker	T1a	N0	MX	IA	Lepidic/BA, Acinar	
30	F	56	Non-smoker	T1a	N0	Mx	IA	Lepidic/BA, Acinar	

1 min at 53 °C, 1 min at 72 °C and a 10 min of final extension at 72 °C.

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

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 standard 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. Immunohistochemistry, FISH and Mutational (PCR) results' concordances were calculated by kappa coefficient. Correlation coefficient *r* was also calculated (Spearman test). The comparison between the existence of different types of mutation and age was realized with Mann-Whitney's test. A significance level of 5% ($p \leq 0.05$) was considered.

Results

Patterns and clinical data

Table 1 shows 29 cases of mixed-type adenocarcinomas with registration of decreasing percentage of the identified

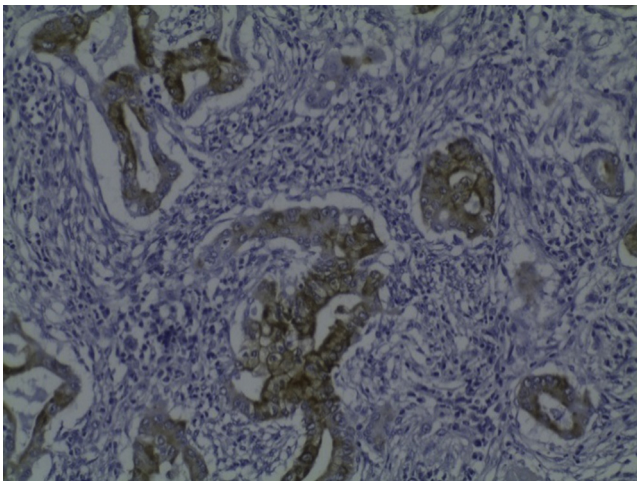


Figure 1 Case 21: 2+ CK20 expression; acinar pattern. 200 \times .

patterns: 28 (93.4%) cases with acinar pattern, 18 (60%) cases with non-mucinous lepidic/BA pattern, 12 (40%) cases with solid pattern, 8 (26.7%) cases with micropapillary pattern, 6 (20%) cases with papillary pattern and 3 (10%) cases with mucinous pattern. Case 28 was the only where only one pattern was present (solid pattern). Two patterns were represented in 16 cases (7 cases had acinar and lepidic/BA non-mucinous patterns, 5 cases had acinar and solid patterns, 1 case had acinar and papillary patterns, 1 case had acinar and micropapillary patterns, 1 case had acinar and mucinous glandular patterns and 1 case had lepidic/BA and solid patterns); 13 cases showed 3 or more histological patterns and of these, the most frequent association was acinar, lepidic/BA non-mucinous and solid patterns ($n=3$), followed by acinar, lepidic/BA non-mucinous and micropapillary patterns ($n=2$), acinar, lepidic/BA non-mucinous and papillary patterns ($n=2$) and acinar, lepidic/BA non-mucinous, papillary and micropapillary patterns ($n=2$).

In this series, 19 (63.3%) cases belonged to female gender, 11 (36.7%) cases to masculine gender, 18 (60%) cases had no smoking habits, 9 (30%) cases belonged to smoker patients and 3 (10%) cases to ex-smoker patients (Table 1); of 18 non-smoking patients (admitting passive smoking), there were 13 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).

Immunohistochemistry

All histological types and patterns identified were CK 5,6,18 and CD56 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 antibodies.

As with normal parenchyma, equivalent results were seen after the application of CK20 except: in two cases of mixed-type adenocarcinoma, where acinar and lepidic/BA patterns had a 2+ expression (moderate intensity) in 50% of the cells; in a third expression (moderate intensity) in 50% of the cells; 2 weak expression (1+) in 10% of the neoplastic cells (Fig. 1). These three cases expressed CK7 3+ and TTF1 3+ (Fig. 1). Lymph node metastasis had no CK20 expression.

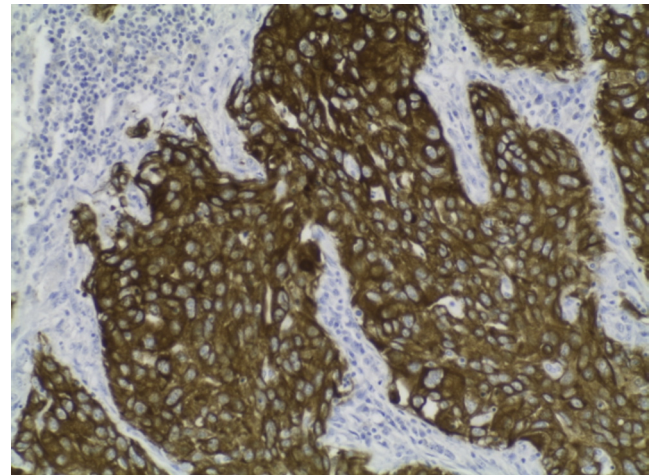


Figure 2 Case 6: CK7 intense expression (3+) in solid pattern. 200 \times .

Mucinous pattern showed lower expression of CK7 than normal pulmonary epithelial cells and respiratory cylindrical epithelial cells ($p=0.09$); acinar pattern ($p=0.0584$), lepidic/BA 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).

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

TTF1 expression was positive in normal alveolar septae cells as expected; it was negative in 6 acinar pattern cases, in 2 of the non-mucinous lepidic/BA pattern cases, in 1 papillary pattern case and in 2 solid pattern cases. Overall expression, taking into account the intensity and the percentage of positive cells, for TTF1 higher expression was seen in lepidic/BA 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 ($p=0.0192$). There were no differences between normal tissue and papillary pattern ($p=0.315$) and lepidic/BA pattern had higher expression than acinar pattern ($p=0.059$) and solid pattern ($p=0.0115$). Micropapillary pattern had higher expression when compared to acinar ($p=0.0413$), solid ($p=0.009$) and mucinous ($p=0.0225$) patterns.

Adenocarcinomas showed higher proliferative index, validated by Ki67 expression, than normal tissues ($p < 0.05$). Ki67 expression was also higher in lepidic/BA (non-mucinous) 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 cases of the studied adenocarcinomas. Cytoplasmic expression was seen in all patterns and in normal tissues adjacent to the neoplasias, with significant statistical differences between alveolar septae and acinar patterns ($p < 0.0001$), lepidic/BA pattern ($p < 0.0001$), papillary pattern ($p=0.004$), micropapillary pattern ($p < 0.0001$), solid pattern ($p=0.0006$) and mucinous pattern ($p=0.0073$).

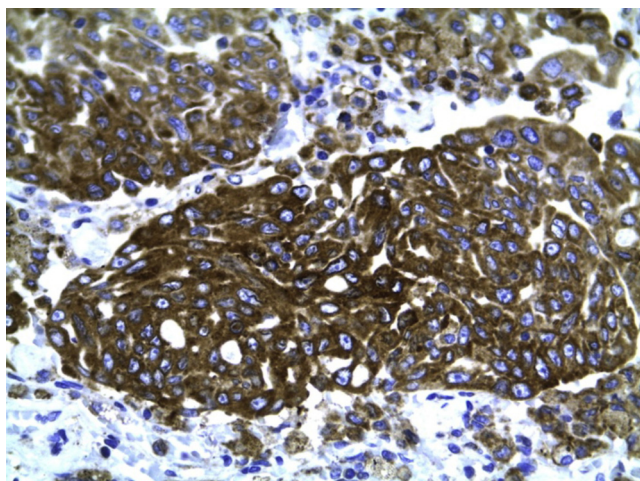


Figure 3 Case 7: ErbB-2 3+ cytoplasmatic expression; solid pattern. ErbB-2, 400 \times .

Solid pattern had lower expression than acinar ($p=0.0034$), lepidic/BA ($p=0.004$), papillary ($p=0.0334$) and micropapillary patterns ($p=0.0089$) (Figs. 3 and 4).

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+. Non-mucinous lepidic/BA pattern showed 2+ membrane expression in 2 cases and 3+ in one case. Solid pattern showed 2 cases with 2+ positivity and 3 cases with 3+ membrane expressions. 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 lepidic/BA pattern (Fig. 5).

Considering immunohistochemical expression between primary adenocarcinomas patterns and lymph node

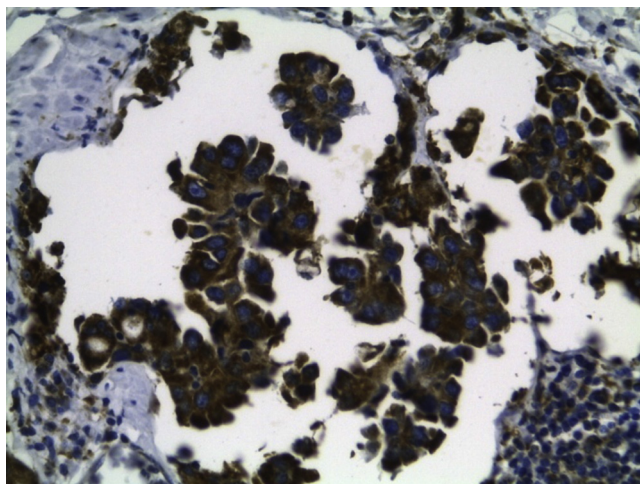


Figure 4 Case 8: ErbB-2 3+ cytoplasmatic expression; micropapillary pattern. ErbB-2, 400 \times .

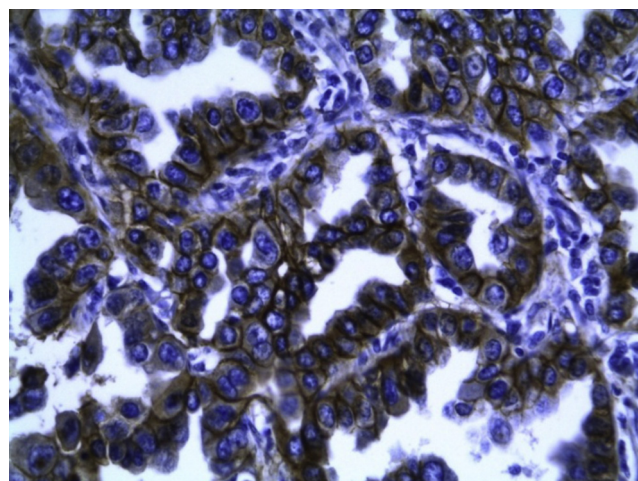


Figure 5 Case 12: Intense (3+) EGFR expression; acinar pattern. EGFR, 200 \times .

metastasis there was no statistical significance for CK7, CK20, CK 5,6,18, CD56, TTF1 and Ki67 proliferative index.

CerbB2 was not expressed as membrane staining in the metastatic (some cytoplasmatic expression was seen) malignant cells. There were no statistical significant differences between primitive patterns in adenocarcinomas and respective metastasis ($p=0.3711$) for the 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 was absent in 30 cases of adenocarcinomas. All the patterns were wild-type (WT).

EGFR mutations

EGFR exon 21 point mutation occurred in 8 cases of the 30 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 (lepidic/BA and solid and lepidic/BA patterns, respectively).

A group of 10 adenocarcinomas showed exon 19 deletions: 6 with mutations in all patterns; one case with mutation in lepidic/BA pattern and acinar pattern WT; the remaining 3 cases had mutations present in one pattern (namely two cases in acinar and one case in lepidic/BA patterns).

EGFR mutations were present in 42.9% of acinar pattern. EGFR mutations were more frequent in lepidic/BA and acinar patterns ($p=0.008$).

Deletion of 9pb (DEL 9pb) was present in 5 cases, deletion 15pb (DEL 15pb) in 4 cases, DEL 18pb in 2 cases and DEL 12pb in one case.

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

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

Case	Lepidic/BA	Acinar	Solid	Micropapillary	Mucinous
7	n.d.	DEL 9 pb	n.d.	n.p.	n.p.
8	n.p.	DEL 9, 15, 18 pb	DEL 15 pb	DEL 15 pb	n.d.
9	n.p.	DEL 9 pb	n.d.	n.p.	n.p.
12	n.p.	DEL 15 pb	DEL 15 pb	n.d.	n.p.
16	DEL 12 pb	DEL 12 pb	n.p.	DEL 12 pb	n.p.
19	n.p.	DEL 15 pb	n.p.	DEL 15 pb	n.p.
20	DEL 15 pb	DEL 15 pb	n.p.	n.p.	n.p.
24	DEL 9 pb	DEL 9 pb	n.p.	DEL 9 pb	n.p.
26	DEL 18 pb	n.d.	n.p.	n.p.	n.p.
30	DEL 9 pb	WT	n.p.	n.p.	n.p.

Table 3 *EGFR* exon 21 mutations (n.p. – not present in pattern; n.d. – not determined).

Case	Lepidic/BA	Acinar	Papillary	Solid	Micropapillary	Mucinous
1	L858R	L858R	n.p.	n.p.	n.p.	n.p.
7	n.d.	L858R	n.p.	n.d.	n.p.	n.p.
14	n.d.	L858R	L858R	n.p.	n.d.	n.p.
18	n.p.	L858R	L858R	n.p.	n.d.	n.p.
23	L858R	L858R	n.p.	n.p.	n.p.	n.d.
24	L858R	L858R	n.d.	n.p.	L858R	n.p.
26	WT	L858R	n.p.	WT	n.p.	n.p.
30	WT	L858R	n.p.	n.p.	n.p.	n.p.

In 4 adenocarcinomas, coexisting L858R exon 21 point mutations and exon 19 deletions were seen. In two of these cases the L858R and Del9pb mutations were present in all patterns (acinar, lepidic/BA and solid patterns in one case and in acinar pattern in another case). In one case, L858R exon 21 point mutation was seen in acinar pattern and exon 19 deletion (DEL 18pb) in lepidic/BA pattern. In another case exon 19 deletion (DEL 9pb) in lepidic/BA pattern and L858R exon 21 point mutation in acinar pattern were observed. [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 tumour. In cases 8 and 30, lepidic/BA, acinar, solid and micropapillary patterns expressed different mutations: in case 8, solid and micropapillary patterns only expressed DEL 15pb, acinar pattern also expressed DEL 9pb and DEL 18pb simultaneously and case 30 expressed differences between lepidic/BA and acinar patterns, as the first one expressed DEL 9pb and the second one was WT.

In case 26, lepidic/BA pattern had DEL 18pb while the acinar pattern was undetermined.

After applying the Mann-Whitney test, patient's age was not statistically related with the existence of *EGFR* mutations (neither *EGFR*-Exon 19 nor *EGFR*-Exon 21 mutations) with $p=0.156$. *EGFR* mutations were more frequent in female patients ($p=0.004$).

There was a statistical relationship between *EGFR* mutations and female gender ($p=0.001$) (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 30 adenocarcinomas studied 5 cases had exon 2 point mutations, 3 of them of the type G12 V (Valine) and 2 of the type G12C (Cysteine).

For 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 (lepidic/BA and acinar) of this mixed-type adenocarcinoma.

In relation 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 age 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 Mann-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](#)).

KRAS mutations were statistically related with male gender ($p=0.001$). (Fisher's Exact Test).

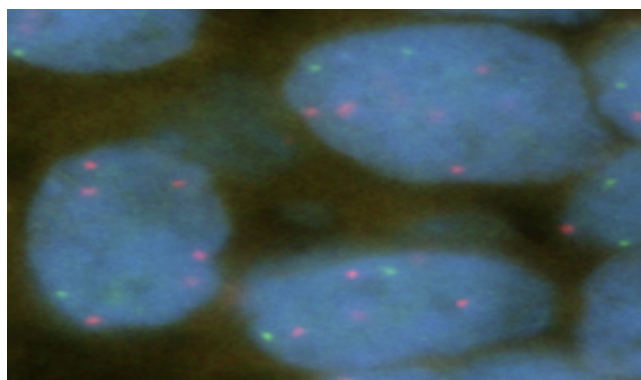
The relationship between smoking habits and *KRAS* mutations was statistically relevant ($p=0.014$) with higher incidence in smokers.

EGFR and HER2 copy number – FISH

For *EGFR* copy number, 12 FISH positive cases (either high polysomy or amplification) ([Figs. 6 and 7](#)) and 18 FISH negative cases were demonstrated. The positive cases showed

Table 4 *KRAS* exon 2 mutations (n.p. – not present in this pattern; n.d. – not determined).

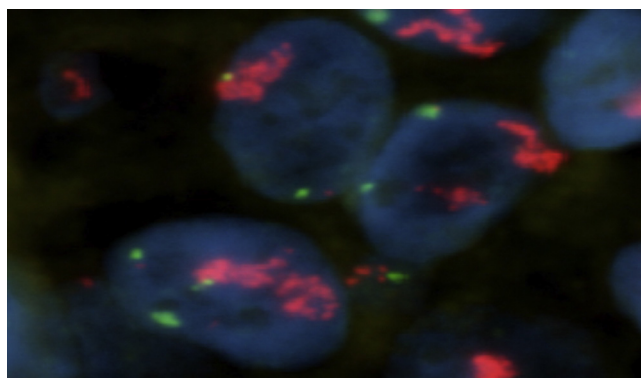
Case	Lepidic/BA	Acinar	Papillary	Solid	Mucinous
13	n.d.	G12C	G12C	n.p.	n.p.
17	n.d.	G12V	n.p.	G12V	n.p.
21	G12V	G12V	n.p.	n.p.	n.p.
23	G12V	G12V	n.p.	n.p.	n.d.
	(<i>EGFR</i> L858R)	(<i>EGFR</i> L858R)			
29	G12C	G12C	n.p.	n.p.	n.p.

**Figure 6** Case 7: FISH positive for *EGFR*/chromosome 7 centromere. Gene high polysomy; *EGFR* – red signal, chromosome 7–green. 1000 \times .

positivity in all the patterns present. In the 12 FISH positive cases, 3 were *EGFR* WT and 9 had *EGFR* mutations. On the other hand, of the 18 FISH negative cases, 13 were *EGFR* WT and 5 had *EGFR* mutations with concordance K Test of $k=0.4595$ (moderate agreement). These results were consistent in all patterns of each tumour.

When considering the K Test for concordance between FISH results and each mutational type (exons 21 and 19) the concordance was only considered as fair ($k=0.2857$ and $k=0.2647$, respectively).

The concordance (K Test) between mutational status and *EGFR* immunohistochemical expression (positive/negative) was considered fair ($k=0.2556$). The concordance (K test)

**Figure 7** Case 12: *EGFR*/chromosome 7. Gene amplification; FISH positive. *EGFR*/chromosome 7 centromere. *EGFR* – red signal, chromosome 7–green. 1000 \times .

between FISH status and IHC results were also considered as fair ($k=0.2635$).

Correlation coefficient between immunohistochemical *EGFR* expression and mutational status was considered not quite significant ($p=0.0561$) with $r=0.3525$. Correlation coefficient between *EGFR* mutational status and FISH status ($r=0.5303$) was considered very significant ($p=0.0026$). No correlation was identified between *EGFR* protein expression and FISH status.

HER-2 FISH positive (mainly by polysomy) cases were identified in 10 cases corresponding mainly to women and 20 cases were *HER-2* FISH negative (Table 5 concise *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 metastases were present, they were *HER2* FISH negative.

Discussion

The predictive importance of making accurate and more specific diagnosis of bronchial-pulmonary carcinoma, in biopsies, 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 CD56) to establish the diagnosis of primary adenocarcinomas towards other pulmonary histological types and metastatic adenocarcinomas, following the proposed classification for lung adenocarcinomas.⁸ IHC was made to validate the pure condition of bronchial-pulmonary adenocarcinomas mainly to validate solid pattern and establish differential diagnosis, as well as characterize proliferation index (Ki67-MIB1 antibody). We tried also to identify differences of expression between the patterns studied in order to understand and validate the importance of differentiating patterns. All morphological patterns were registered for each adenocarcinoma and revealed negativity for CK5,6,18 and for CD56, excluding squamous and neuroendocrine differentiations, as shown by other authors.^{24–26} CD56 is useful for excluding neuroendocrine differentiation, thus confirming that we are dealing with pure adenocarcinomas, not combined adenocarcinomas.

High importance has been given to CKs family, formed by more than 30 polypeptides, distributed by tissue and differentiation-specific peptides, allowing phenotyping by combining particularly CK7, CK20 and epidermoid/basal cells high weight CKs to determine origin of cell types or tissue.^{25–32}

Table 5 EGFR and HER2 FISH Positive Results.

Case	Pattern	EGFR	Estrat	HER2	Estrat
11	Lepidic/BA	Pos	HP	Neg	Dis
	Acinar	Pos	HP	Neg	Dis
333	Papillary	Pos	HP	Pos	HP
	Lepidic/BA	Neg	LP	Pos	HP
	Acinar	Pos	HP	Pos	HP
66	Solid	Pos	HP	Pos	HP
	Acinar	n.d.	n.d.	n.d.	n.d.
777	Solid	Pos	HP	Pos	HP
	Acinar	Pos	HP	Pos	HP
	Lepidic/BA	n.d.	n.d.	n.d.	n.d.
8888	Acinar	Pos	HP	Pos	HP
	Solid	Pos	HP	Neg	BP
	Micropapillary	Pos	HP	Pos	HP
	Mucinous	n.d.	n.d.	n.d.	n.d.
1111	Acinar	Neg	Tris	Pos	Amp
	Lepidic/BA	n.d.	n.d.	n.d.	n.d.
121212	Solid	Pos	Amp	Neg	Tris
	Acinar	Pos	Amp	Neg	Tris
	Micropapillary	Pos	Amp	Neg	Tris
141414	Papillary	Pos	Amp	Pos	HP
	Acinar	Pos	Amp	Pos	HP
	Lepidic/BA	n.d.	n.d.	n.d.	n.d.
1919	Micropapillary	Pos	HP	Neg	Tris
	Acinar	Pos	HP	Neg	Tris
202020	Lepidic/BA	Pos	HP	Neg	Tris
	Acinar	Pos	HP	Neg	Tris
	Micropapillary	n.d.	n.d.	n.d.	n.d.
232323	Lepidic/BA	Pos	HP	Pos	HP
	Acinar	Pos	HP	Pos	HP
	Mucinous	n.d.	n.d.	n.d.	n.d.
262626	Acinar	Neg	Tris	Pos	HP
	Solid	Neg	Dis	Pos	HP
	Lepidic/BA	n.d.	n.d.	n.d.	n.d.
2727	Solid	Pos	HP	Pos	HP
	Lepidic/BA	n.d.	n.d.	n.d.	n.d.
3030	Lepidic/BA	Pos	HP	Pos	HP
	Acinar	Pos	HP	Pos	HP

Estrat. – Estratification for FISH results; DIS – Disomy; LP – Low polysomy; Tris – Trisomy; HP – High polysomy; AMP – Amplification; n.d. – not determined.

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.^{8,27,33,34} CK20 was expressed in 3 cases of primary lung adenocarcinoma, with expression of CK7 and TTF1.

Mucinous glandular pattern showed lower expression of CK7 than the other patterns and one mucinous pattern had CK20 1+ expression in 10% of the cells and 2 of mixed adenocarcinomas showed 2+ expression in 50% of the cells in both acinar and lepidic/BA patterns. At this point it is relevant to make a comment about the recently proposed classification for lung adenocarcinomas.⁸ This classification proposes the replacement of BA – bronchiolo-alveolar pattern carcinoma designation for lepidic. When dealing with classical non-mucinous BAC, 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 seems to mean going for a morphologic description undervaluing carcinogenesis. Mucinous pulmonary adenocarcinoma cells exhibiting positivity for intestinal immunohistochemical markers, like CK20, MUC2 and COX2, fail to express TTF1³⁵ 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, giving life to BA pattern referring to the lepidic non-mucinous type. Predictive subtypes will be described to clarify origin and prognosis, between

glandular and non-glandular mucinous bronchial-pulmonary adenocarcinomas.³⁶

Also 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 the exception of papillary and solid patterns, all the other patterns had intense expression suggesting that some patterns showing decreased expression of TTF1 can explain a small number of cases developing in pure CK7 positive (bronchial) cells.^{27,28} It is a classical concern of pathologists to report a parameter related with tumour 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, which provides information about the portion of active cells in the cell cycle.^{37,38} Ki-67 expression 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.³⁰ 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 behaviour, either reflecting an higher proliferation index or a particular differential origin or as already referred, bronchial development when without expression of either TTF1 and high weight molecular cytokeratin (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 prediction and specific genetic pathways worth exploring.

In the literature, there are numerous references for immunohistochemistry and genetic studies in lung adenocarcinomas and especially under the old nomenclature of NSCLC. The importance of this work lies in the fact that we compared IHC expression between the different patterns of lung adenocarcinomas to identify differences and relevance between them, pioneering the searching of *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 for *EGFR* and *KRAS* mutations. However *EGFR* mutations were more frequent in women and *KRAS* mutations in men. Gender and smoking habits have been significantly related with *EGFR* mutations.^{2,3,30,39}

The commonly reported *EGFR* mutation rate by PCR sequencing and fragment analyses in lung adenocarcinomas is around 20%.⁴⁰ 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^{4,41}; 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.^{3,5,41} More than 70% of NSCLC with *EGFR* mutations are responsive to *EGFR*-TKIs while only 10% of tumours without *EGFR* mutations are responders.⁴

In our study, we had a rate of *EGFR* mutations of 46% gathering all patterns. Female patients had a 57.9% rate and male patients had a 27.3% rate (5 cases in 25) ($p=0.001$). Female lung cancer patients have 6 times more risk (Odds Ratio = 6.286) 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 other studies, based on Japanese subjects, where over 50% of female patients with lung cancer had higher expression of *EGFR* mutations. Although high, our *EGFR* mutation rate is not unique. It may reflect the exhaustive sampling covering all the patterns present, population features, and the selected population may have more advanced surgical cases. Tumour heterogeneity and genetic instability (leading to the accumulation of genetic events) could explain the *EGFR* mutation rate observed as well as the presence of different 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 52.6% (10 in a total of 19 females) while male patients had a rate of 0% (0 in a total of 11 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 26.3% while male patients had a rate of 27.3%. Also, *EGFR* exon 21 mutations were however more frequent in women than in men.

EGFR mutations were described as more frequent in never smokers 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 cannot be sure that smoking can prevent *EGFR* mutations. These findings only suggest that carcinogens contained in tobacco smoke are probably not correlated with the presence of *EGFR* mutations which might be caused by another type of carcinogens than higher numbers' *EGFR* copies.⁴²

Some consider that FISH *EGFR* and *HER2* copy numbers may be used when DNA quantity is not sufficient for *EGFR* mutation search as they showed some degree of TKI responses.^{3,19,39,42-44}

HER2 has strong kinase activity.^{3,12-14} Mutations also occur in the same region (3' of the α C-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)^{10,19}; the majority are insertion mutations in exon 20 and the existence of *HER2* mutations confers resistance to *EGFR*-TKIs but make tumours sensitive to *HER2* target therapies.^{3,10,19,45-47} In our study we did not find *HER2* mutations, indicating lesser importance of *HER2* in lung adenocarcinomas' carcinogenesis.

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 45.5% (5 in 11 male patients) while female had a rate of 0% (0 in 19 female patients). Our findings are similar

to Kim et al. study, who also realized 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%.^{30,42}

KRAS mutations have been associated with mucinous differentiation, goblet cell, poor differentiation adenocarcinomas and with solid patterns.^{48–52} However in our cases we did find this mutation in acinar and lepidic patterns and in only one case with solid pattern. The lower number of poorly differentiated adenocarcinomas, mucinous and solid patterns could in part explain the lower *KRAS* mutation rate.

EGFR mutations and *KRAS* mutations have been described as mutually exclusive but we found 1 case of *KRAS* mutation simultaneously with *EGFR* mutation. So we conclude that although *EGFR* and *KRAS* mutations are generally mutually exclusive, in some cases they can coexist; this has also been described by other authors.^{11,46,53–57} The clinical, therapeutic 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.^{40,42–44,58} We have demonstrated correlation between mutational *EGFR* status and FISH high copy number. We did not find any correlation with IHC, maybe because gene amplification was not the prevalent event but gene polysomy reflecting aneuploidy, a frequent genetic alteration identified in lung carcinomas.

Increased copy number of *EGFR* was present in 40% of our cases which is comparable to the 36% already demonstrated. In our study, *EGFR* mutations had 7 times more risk (Odds Ratio=7.016) 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 ($k=0.4595$) with a significant correlation coefficient ($r=0.5303$; $p=0.0026$). This is relevant because, knowing that some patients having high *EGFR* gene copy number can benefit from TKI, we might consider, specially in cases where mutational studies are inconclusive, using this information in therapeutic decisions.^{43,44} Concordance of the *EGFR* gene copy number by FISH and protein expression by IHC was seen in 7 of 12 IHC-positive cases.⁴⁴

Lepidic/BA non-mucinous pattern has been described as having mutations of *EGFR* more frequently. Comparing our five different patterns (lepidic/BA, 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 lepidic/BA pattern. If we aggregate lepidic/BA and acinar patterns, *EGFR* mutations are more frequent in these two patterns when compared to the other patterns all together ($p=0.008$). Recently other authors showed that *EGFR* and *KRAS* mutations are more frequent in lung carcinoma with adenocarcinoma differentiation based on p63 and TTF1 expression, p63 expression being the most useful immunohistochemical marker since they did not find any mutation in tumours considered p63 positive.⁵¹ In our work we went further correlating the mutational status also with the histological patterns present. Generally, when a mutation is present it is present in all the histological patterns of the

same adenocarcinoma (Tables 2–4). Biopsies, representing only a small portion of a tumour, can be used to determine *EGFR* and *KRAS* mutational status.

The conclusions referred to above are predictive and again raise the controversies explored in the proposed lung adenocarcinomas classification: a small biopsy with a carcinoma CK7 positive and TTF1 positive is an adenocarcinoma (CK 5,6,18 negative) and has to be submitted to *KRAS* mutation search in a male patient, when acinar, solid pattern and lepidic/BA (non-mucinous type) pattern are present and also to *EGFR* mutational search for TKIs prescription decision.

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 probably a particular cell origin and more aggressive biological behaviour and have to be reported as lung adenocarcinomas, avoiding poorly differentiated carcinoma designation.

After this study, it seems that *EGFR* and *KRAS* mutational status can be determined in biopsies representing bronchial pulmonary carcinomas because when a mutation is present it is generally present in all the histological patterns, needing further equivalent studies supported by a practical immunohistochemical panel.

Conclusions

The differences identified between the adenocarcinoma patterns reinforce the necessity to carefully identify the patterns present with implications for diagnosis and pathogenic understanding. Correlation was found in between *EGFR* FISH results and mutational status. There are adenocarcinomas harbouring different *EGFR* mutations in different patterns. In general, *EGFR* mutation is present in all the patterns of the same adenocarcinoma reinforcing the possibility of mutational status determination in biopsies.

Conflicts of interest

The author has no conflicts of interest to declare.

Ethical disclosures

Protection of human and animal subjects: The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data: The authors declare that they have followed the protocols of their work centre on the publication of patient data.

Right to privacy and informed consent: The authors declare that no patient data appear in this article.

Authorship

V.S. was responsible for drafting the manuscript, immunohistochemical interpretation, compilation of clinical data and results as well for the statistical workflow. L.C. was responsible for the orientation, corrections as well for immunohistochemical interpretation. C.R. was responsible for drafting of the manuscript, results interpretation and

also for statistical works. M.S. carried out FISH assays and results interpretation. A.M.A. carried out the immunoassays and mutational evaluation.

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