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ORIGINAL ARTICLE

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

Vitor Sousa\textsuperscript{a,b,*}, Carolina Rodrigues\textsuperscript{a}, Maria Silva\textsuperscript{a}, Ana Maria Alarcão\textsuperscript{a}, Lina Carvalho\textsuperscript{a,b}

\textsuperscript{a} IAP-FMUC-Institute of Pathology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
\textsuperscript{b} Pathology Institute, Coimbra University Hospital, Coimbra, Portugal

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KEYWORDS
Lung; EGFR; KRAS; HER2; Immunohistochemistry; Adenocarcinomas

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...
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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)).\textsuperscript{9,10} may be over-expressed and this correlates with poor prognosis, with aggressive disease and decreased survival.\textsuperscript{3,11-14}

KRAS mutations are associated with poor prognosis, reported since 1990, occurring in 12%, occasionally at codon 13 and rarely at codon 61.\textsuperscript{10,15,16} According to the current data, EGFR and KRAS mutations are mutually exclusive.\textsuperscript{10,17,18} The explanation is related to KRAS-MAPK pathway inserts in the downstream signalling pathway of EGF.\textsuperscript{10} This mutation appears in 30% of Caucasian patients with lung cancer and 10% of East Asian adenocarcinomas.\textsuperscript{16,18,19} KRAS mutations are more frequent in smokers and are related to poor prognosis.\textsuperscript{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.\textsuperscript{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-EGF 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$_2$O$_2$). 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 1/100 for 60 min, TTF1 (clone 8G7G3/1; DakoCytomation, Glostrup, Denmark) at a dilution of 1/100 for 60 min, CD56 (clone CD564; Novacstra Laboratories Ltd, Newcastle, United Kingdom) at a dilution of 1/100 for 60 min, CK7 (clone LP34; Novacstra Laboratories Ltd, Newcastle, United Kingdom) at a dilution of 1/10 for 60 min, and CD56 (clone KS20.8; 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 for 10 min. Primary antibody binding was localized in tissues using peroxidase-conjugated streptavidin (Ultra Vision Kit; TP-125-BN; Lab Vision Corporation; Fremont CA; USA). A signal was generated with *avidin* and peroxidase-diluted ABC reagent slides were counterstained with haematoxylin. 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 m thick, baked overnight at 56°C, deparaffinized in xylol, rehydrated in 100%, 70%, and 50% 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× 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× 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 × 22 mm glass coverslip was placed over probe.

After being sealed with rubber cement and denaturating 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× SSC pH 7) at 46°C and were also washed with 2× SSC 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 polyomy 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 Varela-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 μm 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 μl reaction containing 5 μl of 10× 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 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 electropherograms 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.

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age</th>
<th>Smoking habits</th>
<th>TNM classification</th>
<th>Stage</th>
<th>Histologic patterns</th>
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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
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 case, the mucinous glandular pattern had CK20 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.

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. Cytoplasmatic 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).
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 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**

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.
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 G12V (Valine) and 2 of the type G12C (Cystein).

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...
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 \( (k = 0.2857 \text{ and } k = 0.2647, \text{ respectively}) \).

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 \( (k = 0.2635) \).

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 \( (\text{Table 5 concise HER2 and EGFR positive cases}) \). 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. 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. 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 weights, allowing phenotyping by combining particularly CK7, CK20 and epidermoid/basal cells high weight CKs to determine origin of cell types or tissue.25-31
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.\textsuperscript{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 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.\textsuperscript{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 TTF1\textsuperscript{33} 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

<table>
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Estrat. = Estratification for FISH results; DIS = Disomy; LP = Low polysomy; Tris = Trisomy; HP = High polysomy; AMP = Amplification; n.d. = not determined.
glandular and non-glandular mucinous bronchial-pulmonary adenocarcinomas.\textsuperscript{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.\textsuperscript{37,38} 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.\textsuperscript{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.\textsuperscript{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 the latter 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.\textsuperscript{3,30,39}

The commonly reported EGFR mutation rate by PCR sequencing and fragment analyses in lung adenocarcinomas is around 20%.\textsuperscript{30} 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;\textsuperscript{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.\textsuperscript{2,5,41} More than 70% of NSCLC with EGFR mutations are responsive to EGFR-TKIs while only 10% of tumours without EGFR mutations are responders.\textsuperscript{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% 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.\textsuperscript{32}

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.\textsuperscript{3,19,39,42–44}

HER2 has strong kinase activity.\textsuperscript{12–14} Mutations also occur in the same region (3’ of the α-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)\textsuperscript{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.\textsuperscript{1,10,19,45–47} 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.43-52 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.11,46,51-53 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.40,42,44-48 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.43

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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References

Lung adenocarcinoma: subtyping and EGFR, HER2 and KRAS mutational status


